THE USE OF ANS FLUORESCENCE AS A PROBE FOR IMMUNOGLOBULIN FLEXIBILITY

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1. Introduction

Antigen binding to the combining sites of immunoglobulins results in the activation of various effector systems which interact with the Fc portion of the immunoglobulin molecule. Among the best characterised of these is the classical complement system, activation of which leads to the clearance of IgG and IgM immune complexes from the bloodstream. The binding site for C1q, which is part of the first component of this system (C1), has been located in the CH_2 domains in [1,2] where an immunoglobulin fragment, in which the CH_3 domains were absent, was able to bind C1.

This fragment, designated Facb, was prepared by plasmin digestion of IgG which had been incubated at low pH prior to addition of the enzyme. It was concluded that the low pH treatment converted the IgG into a 'low pH' form which was both unstable at neutral pH and sensitive to plasmin cleavage. As part of our programme aimed at defining the C1q binding site at the molecular level, we have shown that ANS is an inhibitor of C1q binding to rabbit IgG immune complexes [3].

Here we show that ANS in trace amounts may be used as a probe to detect the transition of IgG from its 'low pH' form to that form stable at neutral pH – the 'high pH' form. While the transition rate is faster in reduced and alkylated IgG compared to native IgG, reduction and alkylation had no effect on the transition rate of Fc. This difference suggests that the Fab arms may modulate the flexibility of IgG with cleaved interchain disulphide bonds.

2. Materials and methods

Papain and dithiothreitol were from Sigma (London); iodoacetamide was from Aldrich; pepsin was from Worthington; and 1-anilinonaphthalene-8-sulphonate, Mg-salt (ANS) from BDH.

2.1. *Protein preparation*

Non-immune rabbit IgG was prepared from normal serum as in [4]. The $(Fab')_2$, Fab and Fc fragments of rabbit IgG were prepared according to [5]. Reduction and alkylation of IgG and Fc was carried out as in [6].

2.2. *Fluorescence measurements*

Fluorescence measurements were made on a Perkin-Elmer Hitachi MPF-2A spectrofluorimeter, equipped with a cell compartment thermostatted at 30 ± 1°C. ANS fluorescence was monitored at 480 nm, using an excitation wavelength of 390 nm.

2.3. *Low pH treatment*

Protein samples (4–10 mg/ml, dissolved in 0.01 M sodium acetate, 0.15 M NaCl, pH 5.5) were adjusted to pH 2.2 by addition of 0.1 M HCl and incubated at 30°C for 30 min. The pH was rapidly adjusted to 7.2 by addition of Tris, ANS was added to 0.05 mM, and the fluorescence of the sample monitored as a function of time after addition of the Tris. Control samples were maintained at pH 5.5 during the 30 min incubation.

2.4. *Data analysis*

Where appropriate, data were fitted to eq. (1) using a non-linear regression FORTRAN computer programme written in this laboratory.

3. Results and discussion

The time dependence of the IgG-bound ANS fluorescence of a low pH-treated sample is shown in fig.1.

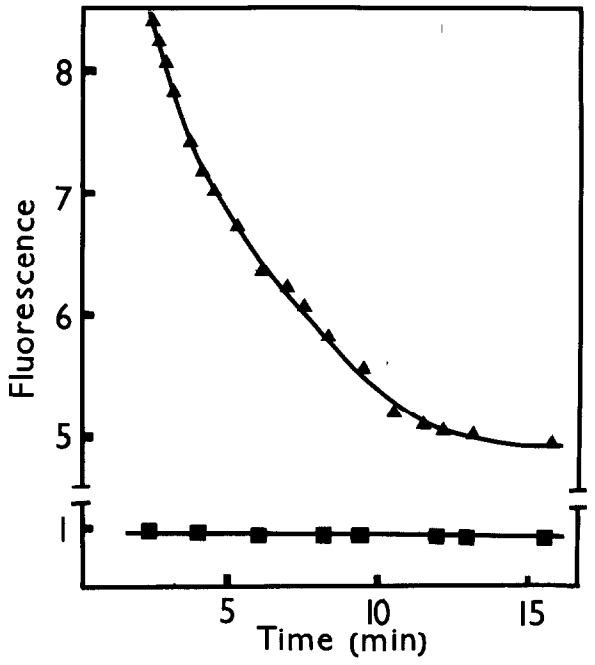

Fig.1. The relaxation behaviour of low pH treated IgG. IgG was subject to the experimental (▲——▲) and control (■——■) low pH treatments in section 2. The final protein concentration was 3.3 mg . ml^{-1}.

The final fluorescence of the low pH-treated sample is not the same as that of the control sample; this may well reflect the irreversible changes in IgG structure which led to the 30–40% loss of C1 fixing ability observed in [1,2]. If it is assumed that the change in fluorescence reflects an irreversible transition from a 'low pH' to a 'high pH' form of the protein, then it can be shown that eq. (1) describes the time dependence of the observed fluorescence (F_{obs}):

$$F_{obs} = F_A \exp(-kt) + F_B (1 - \exp(-kt) \qquad (1)$$

where F_A, F_B, and k represent the fluorescence of the 'low pH' form, the fluorescence of the 'high pH' form and the first-order rate constant for the transition, respectively. Analysis of the data in fig.1 gave values of: F_A, 10.5 ± 0.2; F_B, 4.5 ± 0.1; and k, 0.19 ± 0.01 min^{-1}. When the time dependence of similarly treated samples of $(Fab')_2$, Fab, and Fc was measured the results summarised in table 1 were obtained. Although ANS bound to low pH treated Fab or $(Fab')_2$ exhibited a time-dependent fluorescence change, the low initial fluorescence intensity suggests that the pH-dependent change in IgG structure probed by ANS is essentially confined to the Fc portion of the immunoglobulin molecule.

Table 1
Comparison of the relaxation behaviour of low pH-treated Fab, $(Fab')_2$ and Fc

	$F_A/\mu M$	$F_B/\mu M$	k (min^{-1})
Fab	0.58 ± 0.09	0.32 ± 0.03	0.9 ± 0.3
$(Fab')_2$	0.46 ± 0.12	0.22 ± 0.10	0.81 ± 0.01
Fc	10.8 ± 0.1	3.53 ± 0.04	0.44 ± 0.04

The values shown are the means obtained from duplicate experiments. The values of F_A and F_B have been divided by the protein concentrations used to allow comparison to be made between the different immunoglobulin fragments

When the time dependence of the IgG-bound ANS fluorescence of a low pH-treated sample in which the heavy–heavy and heavy–light interchain disulphide bonds had been reduced and alkylated was measured, the data in fig.2 were obtained. The larger value of k (0.85 ± 0.04 min^{-1}) implies that reduced and alkylated IgG relaxes back to the 'high pH' form more

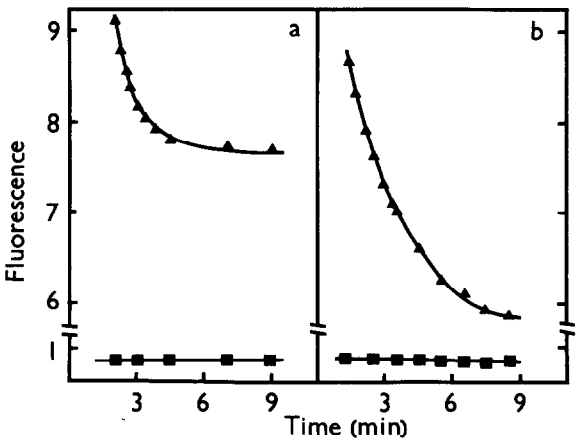

Fig.2. The effect of reduction and alkylation on the relaxation behaviour of IgG and Fc. Reduced and alkylated IgG (A) and Fc (B) were subject to the experimental (▲——▲) and control (■——■) low pH treatments. The final protein concentrations were 3.4 mg . ml^{-1} for IgG and 0.7 mg . ml^{-1} for Fc. Analysis of the data using eq. (1) gave k values of 0.85 ± 0.04 min^{-1} (IgG) and 0.37 ± 0.02 (Fc). The fluorescence intensities cannot be directly compared because different machine sensitivities were used in the two experiments. SDS–polyacrylamide gel electrophoresis [13] showed complete cleavage of the inter-heavy and heavy–light-chain disulphide bonds in the IgG, and the interchain bond in the Fc sample.

rapidly than does IgG with interchain disulphide bonds intact. However, when the fluorescence of Fc-bound ANS was measured after low pH treatment then the mean value of k for native Fc (0.44 ± 0.04 min^{-1}) was not significantly different from that of 0.37 ± 0.02 min^{-1} which was obtained when Fc with cleaved disulphide bonds was used.

An attempt was made to monitor the change in IgG structure in the reverse direction – from the 'high pH' form to the 'low pH' form. Although it was possible to detect an increase in the fluorescence of IgG-bound ANS following adjusting the pH of the sample to 2.2, this change was too rapid to monitor as a function of time.

The mechanism by which formation of an antibody–antigen complex leads to an enhancement of Fc-mediated effector functions is poorly understood. A number of theories (reviewed in [7]) have been advanced, including the suggestion that antigen binding causes a structural change in the Fc portion leading to an enhanced effector interaction with this region of the immunoglobulin molecule. One test of this theory would be unequivocal demonstration of an antigen-induced change in the properties of a probe attached to the Fc region. Given that ANS is an inhibitor of C1q binding [3] and that it can detect structural changes in the Fc portion of the immunoglobulin molecule, then it would appear that this probe may be suitable for detection of putative antigen-induced changes. Preliminary results obtained using an anti-(pneumococcal polysaccharide) antibody suggest that the ANS fluorescence is not altered on addition of hapten (S. B. E.-S., P. Závodszky and J.-C. Jaton, unpublished).

The difference in relaxation behaviour between IgG and Fc on reduction and alkylation of the interchain disulphide bonds may shed some light on the mechanism by which reduction and alkylation of IgG leads to a large decrease in its C1q binding ability. The effect of reduction and alkylation on the relaxation behaviour of IgG may be interpreted in the light of two recent reports on the effect of this procedure on the physical properties of immunoglobulins. It has been suggested [8] that the tensile forces exerted by the Fab arms enhance the dissociation tendency of the two polypeptide chains in Fc with the interchain disulphide bond broken. Evidence consistent with this suggestion was obtained in an electron microscopy study [9] in which it was shown that cleavage of the inter-heavy chain disulphide bond allowed the CH_2 domains to separate under the strain induced by complex formation with a bivalent hapten. The conclusion that the effect of disulphide bond cleavage on complement activation is mediated via the Fab arms' interaction with antigen is supported by the observation that while immune complexes made using reduced and alkylated IgG are unable to activate C1, the ability of monomeric IgG or Fc to bind C1q is unaffected by this treatment [10]. We have also shown that, as judged by high resolution nuclear magnetic resonance spectroscopy, the structure of the Fc fragment is not markedly altered on cleavage of the inter-heavy chain disulphide bond [11].

Thus, when the inter-heavy chain bond is broken, there may be an increase in the flexibility of the domain modules which could explain why reduced and alkylated IgG are able to relax back to the 'high pH' form more rapidly. Fluorescence depolarisation measurements have indicated that the hinge disulphide bond plays a major role in restricting such flexibility in the immunoglobulin molecule [12]. In this context it is interesting to note that cleavage of this bond leads to formation of a new 'hinge' region near the CH_2:CH_3 domain interface [9]. This region contains the labile peptide bond sensitive to plasmin cleavage in low pH-treated IgG [1,2].

These data and the above studies suggest that cleavage of the inter-heavy chain disulphide bonds leads to loss of complement-fixing ability, not via a structural change in the Fc itself, but rather by an alteration of the geometrical relationship between the individual CH_2 domains in an immune complex.

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References

[1] Connell, G. E. and Porter, R. R. (1971) Biochem. J. 124, 53P.
[2] Colomb, M. and Porter, R. R. (1975) Biochem. J. 145, 177–183.
[3] Easterbrook-Smith, S. B., Závodszky, P., Willan, K. J., Gettins, P. and Dwek, R. A. (1978) Biochem. Soc. Trans. 6, 1126–1131.
[4] Wilkinson, J. M. (1969) Biochem. J. 112, 173–185.

[5] Dower, S. K., Dwek, R. A., McLaughlin, A. C., Mole, L. E., Press, E. M. and Sunderland, C. (1975) Biochem. J. 149, 73–87.
[6] Press, E. M. (1975) Biochem. J. 149, 285–288.
[7] Metzger, H. (1978) Cont. Top. Mol. Immunol. 7, 119–152.
[8] Venyaminov, S. Y., Rajnavölgyi, E., Medgyesi, G. A., Gergely, J. and Závodszky, P. (1975) Eur. J. Biochem. 67, 81–86.
[9] Seegan, G. W., Smith, C. A. and Schumaker, V. N. (1979) Proc. Natl. Acad. Sci. USA 76, 907–911.
[10] Goers, J. W., Ziccardi, R. J., Schumaker, V. N. and Glovsky, M. M. (1977) J. Immunol. 118, 2182–2191.
[11] Boyd, J., Easterbrook-Smith, S. B., Závodszky, P., Mountford-Wright, C. and Dwek, R. A. (1979) Mol. Immunol. 16, 851–858.
[12] Chan, L. M. and Cathou, R. E. (1977) J. Mol. Biol. 112, 653–656.
[13] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.