The Interaction of Protein A and the Fc Fragment of Rabbit Immunoglobulin G as Probed by Complement-Fixation and Nuclear-Magnetic-Resonance Studies

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Protein-A–Fc-fragment complexes were observed in sedimentation-velocity experiments by ultracentrifugation. The interaction was studied by protein-fluorescence-quenching titrations of the Fc fragment with protein A, allowing the dissociation constant to be determined under a variety of conditions. The first component of the complement pathway, C1, is activated by complexes of protein A with rabbit IgG (immunoglobulin G), and the structural basis for this interaction was studied by using n.m.r. (nuclear magnetic resonance). The four Fc-fragment-binding sites on protein A were shown to contain aromatic amino acids, and to be connected by mobile hydrophilic regions. Neither n.m.r. nor proton-relaxation-enhancement studies show evidence of a large conformational change of the Fc fragment on binding protein A, and this suggests that the cross-linking of the Fc fragments may be primarily responsible for the activation of component C1. This is supported by the inability of a univalent tryptic fragment of protein A to activate complement fixation by rabbit IgG.

Protein A is covalently bound to the cell wall of *Staphylococcus aureus* (Sjöquist *et al.*, 1972b), and it can be isolated by digestion of the intact bacteria with lysostaphin (Sjöquist *et al.*, 1972a), followed by affinity chromatography on IgG†–Sepharose (Hjelm *et al.*, 1972). It binds to the Fc fragment of normal IgG (Forsgren & Sjöquist, 1966) from several species (Kronvall *et al.*, 1970), resulting in the formation of precipitates with, for example, human IgG. With rabbit IgG, however, soluble aggregates are formed, although precipitation lines can be seen in agar gels (Forsgren & Sjöquist, 1967). This interaction initiates biological activities which are also characteristic of antigen–antibody complex-formation, including activation of the complement series of proteins (Sjöquist & Stålenheim, 1969; Stålenheim *et al.*, 1973).

Protein A has been shown by hydrodynamic studies

to be a very elongated protein, with mol.wt. 43 000 (Björk *et al.*, 1972). By partial tryptic digestion, several low-molecular-weight fragments (6000–8000) are generated which bind to the Fc fragment, but are univalent in this reaction, as shown by the inhibition of haemagglutination of sensitized erythrocytes by protein A (Hjelm *et al.*, 1975). These fragments originate from four highly homologous Fc-fragment-binding regions, each containing approx. 60 amino acid residues, and are consecutively arranged in the peptide chain (Sjödahl, 1976, 1977a). The primary structure of the complete Fc-fragment-binding portion of protein A has been proposed (Sjödahl, 1977b).

We have used spectroscopic techniques to study the mode of interaction of protein A with the Fc fragment, and to detect any conformational changes that may occur. Rabbit Fc fragment was chosen for the n.m.r. studies, since it forms soluble aggregates with protein A. The ability of protein A, and one of its univalent fragments, to initiate complement fixation with rabbit IgG was studied, and in particular their ability to deplete the early components of the complement pathway.

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¶ Abbreviations: IgG, immunoglobulin G; n.m.r., nuclear magnetic resonance; Fc, C-terminal half of the H-chain dimer.

Materials and Methods

Protein preparation

Rabbit non-immune IgG was prepared from pooled normal serum (Wilkinson, 1969). Fc fragments with the inter-heavy-chain disulphide bond intact [Fc(S⁺)] were prepared as described by Dower *et al.* (1975). Electrophoresis in 7% polyacrylamide gels containing sodium dodecyl sulphate showed that the disulphide bond was intact in 80–85% of the molecules in the Fc(S⁺) preparation.

pFc' fragments were prepared by digestion of normal rabbit IgG (20 mg/ml in 100 mM-sodium acetate buffer, pH 4.5) with pepsin, at an enzyme/substrate ratio of 1:50 (w/w) for 16 h at 37°C. The digest was then fractionated on a Sephadex G-75 column (90 cm × 5 cm) in 100 mM-sodium phosphate buffer, pH 6.8.

Protein A, and univalent tryptic fragments, were isolated as described by Hjelm *et al.* (1972) and Sjö Dahl (1976) respectively. The proteins were freeze-dried and stored at –20°C.

Nuclear magnetic resonance

N.m.r. spectra were recorded at 270 MHz by using a Bruker spectrometer with an Oxford Instrument Co. (Oxford, U.K.) superconducting magnet. Chemical shifts are reported downfield of the sodium salt of 3-(trimethylsilyl)propanesulphonic acid.

N.m.r. experiments on Fc-fragment solutions were carried out between pH 4.0 and 4.5 and in the absence of Cl[–] ions, owing to the insolubility of the protein at the high concentrations (1–2 mM) required for n.m.r. studies, except under these conditions. The pH was adjusted by use of NaO²H and [2H₄]-acetic acid. An internal reference of 0.01% acetone was used, and this caused no changes in the properties of Fc-fragment solutions. Solutions of protein A for n.m.r. studies were prepared similarly, and no change in the properties of the solutions was observed after storage of frozen solutions for 3 months. Solutions of lanthanides were prepared by dissolving the nitrates in ²HNO₃ or HNO₃ and adjusting the pH to 4.

Solvent-water proton spin-lattice relaxation times were measured at 20 MHz on a spin echo machine constructed in this laboratory. Disposable plastic tubes and pipettes were used in experiments involving gadolinium, to minimize the effect of Gd(III) binding to glassware.

Fluorescence studies

Fluorescence measurements were made on a Perkin-Elmer-Hitachi MPF 2A spectrofluorimeter, equipped with a cell compartment thermostatically controlled at 25°C. Titrations of Fc fragment with protein A were analysed by fitting the experimental points with curves constructed by assuming various values of the dissociation constant K_D and ϵ_b , the

fluorescence enhancement of the binary complex. ϵ_b is defined as the ratio of the fluorescence intensity of the Fc fragment saturated with protein A to that of free Fc fragments. The binding curves were calculated by assuming two binding sites/Fc fragment (Hjelm, 1974) and four binding sites/molecule of protein A (Sjö Dahl, 1977a).

Sedimentation studies

Ultracentrifugation was carried out at 20°C by using a Beckman model E centrifuge with schlieren optics and a rotor operating at 52 000–56 000 rev./min.

Complement assays

Titration of whole complement and components C1, C2 and C4 activities were carried out as described by Rapp & Borsos (1970). Rabbit haemolysin was prepared from sheep erythrocyte stromata (Mayer, 1961) and the immune sera were heated at 56°C for 30 min and stored at –70°C. Whole complement was prepared by bleeding normal Hartley-strain guinea pigs by cardiac puncture and allowing the blood to clot for 4–5 h at 4°C. Buffers used in complement-fixation assays, e.g. dextrose/gelatine/veronal buffer, are described in Rapp & Borsos (1970).

Materials

Lanthanide nitrates were obtained from Koch-Light, Colnbrook, Bucks., U.K.; ²H₂O, NaO²H and [2H₄]-acetic acid were obtained from Ryvan, Southampton, Hants., U.K. Pepsin was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Results and Discussion

N.m.r. studies on protein A and one univalent fragment

Protein A. The 270 MHz proton n.m.r. spectrum of protein A (2 mM in ²H₂O, at pH 4.5) is shown in Fig. 1, where it is compared with a computed spectrum which is the sum of the spectra of the constituent free amino acids of protein A. Two important features are obvious in this comparison: first, a large proportion of the resonances in the protein-A spectrum coincide with the peaks calculated from the free amino acids, and, secondly, the linewidths of the resonances are much less than those usually observed for a globular protein of mol.wt. about 40 000 (Dwek, 1973). This indicates that many of the amino acid side chains are exposed to the solvent, and are able to undergo motion independently of the tumbling time of the whole protein. This is consistent with the large percentage of hydrophilic amino acid residues present in protein A (Sjöquist *et al.*, 1972a), which would be expected to be exposed to the solvent, and also with the proposed elongated form of the molecule (Björk *et al.*, 1972). It is also noteworthy, however, that

Fig. 1. Comparison of the 270 MHz proton n.m.r. spectrum of protein A (2 mM in $^2\text{H}_2\text{O}$, pH 4.5) with a computer simulation of the constituent amino acids in the random-coil position: $T = 303\text{ K}$
 —, Experimental spectrum; ----, computed spectrum.

Fig. 2. Resonances of protein A as a function of pH (4.5–11.5)
 The protein concentration was 1.5 mM in $^2\text{H}_2\text{O}$. Spectra were recorded at 270 MHz and 303 K.

some areas of the protein-A spectrum indicate ordered structure, for example, the aromatic resonances (6.5–8.5 p.p.m.) and the region where methyl resonances would be expected to occur (0.5–1.5 p.p.m.).

The change of position of resonances of protein A with pH (4–11.5) is shown in Fig. 2. Some of these titrating resonances can be assigned to particular

types of amino acid side chains. The application of Carr–Purcell pulse sequences (Campbell *et al.*, 1976) indicates resonances around 6.7 p.p.m., corresponding to two protons each. Since these resonances begin to titrate at pH 9, they presumably arise from tyrosine residues. This is consistent with the results of spectrophotometric titrations (Sjöholm, 1975),

which showed that the average pK_a value of the four tyrosine residues was 10.25. Two resonances can be seen, at 8.5 and 7.3 p.p.m., which start to titrate at pH 5–6. Both the resonance positions and the titration behaviour are typical of the C-2 and C-4 protons of histidine. No other aromatic residues would be expected to titrate at this pH, so that these two resonances can be assigned to a histidine residue. Only one of the four histidine resonances titrates, indicating that it arises from a histidine residue in a different environment from the other three.

The pK_a of this histidine residue cannot be determined, owing to the discontinuity in the titration curves between pH 7 and 9. This phenomenon is not restricted to the histidine resonances, and the position of most of the proton resonances in the aromatic region cannot be followed through the pH range 7–9 (Fig. 2). Furthermore the aromatic resonances become markedly broader between pH 8.3 and 9.3. This indicates that a structural change must occur in the protein A at pH 7–9. Ultracentrifugation studies have not shown the presence of any aggregates when studied at various pH values, so that a change of the conformation of the protein with pH must be proposed. It is noteworthy that Sjöholm

(1975) did not detect any changes in protein-A structure, over the pH range 1.0–11.0, by using circular-dichroism measurements as a probe for conformational changes. The circular-dichroism studies showed that protein A contains a large proportion of α -helical (48%) and β -structure (21%). It is possible therefore that those regions of the protein-A structure where the side chains are highly mobile may not have a completely random-coil backbone structure. The N–H proton resonances (8–8.5 p.p.m.) can only be observed at pH values below 7. The absence of these resonances at pH values above 7 cannot be due to exchange of the protons with solvent deuterons, because the resonances reappear when the pH is lowered to below 7 from alkaline solutions. This also suggests that a reversible change occurs above pH 7.

Fragment VI". The 270 MHz proton n.m.r. spectrum of the tryptic univalent fragment VI" (1.5 mM in 2H_2O , pH 6.9) originating from region C (Sjödahl, 1976) is compared in Fig. 3 with the spectrum computed from those of the constituent amino acids. It is again apparent that the two spectra are very similar, indicating internal mobility of the protein, and exposure of many of the amino acids to solvent. The

Fig. 3. Comparison of the 270 MHz proton n.m.r. spectrum of fragment VI" of protein A (1.5 mM in 2H_2O , pH 6.9) with a computer simulation of the constituent amino acids in the random-coil position: $T = 303 K$
—, Experimental spectrum; ----, computed spectrum.

narrow linewidths of this protein spectrum allow the assignment of resonances to particular types of amino acid side chains by the technique of *J* decoupling (Campbell *et al.*, 1975). For example, irradiation of the resonance at 6.8 p.p.m. causes the collapse of the doublet resonance at 7.2 p.p.m. to give a singlet. These two resonances can therefore be assigned to the single tyrosine residue in fragment VI'. In this way, all the aromatic resonances of fragment VI' have been assigned, as shown in Fig. 5.

Interaction of protein A and fragment VI' with the immunoglobulin Fc fragment

Ultracentrifugation. Sedimentation-velocity studies have confirmed that a soluble aggregate is formed by protein A and Fc fragment. The sedimentation coefficients ($s_{20,w}$) of fragment Fc (2 mg/ml) and protein A (3.2 mg/ml) alone at pH 5.5 are 3.8S and 2.1S respectively, whereas in a solution containing both proteins at a Fc-fragment/protein-A molar ratio of 2:1, and a total protein concentration of 4.2 mg/ml, at pH 5.5, a broad band was seen giving an average sedimentation coefficient of 10S. Solutions of Fc fragment with fragment VI' at equivalence (Fc-fragment/fragment-VI' molar ratio of 1:2) showed a single peak with a sedimentation coefficient approximately equal to that of the Fc fragment alone, confirming that fragment VI' does not cause cross-linking of Fc-fragment molecules.

Fluorescence. Protein A does not contain any tryptophan residues (Sjöquist *et al.*, 1972a), so that the tryptophan fluorescence of Fc fragment can be readily followed when protein A is added, to obtain the dissociation constant for the interaction. Fc-fragment protein-fluorescence-quenching titrations were performed at pH 4.5 and 7.8, in both the presence and the absence of 0.15M-NaCl. As shown in Table 1, protein A causes the Fc-fragment fluores-

cence to be quenched by approx. 20%. The dissociation constants for the interaction were calculated by assuming the numbers of binding sites on the Fc fragment and protein A to be 2 and 4 respectively. Although the dissociation constant is sensitive to pH, it is evident that under the conditions used for the n.m.r. studies (pH 4.5) the interaction can be considered to be very strong.

Complement activation

The interaction of protein A with the IgG from several species has been shown to lead to complement fixation (Sjöquist & Stålenheim, 1969), and with human IgG this activation is known to involve the early-acting complement components (Kronvall & Gewurz, 1970; Stålenheim *et al.*, 1973). Since the n.m.r. studies were carried out with rabbit IgG, which produces soluble aggregates with protein A, it was necessary to show that in this system also the early-acting components are involved. Incubation of guinea-pig serum as a source of complement (diluted 7-fold in dextrose/gelatine/veronal buffer; 1 ml) with rabbit IgG (50 µg) and/or protein A (5 µg) for 1 h at 37°C shows (Table 2) that rabbit IgG-protein-A complexes do cause a depletion of the early complement components, as well as of whole complement activity. The addition of protein A alone to guinea-pig serum gives complement depletion, although to a lower extent than the addition of protein A and rabbit IgG together. This background extent of complement fixation is presumably due to the presence of immunoglobulins in the guinea-pig serum. The addition of rabbit IgG alone to guinea-pig serum does not cause complement fixation. The depletion of component-C1 activity by protein-A-rabbit IgG complexes suggests that component C1 interacts directly with these complexes, so that n.m.r. studies of the Fc-fragment-protein-A interaction should give information on the structural basis for component-C1 activation by the Fc region of immunoglobulins.

Table 1. *Protein A binding to Fc fragment studied by protein fluorescence quenching*

The excitation wavelength was 290 nm and the emission wavelength 340 nm. The concentration of Fc fragment was approx. 1 µM. Inner filter effects were corrected for by addition of protein A to a solution of tryptophan. The dissociation constant K_D was obtained by curve fitting, as described in the Materials and Methods section.

Conditions	K_D (µM)	Final quenching (%)
pH 4.4	4	15
pH 4.4, +0.15M-NaCl	3	14
pH 7.8	0.05	20
pH 7.8, +0.15M-NaCl	0.01	22

Table 2. *Complement depletion in guinea-pig serum by protein A and rabbit IgG*

Guinea-pig serum (1 ml; 7-fold dilution in dextrose/gelatine/veronal buffer) was incubated with rabbit IgG (50 µg) and/or protein A (5 µg) for 1 h at 37°C, and remaining activity assayed haemolytically as described in the text.

Activity assayed	Activity remaining (%)		
	Protein A	IgG	Protein A + IgG
Whole C'	20	97	1
C1	45	95	5
C4	90	100	65
C2	81	100	62

Incubation of guinea-pig serum with rabbit IgG (100 μ g) and the univalent fragment VI'' (0–100 μ g) does not give depletion of whole complement activity or of any of the early-acting components (C1, C4 and C2).

N.m.r. studies. The 270 MHz proton n.m.r. spectrum of Fc fragment (1 mM) with a stoichiometric amount of protein A (0.5 mM) at pH 4.5 is shown in Fig. 4, along with the spectra obtained for the same concentrations of the two proteins alone. The most notable feature of the spectra is that the peaks characteristic of protein A in the aliphatic region (0–4.5 p.p.m.) still appear in the spectrum of the complex. Since the linewidths of these resonances are not significantly increased on complex-formation, most of the aliphatic amino acid residues in protein A must retain the relative freedom of mobility that they possess in the uncomplexed protein. However, the aromatic region of the spectrum of the protein-A–Fc-fragment complex consists of broad resonances by comparison with the spectra of the two components alone. Thus the aromatic amino acid residues of protein A, which are known to be in regions of greater order than most of the aliphatic residues, as shown above, become further immobilized on complex-formation. This suggests that the sites on the

protein-A molecule responsible for Fc-binding are highly structured and contain most of the aromatic amino acids in the molecule. These sites on protein A appear to be separated by regions of extensive mobility, which is retained in the protein-A–Fc-fragment complexes.

It is difficult to draw any conclusions about the effect of complex-formation on the Fc fragment, because the spectrum of the Fc fragment alone consists of relatively broad lines which cannot be easily recognized among the sharper protein-A resonances. The situation is made even less favourable, since the Fc-fragment resonances appear to be further broadened on complex-formation. This difficulty can be avoided by the use of the univalent fragment VI'', since cross-linking of the Fc fragments, and hence line broadening of the spectrum, does not occur.

The aromatic region of the 270 MHz proton n.m.r. spectrum of the Fc fragment (0.2 mM) in the presence of excess fragment VI'' (1.4 mM) is shown in Fig. 5(b), along with the spectra of fragment VI'' alone (Fig. 5a) and the Fc fragment alone (Fig. 5d). Fig. 5(c) shows the spectrum which results from the subtraction of that of the fragment VI'' alone from that of the fragment VI''/Fc-fragment mixture. A comparison of this spectrum with that of the Fc

Fig. 4. Proton n.m.r. spectra (270 MHz; $T = 303\text{ K}$) of: (a) Fc fragment of rabbit IgG (1.0 mM in $^2\text{H}_2\text{O}$, pH 4.5); (b) Fc fragment with 0.5 mM-protein A in $^2\text{H}_2\text{O}$, at the same pH; (c) protein A alone, at the same pH in $^2\text{H}_2\text{O}$

Fig. 5. Proton n.m.r. spectra (270 MHz; $T = 303\text{ K}$) of: (a) fragment VI" of protein A (1.5 mM in $^2\text{H}_2\text{O}$, pH 4.3); (b) fragment VI" with the Fc fragment of rabbit IgG (0.2 mM in $^2\text{H}_2\text{O}$, pH 4.3); (c) spectrum (a) subtracted by computer from spectrum (b) (d); Fc fragment (0.2 mM in $^2\text{H}_2\text{O}$) alone, pH 4.3

fragment alone shows differences in the regions of the two resonances assigned to the single tyrosine residue of the fragment VI". The broadening of these resonances supports the hypothesis of Sjöholm (1975) that this tyrosine residue may be involved in the site that binds the Fc fragment. However, the most notable feature of Fig. 5(c) is its marked overall resemblance to the spectrum of the Fc fragment alone, indicating that no large conformational changes occur in the Fc fragment on binding of fragment VI". This implies that the circular-dichroism changes observed by Sjöholm (1975) on the interaction of protein A and Fc fragment may arise from aromatic chromophores directly involved in the binding.

Proton relaxation enhancement. The binding of paramagnetic ions to proteins can be followed by

the enhancement of the spin-lattice relaxation rate of the solvent-water protons (Dwek, 1973). In this way the Fc fragment of non-immune rabbit IgG has been shown to have two equivalent Gd(III)-binding sites, with a dissociation constant of approx. $5\text{ }\mu\text{M}$ (Dower *et al.*, 1975). The addition of protein A (0–300 μM) to a solution of Gd(III) (50 μM) does not result in significant changes in the water proton relaxation rate, indicating extremely weak binding of Gd(III) to protein A. Gd(III) can therefore be specifically bound to the Fc fragment, and used as a probe of the Fc fragment on addition of protein A. The addition of protein A up to stoichiometry did not change the enhancement of the water proton relaxation rate by the Gd(III)-Fc-fragment complex, suggesting that no large conformational changes in the Fc fragment appear to be caused by protein A binding.

Neither the high-resolution n.m.r. nor the proton-relaxation-enhancement studies therefore show evidence of conformational changes in the Fc fragment on binding protein A and fragment VI", suggesting that the activation of component C1 by Fc-fragment-protein-A complexes may simply be a result of the cross-linking of the Fc fragments. This is supported by the inability of the univalent protein-A fragment to activate complement fixation by rabbit IgG. Although the primary event triggering complement fixation is cross-linking, it is of course possible that this leads to secondary changes in the Fc-fragment structure.

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