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Formation of complement subcomponent C1q–immunoglobulin G complex

Thermodynamic and chemical-modification studies

Ezekiel J. EMANUEL, Anthony D. BRAMPTON, Dennis R. BURTON* and Raymond A. DWEK
Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

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The interaction between the complement subcomponent C1q and immunoglobulin G was investigated under a variety of experimental conditions. Formation of the subcomponent C1q–immunoglobulin G complex was shown to be an equilibrium process. Thermodynamic studies of the effect of varying the ionic strength indicate that over the salt range 0.15–0.225 M-NaCl the binding of subcomponent C1q to immunoglobulin aggregates releases 9–12 salt ions (Na^+ and/or Cl^-), illustrating the importance of ionic interactions for the formation of the complex. The effects of small peptide and organic ion inhibitors support this conclusion. Chemical modification of carboxylate residues on immunoglobulin G by glycine ethyl ester/water-soluble carbodi-imide (up to 12 residues modified per whole molecule of immunoglobulin G) and of lysine residues by acetic anhydride (3 residues per whole molecule of immunoglobulin G) or methyl acetimidate (19 residues per whole molecule of immunoglobulin G) lowered the binding affinity of immunoglobulin for subcomponent C1q. Modification of arginine residues by cyclohexane-1,2-dione (14 residues per whole molecule of immunoglobulin G) and of tryptophan by hydroxynitrobenzyl bromide (2 residues per whole molecule of immunoglobulin G), however, had little or no effect. The results are consistent with the proposal that the subcomponent-C1q-binding site on immunoglobulin G is to be found on the last two β-strands of the $C_\gamma 2$ domain [Burton, Boyd, Brampton, Easterbrook-Smith, Emanuel, Novotny, Rademacher, van Schravendijk, Sternberg & Dwek (1980) *Nature* (*London*) **288**, 338–344].

Antibodies are the basis of the body's defence against infection. The organization of the antibody molecule into domains allows recognition of an enormous range of antigens by the variable part of the molecule, and the constant (C) portion activates a small number of effector systems culminating in antigen immobilization and elimination. The most thoroughly characterized of these effector systems is the classical complement system.

The triggering event in the classical complement system is the binding of the first component of complement, C1, to the Fc region of aggregated immunoglobulin molecules. C1 is a macromolecular complex consisting of three subcomponents, C1q, C1r and C1s. The C1q subcomponent (mol.wt. 460000; K. B. M. Reid, personal communication) is the protein that actually links the immunoglobulin Fc region with the complement system. Reid & Porter (1976) proposed that C1q has a structure appearing like a bouquet of six tulips. C1q is composed of 18 peptide chains each some 200 amino acid residues long, linked by disulphide bridges into six units of threes at the base. The stalk region forms a collagen-like triple helix and is capped by a globular head region, which is thought to contain the Fc-binding site.

Abbreviations used: the nomenclature of the complement components and subcomponents is that recommended by the World Health Organisation (1968). IgG, immunoglobulin G; Fab fragment, *N*-terminal half of heavy chain and whole of light chain joined by inter-heavy-chain disulphide bond; Fc, *C*-terminal half of heavy-chain dimer; pFc', *C*-terminal quarter of heavy-chain dimer (Fc consists of two $C_\gamma 2$ and two $C_\gamma 3$ domains and the pFc' of two $C_\gamma 3$ domains); C1q, complement subcomponent C1q.

* Present address: Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

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The crystallographic structure of human Fc fragment has been determined to 0.35 nm (3.5 Å) by Huber *et al.* (1976), as has rabbit Fc fragment (B. Sutton & D. C. Phillips, unpublished work). The overall structure has been described as a 'Mickey Mouse', with the $C_\gamma2$ domain forming the ears and the $C_\gamma3$ domains the head. The immunoglobulin fold is the tertiary structure of both domains. The $C_\gamma3$ pair of domains is in close contact with each other [Fab C-like pairing (Beale & Feinstein, 1976)], but the $C_\gamma2$ domains show no similar interaction with each other. There is an asparagine-linked carbohydrate attached to asparagine-297 on the $C_\gamma2$ domain.

Porter and co-workers have shown that the C1q-recognition site on IgG is in the $C_\gamma2$ domain (Colomb & Porter, 1975). We have previously postulated a receptor site on this domain for the C1q molecule. This site is on the two *C*-terminal β-strands of the $C_\gamma2$ domain of IgG. An important feature of this site is its large number of charged amino acid residues. This proposal was based mainly on an analysis of immunoglobulin sequence conservation and accessibility of amino acid residues to solvent (Burton *et al.*, 1980). Some preliminary results supporting this site were obtained from studies of inhibitors of the formation of the C1q–IgG complex and chemical modification of amino acid residues on IgG and C1q. In the present paper we extend our previous experimental studies to the thermodynamics of the formation of the C1q–IgG complex, and also the effect of chemical modification of five different types of residues on the IgG molecule on the formation of the complex. The results reported here support our postulated receptor site and confirm the significant contribution of ionic interactions to C1q–IgG binding.

Materials and methods

Chemicals and reagents

Methyl acetimidate (Pierce Chemical Co.), cyclohexane-1,2-dione (98%; Aldrich Chemical Co.), acetic anhydride (BDH Chemicals), 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (water-soluble carbodi-imide; Sigma Chemical Co.), ovalbumin (grade V; Sigma Chemical Co.), pepsin (Worthington Biochemical Corp.), dinitrophenyl compounds (BDH Chemicals) and gelatin powder (Reeve Angel Scientific Ltd.) were all purchased from the specified chemical companies. Small peptides were gifts from Professor Dennis Stanworth, University of Birmingham, Birmingham, U.K. All other chemicals and reagents were analytical grade.

Antibody production and purification

New Zealand White rabbits were given primary immunizations with ovalbumin emulsified in Freund's complete adjuvant (1:1) into each hind footpad and shoulder muscle. The secondary immune response was elicited 28 days after the primary injection by an intraperitoneal injection of 5 mg of alum-precipitated ovalbumin. Bleedings were taken on days 4, 6, 8 and 10 after secondary immunization. Rabbit IgG was purified on a Sephadex G-200 column and a Whatman DE-52 DEAE-cellulose column (Fanger & Smyth, 1972).

Antibody peptic fragments

Pepsin digestion of rabbit IgG and isolation of $F(ab')_2$ and pFc' fragments on an Ultrogel AcA-44 column in 0.1 M-sodium acetate buffer, pH 5.0, were done essentially as described by Dower *et al.* (1975).

Precipitin reactions

A precipitin curve was obtained with all antibody samples by adding increasing amounts of ovalbumin to constant amounts of IgG. IgG/ovalbumin mixtures were incubated for 1 h at 37°C and then overnight at 4°C. The precipitates were spun down, washed and suspended in 0.5 M-NaOH. The absorbance at 280 nm was used to measure protein concentration (absorption coefficient $A^{1\%}_{280} = 1.4$). Aggregates used in C1q-binding studies were made at equivalence and stored in phosphate-buffered saline (150 mM-NaCl/20 mM-sodium phosphate), pH 7.2 at 4°C.

Human C1q

The C1q was purified from human serum (Reid, 1974) and radiolabelled with ^{125}I (Heusser *et al.*, 1973).

C1q–IgG aggregate binding assay

The C1q-binding assay was essentially as previously described (Lin & Fletcher, 1978), with the following modifications: instead of bovine serum albumin, 0.15% gelatin was added; incubation time was 45 min; total volumes were 400 μl. In inhibition studies, the inhibitor was added to IgG aggregates before the final C1q addition. The IgG aggregates were present at 240 nM, and C1q at 20 nM.

Amino acid analysis of IgG

All amino acid analyses were performed as described by Reid (1974). For cyclohexane-1,2-dione-modified IgG 20 μl of mercaptoacetic acid was added to prevent regeneration of arginine residues (Patthy & Smith, 1975*a*). For carboxylate-modified IgG the extent of modification was calculated from the increase in glycine residues. All amino acid residue values were calculated assuming a value of 84 leucine residues per IgG molecule (Fleischman *et al.*, 1963).

C.d. spectra

The c.d. spectra were obtained as described by Clark *et al.* (1981), except that the wavelength span was 200–340nm.

2,4,6-Trinitrobenzenesulphonic acid assay for lysine residues

The protein was placed in 0.5ml of water, 0.5ml of 4% (w/v) $NaHCO_3$ at pH9.5 and 0.5ml of 10% (w/v) sodium dodecyl sulphate were added and then this sample was incubated at 37°C for 20min. To this 0.5ml of 0.05% 2,4,6-trinitrobenzenesulphonic acid (recrystallized) was added and the sample was incubated at 37°C for 2h. To this mixture 0.25ml of 1M-HCl was added. The absorbance was monitored at 335nm, the absorption coefficient of 2,4,6-trinitroaniline being taken as $1.1 \times 10^4 M^{-1} \cdot cm^{-1}$.

Acetic anhydride modification of IgG

The IgG was suspended in sodium acetate buffer, pH5.0–5.5, at 0°C for 1h. The reaction was run with a 10–1000-fold molar excess of acetic anhydride over IgG. The modified protein was dialysed against phosphate-buffered saline, pH7.2, and then stored at 4°C. The extent of modification was determined by the 2,4,6-trinitrobenzenesulphonic acid assay.

Methyl acetimidate modification of IgG

IgG was suspended in 0.1M-triethanolamine/HCl buffer, pH8.05–8.15, at room temperature. Methyl acetimidate was added to a final concentration of between 100μM and 2.5mM. After 45min the reaction was terminated by lowering the pH to below 8.0. IgG was dialysed against phosphate-buffered saline, pH7.2, and stored at 4°C. The extent of modification was determined by the 2,4,6-trinitrobenzenesulphonic acid assay.

Cyclohexane-1,2-dione modification of IgG

This chemical modification of arginine residues was performed as described by Patthy & Smith (1975*a*) at pH8.9 for 15–60min. The protein was stored in 20mM-sodium acetate buffer, pH5.5. Regeneration was performed as described by Patthy & Smith (1975*b*). The extent of modification was determined by amino acid analysis.

Glycine ethyl ester/water-soluble carbodi-imide modification of IgG

IgG was prepared in 1mM-glycine ethyl ester at pH4.75 at room temperature; a 50-fold molar excess of water-soluble carbodi-imide over IgG was added. The reaction was terminated by addition of 1M-sodium acetate buffer, pH3.6. The modified IgG was dialysed against phosphate-buffered saline, pH7.2, and stored at 4°C. The extent of modification was determined by amino acid analysis.

2-Hydroxy-5-nitrobenzyl bromide modification of IgG

This chemical modification and assay for the extent of tryptophan modification were performed as described by Allan & Isliker (1974*a*). The modified protein was stored in phosphate-buffered saline, pH7.2, at 4°C. The extent of modification was determined from the molar absorption coefficient $\varepsilon_{410} = 1.8 \times 10^{-4} M^{-1} \cdot cm^{-1}$. The concentration of the modified protein was obtained from Lowry and/or biuret assays, which were standardized with non-immune IgG.

C1q binding to chemically modified IgG

Ovalbumin–IgG aggregates were formed at equivalence and stored in phosphate-buffered saline, pH7.2, at 4°C. Increasing amounts of C1q were added to constant amounts of aggregates. IgG was present at 36μg/ml, the C1q at between 1μg/ml and 20μg/ml.

Analysis of C1q-binding data

The data for C1q binding to IgG aggregates were fitted either to a non-linear regression program (Duggleby, 1981) with an equation of the form:

$$C1q_{Bound} = \frac{Capacity \times C1q_{Free}}{K_{diss.} + C1q_{Free}} \quad (1)$$

or to a linear regression program with the inverse of this equation. The capacity is defined as the total number of binding sites available for C1q in the immune aggregates.

Results

Effect of inorganic salts on the formation of the C1q–IgG complex

The binding of C1q to IgG aggregates was optimal at 170mM-NaCl. Higher concentrations of the salt inhibited the interaction. Testing of other inorganic salts, e.g. NaSCN, NaI and NaF, revealed that there was a significant variation in the inhibition with the variation of anions [Fig. 1(*a*) and Burton *et al.* (1980)]. This inhibition correlates with the empirical Hofmeister or lyotropic series, in which those anions best at dissociating and denaturing proteins are best at inhibition of protein interactions $SCN^- > I^- > Cl^- > SO_4^{2-} > F^-$ (Record *et al.*, 1978; Jencks, 1969). Similarly we examined the ability of cations to inhibit the C1q–IgG interaction. The cation effects are much smaller than the anion effects, although bivalent cations were slightly better inhibitors than univalent cations (Fig. 1*b*). This is consistent with observations on other protein systems (Record *et al.*, 1978).

The inhibition by inorganic salts of the C1q–IgG interaction is reversible. The inhibition of the

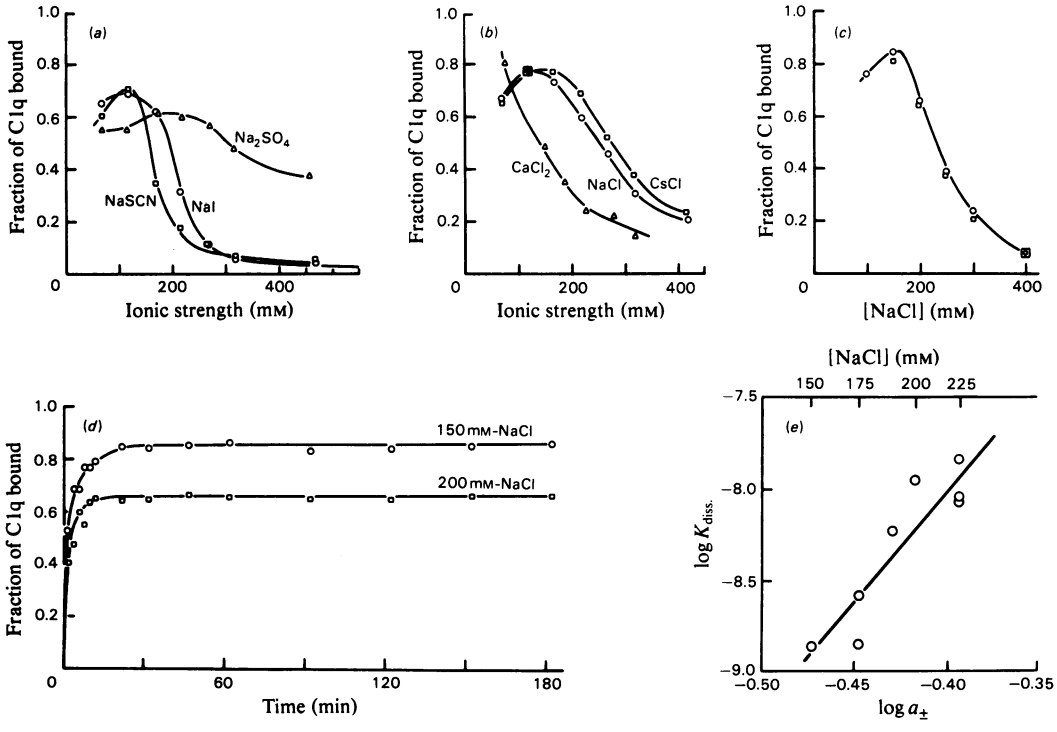

Fig. 1. *Effects of inorganic ions on the formation of the C1q–IgG complex*

(*a*) Inhibition by anions: NaI (○), NaSCN (□) and Na_2SO_4 (△). (*b*) Inhibition by cations: NaCl (○), CsCl (□) and $CaCl_2$ (△). (*c*) Reversibility of inhibition. The control samples (○) were incubated at 37°C for 45 min with various concentrations of NaCl. The test samples (□) were obtained by initial incubation with 400 mM-NaCl at 37°C for 45 min followed by dilution, with buffer, to the appropriate NaCl concentration. (*d*) Time course of formation of the complex. All protein samples were incubated at 37°C for 3 h. At appropriate times C1q was added from a stock C1q solution (also at 37°C). NaCl concentrations are 150 mM (○) and 200 mM (□). (*e*) Variation of the logarithm of dissociation constant ($\log K_{diss.}$) of the C1q–antibody-aggregate complex as a function of $a_{\pm}$, the ionic activity of NaCl ($a_{\pm}$ is defined as the square root of the NaCl activity). In (*a*)–(*d*) the IgG aggregates were present at 240 nM and C1q was at 20 nM.

formation of the C1q–IgG complex by 400 mM-NaCl can be overcome by diluting the NaCl (Fig. 1*c*). Further, this salt inhibition does not affect the kinetics of the C1q–IgG interaction, but affects only the position of the final equilibrium (Fig. 1*d*). This interaction proved to be temperature-independent in the temperature range 4–37°C (results not shown). The above data suggest that the C1q–IgG interaction is an equilibrium process with a significant ionic component.

By using various NaCl concentrations, it is possible to determine dissociation constants for C1q–IgG aggregates over a large salt concentration range. The dissociation constants increase with increasing salt concentration (Fig. 1*e*). A quantitative measure of the ionic effect on a macromolecular equilibrium, can be obtained from the following equation:

$$\log K_{obs.} = \log K^0 - \Delta n_{ion} \cdot \log a_{\pm} \qquad (2)$$

In this equation $a_{\pm}$ is the square root of the NaCl activity, Δn_{ion} is the number of ions released per molecule of complex formed, $K_{obs.}$ is the phenomenological equilibrium quotient and K^0 that at unity activity (Record *et al.*, 1978). This equation is equivalent to:

$$\log K_{diss.} = \Delta n_{ion} \cdot \log a_{\pm} - \log K^0 \qquad (3)$$

In this equation $K_{diss.}$ is the observed dissociation constant. Thus a graph of the logarithm of the dissociation constants versus the logarithm of the square root of the NaCl activities affords a measure of Δn_{ion}, the number of ions displaced in the C1q–IgG complex interaction on a per-molecule basis. This analysis assumes that the change in the

number of ions is constant over the range of salt activities. From the slope in Fig. 1 $\Delta n = 12 \pm 2$.

Effect of organic ions and sugars on the formation of the C1q–IgG complex

The ability of a variety of organic ions to inhibit the formation of the C1q–IgG complex was tested. A qualitative measure of the relative potency of the inhibitor is provided by the concentration of the inhibitor that results in a 50% decrease in the concentration of the initial C1q–IgG aggregate complex, i.e. the binding of C1q is inhibited by 50%. The most potent inhibitors were those with charged groups attached to large hydrophobic groups, such as dinitrophenyl-lysine. All aliphatic inhibitors, e.g. 1,4-diaminobutane, whether with charged groups or not, were less potent inhibitors than those with hydrophobic rings (Table 1).

Several sugar moieties were tested as inhibitors. None of the sugars tested showed any significant inhibition (Table 1). A small amount of inhibition may possibly arise from the large increase in viscosity of concentrated sugar solution rather than from inhibition of a specific site on C1q or IgG.

Effect of peptides on the formation of the C1q–IgG complex

We explored the nature of the C1q–IgG interaction by examining the inhibitory effect of four small peptides. Three of the peptides correspond to fragments from human IgG1: peptide A, Thr-Lys-Pro-Arg (residues 289–292), peptide B, Trp-Tyr-Val-Asp-Gly (residues 277–281), and peptide C, Pro-Arg (residues 291–292). The fourth peptide is peptide D, Asp-Ser-Asp-Pro-Arg. Peptide B (with a tryptophan residue) precipitates C1q in the absence of IgG immune aggregates. This precluded consideration of it as an inhibitor of the C1q–IgG interaction. The values of K_{50} are >6 mM for peptide A, 4.5 mM-for peptide C and 2.5 mM for peptide D. In each case the inhibitory ability is not better than for the dinitrophenyl compounds (see Table 1 for comparison).

Effect of amino acid modifications on the formation of the C1q–IgG complex

Detailed characterization of the molecular interaction in the C1q–IgG interaction requires identification of the amino acid residues that participate in

Table 1. *Effects of inorganic ions, organic ions and sugars on the formation of the C1q–IgG complex*

K_{50} indicates the concentration of inhibitor at which formation of the C1q–IgG complex is decreased by 50% from the maximum value. For the inorganic ions NaCl was added to maintain a constant ionic strength of 170 mM. For the polyions and sugars the NaCl concentration was constant at 170 mM. The IgG aggregates were present at 240 nM and C1q at 20 nM. > indicates that a full inhibition curve was not obtained and the value cited is an extrapolation; ⩾ indicates that no inhibitory effect was observed up to the cited concentration. Abbreviation used: Dnp, 2,4-dinitrophenyl. For full experimental details see the text.

Inorganic ions	K_{50} (mM)
NaSCN	170
NaI	200
NaCl	260
Na_2SO_4	450
NaF	>450
KI	175
RbI	200
$MgCl_2$	145
$CaCl_2$	165
CsCl	300
Polyions	
Polylysine	<0.1
Suramin	3.5
Heparin	1.0
Sugars	
Galactose	>370
N-Acetylglucosamine	450
Raffinose	575
Xylose	575
Fucose	600
Glucose	>600
Mannose	700
Glucose 6-phosphate	⩾19
Lactate	⩾18

Organic ions	K_{50} (mM)
2-*N*-(*p*-Nitrophenyl)-2-aminoethyl phosphate	2.0
2-*N*-(Dnp)-aminoethyl phosphate	2.5
Dnp-lysine	2.5
Dnp-glycine	12.5
Dnp-leucine	>22.5
Dnp-OH	⩾10.5
2,4-Dinitronaphthol-7-sulphonate	2.0
ATP	10.0
ADP	9.0
MnATP	11.0
AMP	13.0
Adenosine	⩾12.0
1,4-Diaminobutane	55.0
Glycine	⩾50.0

the binding. There have been many reports of the effects of chemical modification of residues on IgG and various IgG fragments on the binding of C1q. Many of these experiments were done without adequate controls for the integrity of the molecular conformation after chemical modification. We have tested the effect of modification of five types of residues on IgG for the formation of the C1q–IgG complex. Our chemical-modification experiments contained four levels of control to ensure that the functional changes examined were related to the formation of the C1q–IgG complex and not to non-specific conformational or configurational changes: (1) all experiments contained a control sample of IgG obtained from the same batch of immune IgG; (2) the control samples were processed identically with the modified samples except for the addition of modifying reagents; (3) the precipitin curves obtained for both control and modified IgG had to be identical up to the equivalence point to ensure that the modification had not altered either the antigen-binding site or possible Fc–Fc interactions (Rodwell *et al.*, 1980); (4) the IgG was subject to c.d. measurements to monitor conformational integrity after modification (Dorrington & Smith, 1972; Cathou & Dorrington,

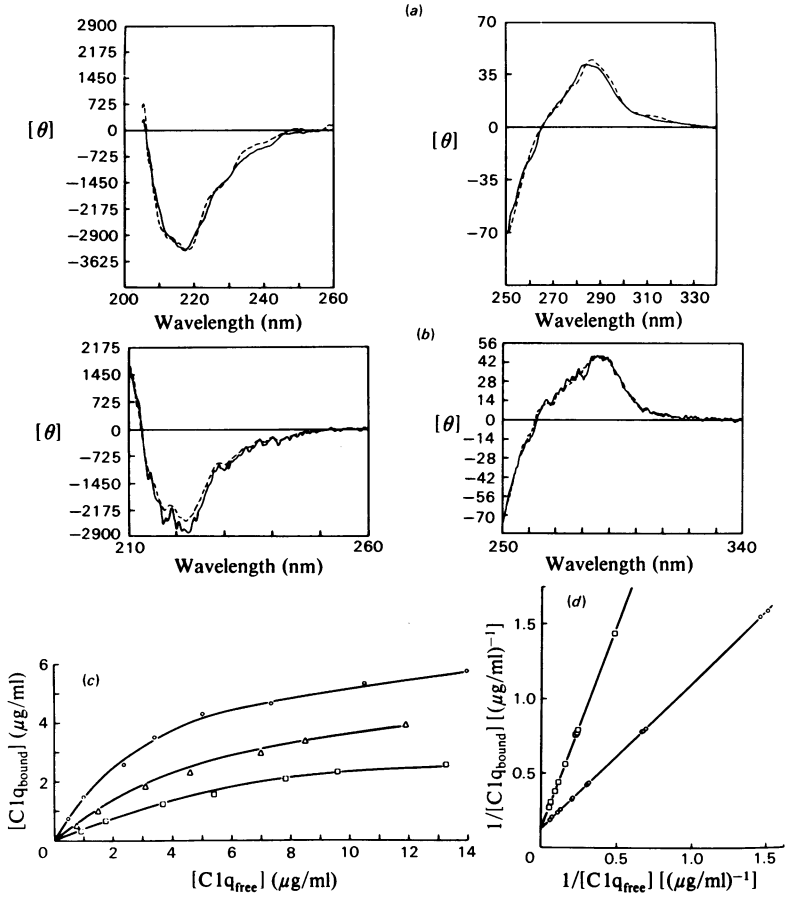

Fig. 2. *Effects of modification of carboxy groups and lysine residues on the c.d. spectra and on C1q binding*
(*a*) and (*b*) show the c.d. spectra ([θ] = molar ellipticity) of normal (——) and modified (– – – –) anti-ovalbumin IgG. In (*a*) 11 ± 1 carboxy groups per molecule were modified with water-soluble carbodi-imide and glycine ethyl ester. In (*b*) 2.18 ± 0.32 lysine residues per molecule were modified with acetic anhydride. (*c*) and (*d*) are the results of C1q binding to aggregates from the modified IgG. In (*c*) the results are shown for the control sample (○), and for those with 6 ± 1 (△) or 12 ± 2 (□) carboxy groups modified per IgG molecule. In (*d*) the results are again for the control sample (○) and for one with 3 ± 1 lysine residues modified per IgG molecule (□). The curves are computed in (*c*) and (*d*) as described in the Materials and methods section. The two methods of plotting emphasize the decreases in the capacity in each case in (*c*).

Table 2. *Changes in $K_{diss.}$ and relative capacity on binding of C1q to modified aggregates*
The modifications were performed as described in the Materials and methods section. In each case the control and modified samples were processed in an identical manner except for the addition of modifying reagent. The C1q-binding assay was performed on anti-ovalbumin immune aggregates. The $K_{diss.}$ and relative capacity are derived from the plots of eqn. (1) or its inverse (see Figs. 2*c*, 2*d*, 3*a*, 3*b* and 3*c*). The modified IgG samples are as follows: carboxylate is glycine ethyl ester/water-soluble carbodi-imide; MAI is methyl acetimidate; AA is acetic anhydride; DHCH is cyclohexane-1,2-dione; HNBB is 2-hydroxy-5-nitrobenzyl bromide.

Type of aggregate	Extent of modification (residues/molecule)	$K_{diss.}$ (M^{-1})	Relative capacity
Carboxylate control IgG	—	7.95×10^{-9}	
Carboxylate-modified IgG	6 ± 1	1.85×10^{-8}	22% decrease
Carboxylate-modified IgG	12 ± 1	1.72×10^{-8}	34% decrease
MAI (lysine) control IgG	—	6.22×10^{-9}	
MAI (lysine) modified IgG	19 ± 1	2.33×10^{-8}	No change
AA (lysine) control IgG	—	1.9×10^{-8}	
AA (lysine) modified IgG	3 ± 1.5	5.5×10^{-8}	No change
DHCH (arginine) control IgG	—	3.1×10^{-9}	
DHCH (arginine) modified IgG	14 ± 1	3.2×10^{-9}	15% decrease
HNBB (tryptophan) control IgG	—	1.7×10^{-8}	
HNBB (tryptophan) modified IgG	2	1.1×10^{-8}	10% increase

1975; Stewart *et al.*, 1977). Duplicate controls are provided for the carboxylate- and acetic anhydride-modified IgG (Figs 2*a* and 2*b*). Similar results were obtained for all of the modified samples. In all cases the effects of chemical modifications on C1q binding to the IgG were measured by the changes in binding affinity and the capacity relative to the control.

Modification of the carboxylate residues

With 12 carboxylated residues per whole molecule of IgG modified the binding affinity of C1q to immune aggregates is lowered 3.5-fold. A non-linear regression analysis of the binding data revealed a 33% decrease in the capacity of the modified aggregates compared with the control aggregates. Six modified carboxylated groups per whole molecule of IgG lowered the binding affinity 2-fold, with a 22% decrease in capacity (Fig. 2*c* and Table 2).

Modification of lysine residues

In IgG modification of lysine residues by acetic anhydride significantly affected C1q binding to IgG (analysed by a linear regression). Modification of 3 ± 1.5 lysine residues in the whole molecule of IgG by acetic anhydride, which eliminates the charge of the amino group, results in a decrease in binding affinity of 2.9-fold without a change in relative capacity (Fig. 2*d* and Table 2). In Fig. 3(*a*) and Table 2 the methyl acetimidate-modified aggregates show a decrease of 3.7-fold in binding affinity for C1q. This effect in the binding affinity exists even though the positive charge on the lysine residues is not destroyed by the modification (Fig. 3*a*).

Modification of arginine residues

Cyclohexane-1,2-dione reacts specifically, and reversibly, with arginine residues (Patthy & Smith, 1975*a*). Modification of 13–14 arginine residues per whole molecule of IgG produced no significant change in binding affinity and a 15% decrease in the capacity compared with the control (Fig. 3*b* and Table 2). Regeneration of the native arginine residues, by treatment with hydroxylamine for 12h at 37°C (Patthy & Smith, 1975*b*), resulted in no change in the precipitin curve and C1q-binding affinity compared with the control IgG (Fig. 3*b*).

To determine the number of modified arginine residues in the $C_\gamma 2$ domain we cleaved the modified IgG with pepsin. Since the cyclohexane-1,2-dione modification is stable in acetate buffers at pH values below 6.0 (Patthy & Smith, 1975*a*), this enzyme treatment and purification of the fragments on an Ultrogel AcA-44 column equilibrated in sodium acetate buffer should not have regenerated any of the modified arginine residues. After isolation of both the $F(ab')_2$ and pFc' fragments, the fragments were subject to amino acid analysis to determine the number of modified arginine residues. Table 3 indicates that the $F(ab')_2$ fragment had four modified arginine residues, and that the pFc' fragment had two to three modified residues. Thus of the 13–14 modified arginine residues per molecule between seven and eight were in the $C_\gamma 2$ domain. Therefore over half of the 14 arginine residues in the $C_\gamma 2$ dimer of rabbit IgG were modified with cyclohexane-1,2-dione with little or no effect on the C1q-binding affinity but with a 15% decrease in relative capacity.

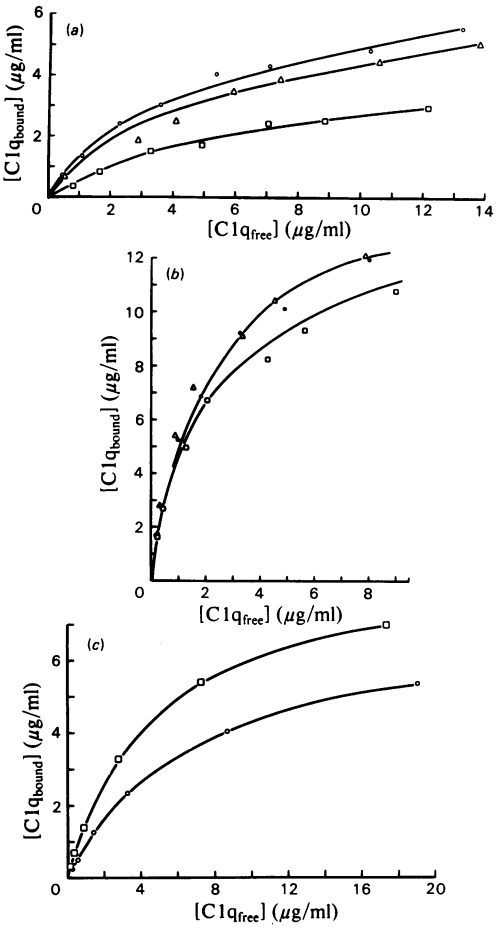

Fig. 3. *C1q binding by chemically modified IgG aggregates*

(*a*) Methyl acetimidate-modified IgG aggregates. Control IgG (○) and 4 ± 1 (△) and 19 ± 1 (□) modified lysine residues per molecule of IgG. (*b*) Cyclohexane-1,2-dione-modified IgG aggregates. Control IgG (○), IgG regenerated after modification (△) and 14 ± 1 (□) modified arginine residues per molecule of IgG. (*c*) 2-Hydroxy-5-nitrobenzyl bromide-modified IgG aggregates. Control IgG (○) and 2 (□) modified tryptophan residues per molecule of IgG. The modification procedures, extents of modification, C1q-binding assays and analysis of binding data are all as described in the Materials and methods section.

Modification of tryptophan residues

To test the importance of tryptophan residues for the C1q-binding site, we modified rabbit IgG with 2-hydroxy-5-nitrobenzyl bromide. Two tryptophan residues per whole molecule of IgG were modified. This slight extent of modification resulted in a small enhancement of C1q binding and a small increase in relative capacity (Fig. 3*c*).

Discussion

This study is an attempt to define in a quantitative manner at the molecular level the interactions involved in the formation of the C1q–IgG complex. Three types of analysis have been used. Firstly, an important ionic contribution to the interaction was established through the effects of inorganic ions on C1q–IgG interaction. This was largely confirmed in the second part by the use of sugars and small peptides as inhibitors. Thirdly, the effects of chemical modifications of selected amino acid residues on IgG was studied to establish the nature of the residues involved in the C1q—IgG interaction.

The assay system of ovalbumin–IgG immune aggregate binding to soluble C1q used in the present studies has several advantages over the systems previously used to study the formation of the C1q–IgG complex. Firstly, formation of these aggregates does not require extremes of temperature, solvents or cross-linking reagents. Possible denaturing effects, conformational distortions or blocking of amino acid residues through heating (Lin & Fletcher, 1978), dioxan precipitation (Allan & Isliker, 1974*b*) or glutaraldehyde cross-linking have therefore been avoided. Such treatments can affect C1q binding by destroying the C1q-binding sites on IgG or by altering the relative disposition of the Fc portions of the IgG molecules so as to diminish the multivalent binding of C1q.

Secondly, we used immune aggregates with unmodified and modified residues on IgG to assay the formation of the C1q–IgG complex to determine quantitative binding constants over a wide range of C1q concentrations. Most other investigators determine binding constants by using fragments of IgG (rather than immune aggregates) and assay for inhibition of either C1q–IgG binding or complement fixation (Lee & Painter, 1980). However, such studies often depend on small changes in the assay systems. The trypsin-digest fragment of pFc′ fragment, at $C_\gamma 3$, used by Painter *et al.* (1981) to inhibit the C1q–IgG interaction, has a binding constant of 2×10^4–4×10^4 M, comparable with that for monomer IgG. This result, however, is an extrapolation from very small changes in the fraction of C1q bound to IgG aggregates. This change is near the limit of sensitivity of the assay system used, and therefore binding constants determined in this manner must be viewed with extreme caution.

Thirdly, many other investigators interpolate C1q-binding affinity from assays of C1 consumption or fixation of whole complement (see, e.g., Vivanco-Martinez *et al.*, 1980). However, inter-

Table 3. *Distribution of arginine and of cyclohexane-1,2-dione-modified arginine residues in rabbit IgG and its peptic fragments*

The cyclohexane-1,2-modified rabbit IgG was digested by pepsin; the peptic fragments were purified at low pH to prevent regeneration of modified arginine residues, and the fragments were then subjected to amino acid analysis with 20 μl of mercaptoacetic acid to determine the number of modified arginine residues in the $C_\gamma 2$ domain.

IgG	42.0 ± 0.1*
Modified IgG	28.2 ± 0.2
No. of modified arginine residues in IgG	13.8 ± 0.3
Regenerated IgG	41.8 ± 0.3
$F(ab)_2$	20.0 ± 0.1
$(Fab')_2$ from modified IgG	15.8 ± 0.4
No. of modified arginine residues in $(Fab')_2$	4.2 ± 0
Regenerated $(Fab')_2$	18.8 ± 0.2
pFc	8.0†
pFc' from modified IgG	5.6 ± 0.1
No. of modified arginine residues in pFc'	2.4 ± 0.1
Regenerated pFc'†	8.0 ± 0.2
$(C_\gamma 2)_2$	14.0†
No. of modified arginine residues in $(C_\gamma 2)_2$	7.2 ± 0.7‡

* Average of all rabbit IgG amino acid determinations, assuming 84 leucine residues per IgG molecule (Fleischman *et al.*, 1963).

† The numbers of arginine residues in the pFc' and $C_\gamma 2$ domains were obtained from Dayhoff (1978). Peptic fragments of modified IgG were regenerated by the method of Patthy & Smith (1975*b*).

‡ Determined by difference between modified IgG and its peptic fragments.

preting effects on C1q binding from changes in either anti-complementary activities or inhibition of erythrocyte lysis have proved misleading in the past. Allan & Isliker (1974*a*,*b*) have shown that modification of tryptophan residues does not affect C1q binding, but results in marked anti-complementary activity. Similarly Hughes-Jones and co-workers conclude that 'there was no correlation between the extent of [C1] activation and the values of functional affinity constants for the reaction between C1q and the C1 binders' (Folkerd *et al.*, 1980). This serves to highlight the necessity of studying C1q binding directly to IgG immune aggregates if conclusions about the molecular interactions of C1q and IgG are to be drawn.

Fourthly, immune aggregates are used directly in the present studies rather than assays that are based on modified IgG or IgG fragments, and therefore the problem of undetected aggregates is avoided. Aggregates are more efficient than monomers at binding C1q (Wright *et al.*, 1980). Chemical modifications of IgG or its fragments may dramatically alter aggregation properties of the sample. These changes in aggregation properties can in turn dramatically change the efficiency of C1q binding. For example, if IgG at 3 mg/ml is titrated in as an inhibitor in a C1q–IgG binding assay, and if we assume aggregation occurs to dimers, which have a binding constant of $10^6 M^{-1}$, then only 1% aggregation could account for a $2 \times 10^4 M^{-1}$ binding constant of the sample. Performing the C1q–IgG binding assay with immune aggregates avoids the problem of aggregation.

We have shown that the effects of inorganic ions on the formation of the C1q–IgG complex parallel the lyotropic series in both anions and cations. Binding affinity increases with a decrease in salt concentration. According to Record *et al.* (1978), 'The observed equilibrium quotient (and extents of complex formation) increases with a decrease in the activity of electrolyte MX' when 'non-covalent association reactions are accompanied by the release of large numbers of low molecular weight ions'. Hydrophobic interactions show the opposite relation between electrolyte activity and binding constants. Thus, from the above data, we conclude that the C1q–IgG interaction contains a significant ionic component, and is not simply a result of shielding exposed hydrophobic regions as has been proposed (Dorrington, 1978).

In an attempt to quantify the number of ions involved in the C1q–IgG aggregate interaction, we plotted $\log K_{diss.}$ versus $\log a_\pm$ (Record *et al.*, 1978). The slope of this plot gives a measure of the number of ions displaced, Δn_{ion}, on binding of one C1q molecule to the aggregates of IgG. The number is 12 ± 2. This plot was conducted over a small range of NaCl concentrations in the physiological range,

namely 0.15–0.225 M. Therefore extrapolation to all NaCl concentrations may not be valid, since the Δn_{ion} may vary with ion activity, making the plot of $\log K_{diss.}$ versus $\log a_{\pm}$ non-linear. [The implication of this is that all C1q–IgG binding interactions are not identical and may change with NaCl concentration (Lin & Fletcher, 1978; Hughes-Jones, 1977).] However, Hughes-Jones & Gardner (1978) also measure $K_{diss.}$ of the C1q–IgG interaction at several NaCl concentrations extending from 0.11 M to 0.20 M. A re-plot of their data (Fig. 1 of the cited paper) according to eqn. (3) gives a slope of 9 ± 1. It seems reasonable to conclude that formation of the C1q–IgG aggregate complex is accompanied by release of 9–12 ions per C1q molecule bound.

The binding studies on organic compounds confirm that the C1q–IgG interaction is predominantly ionic. The best organic inhibitors tested were those with charged moieties. This is consistent with the inhibition of the formation of the C1q–IgG complex by salts. Thus the inhibitory power of 1,4-diaminobutane (Sledge & Bing, 1973) and heparin (Lin & Fletcher, 1978) in C1q–IgG interaction is probably due to the ionic charges of these molecules.

The $C_\gamma 2$ domain of IgG both binds C1q and contains the asparagine-linked complex carbohydrate of IgG. Thus it is conceivable that the $C_\gamma 2$-domain carbohydrate is involved in the formation of the C1q–IgG complex (Winklehake *et al.*, 1980). To test this hypothesis we assayed the inhibitory potential of several sugar moieties on C1q–IgG binding. The lack of inhibition by any of the sugars tested, including galactose and fucose, indicates that a single sugar moiety is not the recognition site, as in the case of the hepatic galactose receptor (reviewed by Neufeld & Ashwell, 1980). The ability of the purified complex carbohydrate to inhibit C1q–IgG binding was tested and shown to be negligible (van Schravendijk & Dwek, 1981). This agrees with inhibition data published on a peptide that contains the $C_\gamma 2$-domain carbohydrate (Smiley & Horton, 1965). These results suggest that the *N*-linked complex sugar is not involved in the binding site for C1q.

Small peptides have at least two charged termini, and may have additional charged residues. Because of the results indicating the ionic nature of the C1q–IgG interaction, the possibility arose that previous demonstrations of inhibition by peptides (Kehoe & Fougereau, 1969; Waldesbuhl *et al.*, 1970; Johnson & Thames, 1976; Boackle *et al.*, 1979; Lee & Painter, 1980) can be interpreted to be a consequence of either the charged interactions between the peptides and C1q and/or IgG, or the ability of the peptides to precipitate C1q. Indeed, our studies on ionic inhibitors suggest that for many of the peptides used as inhibitors one of the main factors in their inhibition is the charged nature of the peptides rather than their specific sequence. Our experiments on small peptides confirm this. In three cases the K_{50} values of the peptides are similar to those for the dinitrophenyl compounds assayed for inhibition, which bear no similarity to the amino acid residues involved in C1q–IgG interaction. In addition, peptides examined by the investigators cited above, especially those with tryptophan residues, may have anti-complementary activity, because (like peptide B) they precipitate C1q. In either case the peptides' primary sequences need not necessarily correspond to the IgG binding site for C1q. Thus testing peptides for their ability either to bind complement or to inhibit formation of the C1q–IgG complex is unlikely to provide clear evidence for the amino acid residues on IgG to which C1q binds.

In an effort to localize the binding site on IgG, we modified five types of residues under controlled conditions and then examined the effect on the formation of the C1q–IgG complex. Modification of both carboxylate and lysine residues resulted in significant lowering of the binding affinity of C1q for IgG. Thus the C1q-binding site on IgG should contain lysine residues and glutamic acid and/or aspartic acid residues. This is consistent with the results obtained by Vivanco-Martinez *et al.* (1980), who find that carboxylate modification of 16 residues on human Fc region results in a loss of complement fixation. However, they did not test C1q binding directly but inferred the effect on C1q from anti-complementary activity. Although the carboxylate residues are distributed throughout the $C_\gamma 2$ domain of IgG, six out of eight lysine residues in the rabbit $C_\gamma 2$ domain are concentrated in one stretch between lysine-317 and lysine-338. The remaining two lysine residues are close to the $C_\gamma 2/C_\gamma 3$ interface. This lysine-rich region, strands fy2 and fy3 in the Beale & Feinstein (1976) nomenclature (Fig. 4), also contains several carboxylate groups, and thus has been postulated to be the C1q-binding site (Burton *et al.*, 1980).

As regards arginine residues, our results indicate that modification of seven or eight arginine residues in the $C_\gamma 2$ domain, with the large modifying group cyclohexane-1,2-dione, does not have any effect on the formation of the C1q–IgG complex. Five of the seven arginine residues in the rabbit IgG $C_\gamma 2$ domain are on the outside of the molecule, namely strands b1, fx3, b4 and fx4 (this is on the opposite side from strands fy2 and fy3). This region is the area suggested by Brunhouse & Cebra (1979) as a C1q-binding site containing residues 288–293, which in rabbit IgG are Arg-Pro-Pro-Leu-Arg-Glu. Our findings argue against these residues being in the C1q-binding site, since in rabbit IgG (1) lysine residues are probably involved in formation of the C1q–IgG complex and there are no lysine residues

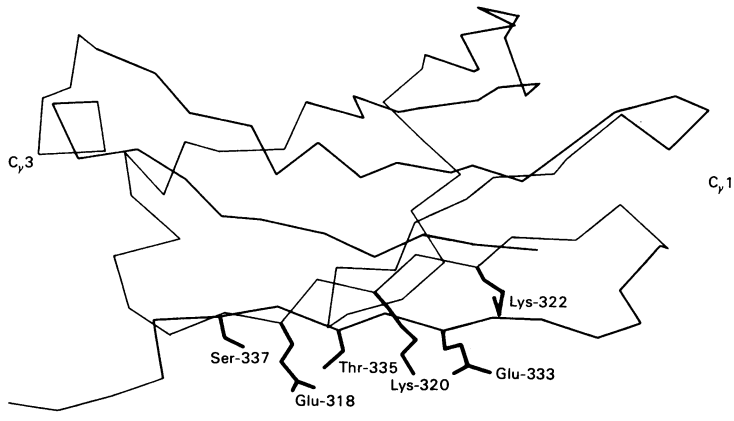

Fig. 4. *Postulated C1q-receptor site on the $C_\gamma 2$ domain of rabbit IgG*

The α-carbon backbone of the $C_\gamma 2$ domain of rabbit IgG on which the side chains of main residues implicated in the C1q-recognition site are indicated. The Figure was obtained from unpublished work by B. Sutton & D. C. Phillips on the rabbit Fc fragment. The locations on the adjacent $C_\gamma 3$ and $C_\gamma 1$ domains are shown (the carbohydrate moiety that is attached to the $C_\gamma 2$ domain is not shown).

in or near this region, and (2) chemical modification of arginine residues would be expected to inhibit formation of the C1q–IgG complex and it does not.

Our results also indicate that modifications of tryptophan residues in the $C_\gamma 2$ domain do not change the C1q–IgG binding affinity. This result is in agreement with Allan & Isliker (1974*a*,*b*), who demonstrated that IgG and Fc fragment with modified tryptophan residues bind C1q normally even though they possess anti-complementary activity. The previous experimental studies that have implicated tryptophan-277 as being in the C1q-binding site on the IgG $C_\gamma 2$ domain (Griffin *et al.*, 1967; Johnson & Thames, 1976; Lee & Painter, 1980) have all assayed complement fixation or anti-complementary activity and not C1q–IgG binding directly. (This controversy over the role of the tryptophan residues must be taken as a warning: studying C1q–IgG interactions on a molecular basis requires assay of C1q binding and not anti-complementary activity or fixation of whole complement.)

Others (Dorrington, 1978; Isenman *et al.*, 1977) have suggested that tryptophan-277 is the centre of the C1q-binding site on the $C_\gamma 2$ domain because of its exposure to solvent. Analysis of residue accessibility to a water molecule by using the X-ray-crystallographic models of rabbit Fc fragment (B. Sutton, personal communication) and of the human Fc fragment (Diesenhofer, 1981) indicates clearly that the whole residue tryptophan-277 has less than 0.05 nm^2 area of total accessibility to solvent. Thus it seems quite clear that tryptophan-277 is not exposed to solvent in the native IgG and therefore is unlikely to be involved in the formation of the C1q–IgG complex, although it may play a yet unspecified role in complement activation.

In summary, the following points are to be made. (1) C1q binding (as such) to IgG immune aggregates must be assayed if functional conclusions are to be drawn about the C1q–IgG interaction. (2) Formation of the C1q–IgG complex is an equilibrium process in which ionic interactions are predominant, and is not entirely a result of hydrophobic shielding. Over the salt range 0.15–0.225 M-NaCl the binding of one molecule of C1q to IgG aggregates releases 9–12 ions. (3) The $C_\gamma 2$-domain carbohydrate is probably not the site for C1q binding. (4) Lysine, glutamic acid and/or aspartic acid residues are in the C1q-binding site on IgG, whereas arginine and tryptophan residues are not. The chemical-modification studies are consistent with the proposal that the C1q-binding site is on the terminal two β-strands of the $C_\gamma 2$ domain of IgG (Burton *et al.*, 1980).

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