Quantitative analysis of the interaction between immune complex and Clq complement subcomponent

The role of interdomain interactions in rabbit IgG in binding of Clq to immune precipitates

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A novel method was developed for the analysis of the interaction of large multivalent ligands with surfaces (matrices) to analyse the binding of complement subcomponent Clq to immune precipitates. Our new evaluation method provides quantitative data characteristic of the Clq-immune-complex interaction and of the structure of the immune complex as well. To reveal the functional role of domain-domain interactions in the Fc part of IgG the binding of Clq to different anti-ovalbumin IgG-ovalbumin immune complexes was studied. Immune-complex precipitates composed of rabbit IgG in which the non-covalent or covalent bonds between the heavy chains had been eliminated were used. Non-covalent bonds were abolished by splitting off the CH3 domains, i.e. by using Facb fragments, and the covalent contact was broken by reduction and alkylation of the single inter-heavy-chain disulphide bond. The quantitative analysis of the binding curves provides a dissociation constant (K) of 200 nM for the interaction between Clq and immune precipitate formed from native IgG. Surprisingly, for immune precipitates composed of Facb fragments or IgG in which the inter-heavy-chain disulphide bond had been selectively reduced and alkylated, stronger binding ($K = 30$ nM) was observed. In this case, however, changes in the structure of the immune-complex matrix were also detected. These structural changes may account for the strengthening of the Clq-immune-complex interaction, which can be strongly influenced by the flexibility and the binding-site pattern of the immune-complex precipitates. These results suggest that domain-domain interactions in the Fc part of IgG affect the segmental mobility of IgG molecules and the spatial arrangement of the immune-complex matrix rather than the affinity of individual Clq-binding sites on IgG.

INTRODUCTION

The initial step of the classical pathway of complement activation is the interaction of immune complexes and the q subcomponent of component C1 (Müller-Eberhard, 1975). Clq binds to the Fc part of complexed IgG. The nature of this interaction has been intensively studied with the aim of localizing the Clq-binding site on the Fc, to identify the residues participating in the interaction (Cohen & Becker, 1968*a,b*; Sledge & Bing, 1973; Allan & Isliker, 1974*a,b*; Isenman *et al.*, 1975; Vivanco-Martinez *et al.*, 1980; Burton *et al.*, 1980; Painter, 1984) and to clarify the conformational background of binding (Isenman *et al.*, 1975; Lin & Fletcher, 1978; Boyd *et al.*, 1979; Liberti *et al.*, 1982). It has been proposed that the CH2 domain of IgG is involved in Clq binding (Augener *et al.*, 1971; Connell & Porter, 1971; Colomb & Porter, 1975; Závodszy *et al.*, 1979); however, the influence of other domains has also been suspected (Ovary *et al.*, 1976).

There is no contact surface between the CH2 domains, which are held together by disulphide bonds in the hinge region of the molecule, but the CH3 domains are connected by strong non-covalent interactions (Huber, 1980, and references cited therein). The elucidation of the role of the four structural domains in Fc and their

interactions in the binding of C1 is of fundamental importance in understanding the mechanism of triggering and regulating the classical complement pathway. It has been suggested that integrity of both covalent and non-covalent bonds between the heavy chains of rabbit IgG is essential for C1 binding to immune complexes (Press, 1975).

Colomb & Porter (1975) have shown that rabbit IgG divested of its CH3 domains (Facb fragment) is characterized by decreased complement-fixing ability (to an extent of 60%) compared with native IgG, as measured by the haemolytic assay described by Ishizaka *et al.* (1965). Much attention has been devoted to the functional significance of the covalent interaction between the two CH2 domains, i.e. of the inter-heavy-chain disulphide bond, but the results obtained in various laboratories are contradictory. The results have been summarized by Johnson & Hoffmann (1981, 1984), and it was concluded that 'the observed functional effect of reduction and alkylation depends strikingly on the assay used to evaluate that effect'. To avoid the pitfalls of indirect evaluation in functional assays use of direct Clq- or C1-binding assays appears to be the solution. There are several studies of this type in the literature (Thompson & Hoffmann, 1974; Hughes-Jones, 1977; Lin & Fletcher, 1978; Leatherbarrow & Dwek, 1984);

Abbreviation used: R/A IgG, IgG in which the inter-heavy-chain disulphide bond is reduced and alkylated.

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however, the lack of an adequate method for quantitative evaluation of the experimental binding data is responsible for the divergence of the results.

In the present study we have examined the effect of both covalent and non-covalent interactions between domains in the Fc piece of the IgG molecule on Clq binding. Elimination of covalent interactions was achieved by reduction of the only inter-heavy-chain disulphide bond in rabbit IgG; non-covalent interactions were abolished by splitting off the CH3 domains, i.e. by using Facb fragments. We measured the binding of Clq to immune complexes. Since the well-known and widely used linearization processes (Keleti, 1986) are applicable for binding of point-like ligands and are meaningless in the case of large ligands (Stankowski, 1983), we developed a novel method for quantitative analysis of interaction of large multivalent ligands with surfaces. Our new evaluation method provides quantitative data characteristic of the Clq-immune-complex interaction and of the structure of the immune complex as well.

MATERIALS AND METHODS

Purification of rabbit anti-ovalbumin IgG

Anti-ovalbumin immunoglobulins were raised in rabbits, and IgG was isolated from pooled rabbit sera by using ovalbumin-Sepharose 4B immunosorbent (Fuchs & Sela, 1978).

Preparation of Facb fragment

The method described by Colomb & Porter (1975) for preparation of Facb fragments of rabbit anti-ovalbumin IgG by plasmin cleavage was followed with minor modifications as described previously (Závodszy *et al.*, 1979). Plasminogen was prepared by the method of Deutsch & Mertz (1970). The plasmin-treated sample was applied to a Protein A-Sepharose CL-4B (Pharmacia) column equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, containing 140 mM-NaCl. The Facb fragment does not bind to the resin, and appears in the first peak mixed with the peptide fragments. Further purification of Facb fragment was achieved by Sephacryl S200 (Pharmacia) chromatography. The homogeneity and molecular mass of the Facb fragment were evaluated by SDS/polyacrylamide-gel electrophoresis and analytical ultracentrifugation.

Reduction and alkylation of the inter-heavy-chain disulphide bond of rabbit anti-ovalbumin IgG

The reduction of IgG was carried out by a slight modification of the method of Palmer & Nisonoff (1964). A 3 ml portion of protein solution (3–30 mg/ml) was incubated in 100 mM-sodium acetate buffer, pH 5.0, containing 30 mM-NaCl and 50 mM-2-mercaptoethylammonium chloride under N₂ atmosphere at 37 °C for 80 min. The mixture was then passed through a Sephadex G-25 column under N₂ atmosphere at pH 5.0 to remove the reducing agent. The reduced IgG was pooled under N₂ into the solution of alkylating agent, either methyl iodide or iodoacetamide (1–10 mM). The excess of alkylating agent was removed by dialysis against 30 mM-NaCl. The homogeneity of samples was examined by SDS/polyacrylamide-gel electrophoresis and by sedimentation-velocity experiments carried out at pH 2.5, where the non-covalently associated 'half' IgG molecules dissociate.

Immune complexes

Anti-ovalbumin IgG-ovalbumin immune-complex precipitates were prepared by mixing antigen (1 mg/ml) and antibody (3–5 mg/ml) in 1:3 molar ratio for intact IgG, R/A IgG or Facb fragment. The mixture was incubated for 1 h at 37 °C and overnight at 4 °C. For Clq-binding experiments the immune-complex precipitates were washed three to six times with phosphate-buffered saline (50 mM-sodium phosphate buffer, pH 7.5, containing 150 mM-NaCl).

The molar ratio of IgG and ovalbumin in the precipitates was determined by two alternative methods: (1) acid-dissociated samples were subjected to SDS/polyacrylamide-slab-gel electrophoresis, and the quantity of proteins in the bands was estimated by densitometry; (2) ¹²⁵I-labelled ovalbumin was used in the preparation of the immune complex, and the amount of ovalbumin in the precipitates was determined from the radioactivity, the total protein being measured spectrophotometrically after dissolution of the precipitates in 0.1 M-NaOH.

Purification of human Clq

Clq was isolated from human serum by a slight modification of the procedure used by Tenner *et al.* (1981). The Clq-containing fractions [selected by immunodiffusion against anti-(human Clq) (Hyland)] eluted from a Bio-Rex 70 column (5.6 cm × 10 cm) were concentrated on an Amicon UM 20 membrane and applied to a Sepharose 6B column (3 cm × 100 cm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.55, containing 200 mM-KCl and 2 mM-EDTA. Clq was eluted as the second peak and was concentrated on an Amicon UM 20 membrane. The purified protein was frozen in liquid N₂ and stored at –18 °C. The purity and homogeneity of Clq samples were checked by SDS/polyacrylamide-gel electrophoresis and analytical ultracentrifugation.

Radioactive labelling of Clq

Clq was labelled with ¹²⁵I with the use of lactoperoxidase according to the method of Heusser *et al.* (1973). The specific radioactivity of the labelled Clq was 3 TBq/mol of Clq.

Clq-binding assay

Radioiodinated Clq (2 µg portions) was added to different amounts of immune precipitates (2–200 µg) thoroughly suspended in phosphate-buffered saline. The final volume was 200 µl. The protein content of added immune precipitates was determined as follows. The immune precipitates were dissolved in 0.1 M-NaOH and their absorption was measured at 280 nm. In the calculations the weighted sum of absorption coefficients of IgG and ovalbumin was used according to their molar ratio found in the precipitates (see the Results section).

The mixtures of Clq and immune precipitates were incubated for 1 h at 37 °C with stirring and then overnight at 4 °C. The samples were then centrifuged, the supernatant was divided into halves, and the radioactivities of the half-supernatant and the precipitate + half-supernatant were counted with a Beckman type 300 γ-radiation counter. The volume of the precipitate was considered to be negligible in the evaluation.

Fig. 1. Representative sedimentation-velocity patterns of IgG samples

All experimental conditions were identical in the three runs, and the exposures were taken 18 min after reaching the maximum rotor speed, 60 000 rev./min. (a) Native IgG at pH 7.5; (b) IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated by methyl iodide, at pH 7.5; (c) sample (b), at pH 2.5.

Analytical ultracentrifugation

Sedimentation-velocity experiments were performed in an MOM type 3170/b analytical ultracentrifuge equipped with Philpot-Svensson and Rayleigh interferometer

optics. The accuracy of speed control was ± 10 rev./min, and the temperature was controlled with an accuracy of 0.1 °C. All measurements were carried out in single-sector polyamide centre pieces, at a rotor speed of 60 000 rev./min, at 293 K temperature. The concentration of the protein solutions was in the range 0.5–10 mg/ml.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out as described by Neville (1971) with 15% and 4% gels for different IgG preparations and Clq respectively. IgG and immune-complex samples were applied on gels without further reduction.

RESULTS

The Clq-binding affinities of immune-complex precipitates containing as antibody the following five IgG samples were measured and compared: (I), native rabbit anti-ovalbumin IgG; (II), Fabc fragment of rabbit anti-ovalbumin IgG; (III), acid-treated IgG; since the preparation of the Fabc fragment includes a step of exposure of IgG to pH 2.3 for 30 min, which may lead to certain irreversible changes in the structure and Clq-binding properties of the molecule, these samples were handled like the Fabc preparations, but with omission of the plasmin digestion, to give a control; (IV), IgG with reduced inter-heavy-chain disulphide bond and alkylated with iodoacetamide (R/A_I IgG); (V), IgG with reduced inter-heavy-chain disulphide bond and alkylated with methyl iodide (R/A_{II} IgG) (a non-polar group with minimum space requirement between the two CH₂ domains). Reduction and alkylation result in a product not differing in sedimentation coefficient from the native

Fig. 2. SDS/polyacrylamide-gel-electrophoresis pattern of rabbit IgG samples

Sample 1, IgG in which all disulphide bonds were reduced; sample 2, acid-treated IgG; sample 3, native IgG; sample 4, IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated by iodoacetamide; sample 5, IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated by methyl iodide. Sample 1 was run in the presence of reducing agent, whereas samples 2–5 were run without it. H and L denote heavy and light chains respectively.

Fig. 3. Clq binding to various ovalbumin-anti-ovalbumin IgG immune-complex precipitates

Increasing amounts of immune complexes were added to constant amount (2 μ g) of 125 I-labelled Clq. The final volume was 200 μ l. Immune complexes were formed from IgG samples as indicated: $\circ$, native IgG; $\bullet$, acid-treated IgG; $\triangle$, Facb fragment; $\square$, IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated by iodoacetamide; $\blacksquare$, IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated by methyl iodide.

molecule at neutral pH (Figs. 1a and 1b). At acidic pH dissociation of the non-covalently bound 'half-molecules' is observed (Fig. 1c) and the dissociation is quantitative, revealing the breakage of practically all the inter-heavy-chain disulphide bonds. The boundary of the sedimenting sample was symmetrical and the $s_{20,w}$ value was decreased to 4.1 S from the 6.9 S value observed at neutral pH. SDS/polyacrylamide-gel-electrophoresis patterns are shown in Fig. 2. The R/A IgG samples were run together with IgG samples where all disulphide bonds had been reduced. These latter samples dissociate into heavy and light chains in SDS, whereas samples containing 'native-like' loops (Fig. 2: R/A_I IgG and R/A_{II} IgG) stabilized by intrachain disulphide bonds run faster than would be expected for a completely reduced molecule of the same mass. 'Selective' reduction of the inter-heavy-chain disulphide bond results (to a

slight extent) in reduction of disulphide bonds between heavy and light chains. Free heavy chain (H) is therefore visible in Fig. 2, samples 4 and 5. From the SDS/polyacrylamide-gel-electrophoresis patterns the amount of 'half-molecules' (H-L) can be estimated quantitatively. According to the densitometric evaluation the H-L 'half-molecules' come to 80% of the total quantity of IgG in the sample.

Comparison of Clq-binding affinities

Increasing amounts of immune-complex precipitates were added to a constant concentration (25 nM) of Clq until no change was observed in the amount of bound Clq. The binding curves are shown in Fig. 3. A small (about 10%) decrease was observed in Clq-binding capacity of immune precipitates containing either Facb fragment or R/A IgG compared with those of native or acid-treated IgG.

The molar ratio of IgG and ovalbumin in the precipitates was found to be between 1:0.7 and 1:1 for all of the preparations except for the IgG sample alkylated by methyl iodide, this latter ratio being between 1:0.4 and 1:0.5. To convert the weight concentrations of immune complexes into molar ones the following molecular masses were used: 195000 Da for immune complexes formed from native IgG, acid-treated IgG and reduced IgG alkylated by iodoacetamide; 170000 Da for immune complexes prepared from Facb fragment, taking the nearest integer value for the antigen/antibody ratio in the calculations; 167000 Da for immune complexes formed from reduced IgG alkylated by methyl iodide.

Since Clq is a large multivalent ligand covering more than one binding site on the surface of the immune-complex matrix, the binding data could not be analysed by conventional methods (Keleti, 1986) developed for univalent point-like ligands. Stankowski (1983) suggested a formalism to treat the adsorption of large univalent ligands on surfaces. Starting from his results we developed a new evaluation procedure for the binding of large multivalent ligands to surfaces, which is presented in detail in the Appendix. With the aid of this method [see eqn. (1) in the Appendix] Clq-immune-complex-precipitate binding curves were analysed. Parameter combinations K/m and n/m were obtained, where K is the dissociation constant, n is the average number of available binding sites per one IgG in the immune-complex matrix, and m is the number of binding sites on the surface of immune matrix covered by one bound

Table 1. Characteristic parameters for immune-complex precipitates and their interactions with Clq

Immune complex formed from	m/n	K (nM) if $m = 6$
Native IgG	8	200
Acid-treated IgG	8	200
Facb fragment	30	30
Reduced IgG alkylated by methyl iodide	38	30
Reduced IgG alkylated by iodoacetamide	45	30

ligand (Clq). On the basis of structural data (Shelton *et al.*, 1972; Turner, 1981) we estimated that one bound Clq molecule might cover about four to eight binding sites on the surface of the immune matrix. For the case of $m = 6$ we calculated the values of K and n ; the latter provides information about the structure of immune complex. The parameters determined for the various immune-complex precipitates are listed in Table 1.

DISCUSSION

The mechanism of the activation of the complement cascade by immune complexes is a complicated procedure. In the first step, i.e. in C1 binding and activation, several factors may be of equal importance. It was suggested by Borsos *et al.* (1980) that the geometric array of IgG on the cell surfaces or in immune complexes is of crucial importance in the generation of C1-binding sites and in the interaction between IgG and Clq. The individual Clq-binding sites should be available (exposed), arranged in a flexible way in the immune complex to provide multiple binding sites (clusters) for the related Clq heads. Interactions among antigen, IgG and Clq are interdependent: intermolecular interactions (Isenman *et al.*, 1975; Press, 1975; Borsos *et al.*, 1980; Tschopp *et al.*, 1980; Wright *et al.*, 1980; Painter, 1984) as well as subtle conformational changes (Jaton *et al.*, 1975; Lancet & Pecht, 1976; Füst *et al.*, 1978; Pecht, 1982) are present in this complex, which all may be of importance in the regulation of C1 activation, and thus the quantitative evaluation of the effects of various contributions to this complicated phenomenon is the only promising approach.

The purpose of the present study was to obtain quantitative data on the interaction between IgG and Clq, and to correlate the Clq-binding affinity of immune complexes with domain-domain interactions within the Fc piece of IgG molecules.

Rabbit anti-ovalbumin IgG complexed with ovalbumin has provided a suitable system to accomplish these objectives, since (a) rabbit IgG contains only one inter-heavy-chain disulphide bond, the reduction of which eliminates covalent contact between the two heavy chains, (b) limited proteolysis of rabbit IgG by plasmin splits off the CH3 domains, resulting in the F_{ab} fragment (Colomb & Porter, 1975), in which the non-covalent contacts between the heavy chains and the longitudinal CH2-CH3 interactions are abolished, and (c) the immune complex formed from anti-ovalbumin rabbit IgG has high affinity for ¹²⁵I-labelled Clq. In the present study we measured the binding of ¹²⁵I-labelled Clq to immune precipitates to avoid the pitfalls of indirect evaluation by haemolytic assay.

There are a number of studies of Clq binding to IgG in solution or to immune complexes, and most authors derive numerical data concerning dissociation constants and so-called 'affinity constants' (Thompson & Hoffmann, 1974; Hughes-Jones, 1977; Lin & Fletcher, 1978; Leatherbarrow & Dwek, 1984). However, these calculations do not consider the large size and multivalent nature of the Clq ligand, therefore the results obtained are not necessarily appropriate.

The method presented here is suitable for the derivation of quantitative and comparable parameters in the case of complicated processes such as the binding of the large multivalent ligand Clq to a matrix, i.e.

immune-complex precipitate. In addition, it supplies information about the structure of the immune complex in connection with the binding affinities.

Parameters characteristic for the structure of immune-complex precipitates and their interaction with Clq were determined from the saturation curves by our novel evaluation method described in the Appendix. The dissociation constant of Clq-immune complex formed from native and acid-treated IgG was found to be 200 nM. Splitting off the CH3 domains or reduction and alkylation of the inter-heavy-chain disulphide bond do not abolish the Clq binding irrespective of the size and polarity of the alkylating group. The lack of either the covalent bond between the two symmetrical halves of Fc or the CH3 domains in the F_{ab} fragment results in about 6-fold decrease in the Clq-immune complex dissociation constant (Table 1). The slightly stronger binding of Clq to immune complex formed from F_{ab} or IgG in which the inter-heavy-chain disulphide bond was reduced and alkylated is surprising. However, it is in accordance with the suggestion that, because of steric effects, the Clq-binding site on the CH2 domains is not fully accessible in the native IgG molecules (Isenman *et al.*, 1975; Oi *et al.*, 1984; Sutton *et al.*, 1984). As a consequence a considerable portion of Clq-binding sites is not accessible on the surface of immune-complex precipitates formed from native IgG. The removal of either the covalent or the non-covalent interactions between the two heavy chains brings about an enhanced segmental flexibility of the IgG molecules (Sutton *et al.*, 1984; Schumaker *et al.*, 1986), which may result in an increased effective binding-site density on the surface and in changes in the spatial arrangement of the immune-complex matrix.

An increased effective binding-site density leads to an increase in the values of m and n , which represent the number of effective binding sites covered by one ligand (Clq) and the average number of available binding sites per one IgG in the immune-complex matrix respectively. The m/n ratio also increases, since m changes parallel to the binding-site density on the surface whereas n does not, since it is mainly determined by the fraction of binding sites hidden inside the immune-complex precipitates. As Clq is a very flexible molecule (Schumaker *et al.*, 1981; Hanson *et al.*, 1985; Perkins, 1985), a slightly increased effective binding-site density on the surface of immune-complex matrix can significantly increase the Clq-immune-complex interaction and decrease the dissociation constant (K) too.

On the other hand, more-flexible IgG molecules may form larger immune-complex aggregates as a consequence of the released steric constraints. This results in a greater proportion of Clq-binding sites being hidden inside the immune precipitates, which decreases n but increases m/n again.

In summary, abolition of the covalent or non-covalent interactions between the domains of the Fc part in the IgG molecule results in an increase in the value of m/n and a decrease in the dissociation constant (cf. Table 1), which can be explained by increased flexibility of IgG molecules in immune precipitates. Accordingly, the supposed structural changes can account for the strengthening of the Clq-immune-complex interaction alone, although domain-domain interactions may influence the affinity of Clq-binding sites.

Our observations suggest that domain-domain inter-

actions in the Fc part of IgG exert their effect both on the segmental flexibility of the IgG molecule and on the spatial arrangement of the immune-complex matrix, but do not influence essentially the affinity of individual Clq-binding sites on IgG.

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APPENDIX

In describing the interactions between multivalent large molecules (Clq) and surfaces (immune-complex precipitates), the conventional formalisms fail. To provide means for the quantitative treatment of problems like the one outlined in the accompanying main paper, a model is suggested. The binding of large univalent ligands to surfaces has been analysed by Stankowski (1983), and the method presented here is an extension to multivalent ligands. The model is demonstrated on the Clq-immune-complex matrix system; however, it has general validity and can be applied to related problems.

The following assumptions were made in the treatment.

(1) There are a large number of homogeneously distributed ligand (Clq)-binding sites on the surface (of

immune-complex precipitates); (2) there is no cooperativity in the binding of ligands; (3) the area covered by one particular ligand is independent of the number of actual attachment points; (4) the covered area is represented by a disc (since Clq is a symmetrical molecule, this assumption is not far from reality).

If all these assumptions are fulfilled, then the environment of every bound ligand molecule is the same on the surface, and therefore the probability of multiple binding becomes independent of the concentration of the free ligand, i.e. it depends only on the structural features of the matrix and the Clq. In this case the average free-energy gain per mol of bound ligand can be defined, i.e. a global dissociation constant of the binding, K , does exist. Since steric effects dominate, the equilibrium

constant can be written similarly to that for larger univalent ligands (Stankowski, 1983), as follows:

$$K = \frac{[\text{Clq}_f] \cdot (n \cdot [\text{IC}] - f \cdot m \cdot [\text{Clq}_b])}{[\text{Clq}_b]} \quad (1)$$

where K is the dissociation constant, $[\text{Clq}_f]$ and $[\text{Clq}_b]$ are the free and bound ligand concentrations respectively, and $[\text{IC}]$ is the molar concentration of immune complex. A considerable proportion of the Clq-binding sites is hidden inside the immune-complex precipitates, and therefore the interaction between Clq and immune complex is dependent only on the $n \cdot [\text{IC}]$ binding sites available on the surface. In calculating the molar concentration of immune complex we chose entities containing exactly one IgG molecule (see the Results section of the main paper), and thus n is here the average number of available binding sites per one IgG in the immune-complex matrix. The term $f \cdot m \cdot [\text{Clq}_b]$ represents the excluded binding sites by bound ligands: m is the number of binding sites covered by one ligand when bound, and f is the excluded-binding-area function. Since bound ligands cannot overlap each other, the area that is not accessible for free ligands on the binding surface, i.e. the excluded area, is larger than the area covered by bound ligands. The f function is the ratio of the excluded and the covered surface. Since the f function is concentration-dependent, eqn. (1) cannot be linearized. In addition to that, the f function cannot be expressed analytically. If the surface covered by a ligand is represented by a disc, f approximates limiting values of $2\sqrt{3}/\pi$ and 4 at saturation by the ligand and at infinite dilution of the ligand respectively (Stankowski, 1983). Therefore at low saturation ($f = 4$) it is correct to use, for example, the Scatchard representation to interpret the binding curves:

$$\frac{[\text{Clq}_b]}{[\text{IC}]} \cdot \frac{1}{[\text{Clq}_f]} = \frac{1}{K} \cdot \left(n - 4m \cdot \frac{[\text{Clq}_b]}{[\text{IC}]} \right) \quad (2)$$

It is worth mentioning that parameters of $-4m/K$, n/K and $n/4m$ are obtained from the slope and intercepts of the plot, unlike the conventional linearization, which yields $-1/K$, n/K and n . Formal usage of the original Scatchard or Wu-Hammes plots may result in an error of several orders of magnitude in the values of K and n . The real K value can be estimated with knowledge of the value of m . This latter can be estimated from independent structural information.

Since there is some uncertainty in the plateau region of our binding curves (see Fig. 3 of the main paper), which could lead to an increased error in the calculated

Fig. 1. Example of obtaining n/m and K/m from binding data by fitting curves according to eqn. (1)

The upper and the lower curves correspond to $f = 2\sqrt{3}/\pi$ and $f = 4$ respectively. The points are taken from Fig. 3(b) of the main paper.

$[\text{Clq}_f]$ values in the low-saturation region, we replaced the above-described linearization by a curve-fitting procedure.

We used eqn. (1) to fit the measured points. We varied K/m , n/m and plateau values to fit the experimental points for $f = 2\sqrt{3}/\pi$ in the high-saturation region and $f = 4$ at low saturation. It was also required that the two curves fitted this way would enclose the experimental points in the intermediate region (Appendix Fig. 1). Assuming $m = 6$ from structural data (Shelton *et al.*, 1972; Turner, 1981), the parameters obtained for the various immune-complex precipitates are listed in Table 1 of the main paper.

This treatment was developed to carry out quantitative analysis of Clq-immune-complex-precipitate interactions. The complexity of the system is reflected in the somewhat complicated evaluation procedure, and the results are not explicit for the dissociation constant. On the other hand, the procedure has its advantages by supplying information on the structure of the interacting components. By defining one of the three parameters (K , n and m) from independent experiments or by suggesting plausible models, the other two can be calculated for the given case.

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