Dissociation Kinetics of Bivalent Ligand–Immunoglobulin E Aggregates in Solution†

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ABSTRACT: We study the dissociation of preformed bivalent ligand–bivalent receptor aggregates in solution, where the ligand is a symmetric bivalent hapten with two identical 2,4-dinitrophenyl (DNP) groups and the receptor is a fluorescein-labeled monoclonal anti-DNP IgE. We promote dissociation in two ways: by the addition of high concentrations of a monovalent hapten that competes for IgE binding sites with the bivalent hapten and by the addition of high concentrations of unlabeled IgE that binds almost all ligand binding sites that dissociate from labeled IgE. We investigate both theoretically and experimentally the two types of dissociation and find them to be quite different. Theory predicts that their kinetics will depend differently on the fundamental rate constants that characterize binding and aggregation. Using monovalent ligand to promote dissociation, we find that the fraction of labeled IgE sites bound to bivalent ligand decays with a slow and fast component. The fast decay corresponds to the dissociation of a singly bound DNP hapten. The interpretation of the slow decay depends on the detailed way in which ligand–receptor aggregates break up. We show that one possible explanation of these data is that small stable rings form before the addition of monovalent ligand. Other possible explanations are also presented.

On the surface of basophils, mast cells, and transformed cells derived from these are Fc_ϵ receptors that bind immunoglobulin E (IgE)[1] with high affinity. When an IgE is bound to an Fc_ϵ receptor on the surface of a cell it in turn acts as a bivalent receptor for the ligand it is capable of binding. This means that one can study the binding properties of IgE for a ligand both in solution and on cell surfaces. This is a major advantage since binding to receptors uniformly distributed in solution is much easier to understand than binding to receptors confined to cell surfaces (Goldstein et al., 1989). Seeing what aspects of the binding are the same and what aspects differ gives insights into the processes that occur during cell surface binding. In this and the following paper (Erickson et al., 1991) we use a monoclonal, 2,4-dinitrophenyl- (DNP-) specific murine IgE (Liu et al., 1980) to carry out such a study. This antibody has two identical binding sites for DNP, one in each Fab segment. The ligand we use is a symmetric bivalent hapten *N*,*N*′-bis[[ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl]cystine [$(DCT)_2$-Cys] that has been shown to cross-link IgE both in solution and on cell surfaces (Erickson et al., 1986; Kane et al., 1986).

The cross-linking of immunoglobulin by multivalent ligands (antigens) occurs in a variety of contexts during an immune respone. On B lymphocytes, mast cells, and basophils the cross-linking of surface immunoglobulin triggers a spectrum of cellular responses (Cambier et al., 1987; Metzger et al., 1986). In solution the formation of immune complexes (ligand–antibody aggregates) is a natural consequence of the humoral immune response, where antibodies specific for the pathogen that induced the response are secreted.

We have chosen to use a bivalent ligand in our study because it is the simplest ligand that can aggregate receptors. A bivalent ligand can interact with a bivalent receptor to form only two types of aggregates, linear chains (open structures) and rings (closed structures). For example, an open aggregate containing n IgE molecules will contain $n - 1$ doubly bound ligands and either no, one, or two singly bound ligands, depending on whether both ends of the chain are free, one is bound and one is free, or both are bound. A ring containing n IgE molcules will contain n doubly bound ligands and no singly bound ligands.

In these studies we focus on the dissociation of ligand–receptor aggregates. To follow the kinetics of dissociation we use fluorescein-modified monoclonal anti-DNP IgE. The IgE is labeled with fluorescein isothiocyanate (FITC), and the kinetics of binding is monitored by measuring the fluorescence quenching that accompanies DNP binding to FITC-IgE. Dissociation is monitored by following the fluorescence recovery that occurs when the ligand separates from the FITC-IgE binding site. We find that the dissociation has both a rapid and slow component. The rapid decay is due to the dissociation of singly bound ligands. Identifying the origin of the slow decay is more difficult. We show that the data are consistent with at least two possible mechanisms: the presence of small stable rings; the existence of interactions that cause a bond on a doubly bound ligand, i.e., a ligand that bridges two antibody binding sites, to be more stable than a bond on a singly bound ligand.

We probe the dissociation of IgE–bivalent ligand complexes in two ways: (1) We add a large excess of monovalent ligand that competes for binding sites on the IgE with the bivalent ligand. When the concentration of the monovalent ligand is sufficiently high it essentially blocks all free binding sites on the IgE and prevents the rebinding of any bivalent ligand that has dissociated. (2) We add a large excess of unlabeled IgE.

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[1] Abbreviations: IgE, immunoglobulin E; DNP, 2,4-dinitrophenyl; $(DCT)_2$-Cys, *N*,*N*′-bis[[ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl]cystine; FITC, fluorescein 5-isothiocyanate; DCT, [ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine.

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Here the probe acts as a sink for dissociating bivalent ligand; i.e., when the concentration of unlabeled IgE is sufficiently high, any ligand binding site that dissociates from a labeled IgE will bind to an unlabeled IgE.

When we use unlabeled IgE to study the dissociation of bivalent ligands bound to labeled IgE, the dissociation properties of the ligand appear quite different if the labeled IgE is on cell surfaces rather than in solution. However, when we use excess monovalent ligand to induce dissociation, the dissociation properties of the ligand are the same whether the labeled IgE is in solution or on a cell (Erickson et al., 1991). Why this is so is discussed in the following paper. Here we look at only the dissociation of ligand–receptor aggregates in solution and investigate how the dissociation processes differ, depending on whether a competitive inhibitor (monovalent ligand) or a sink (unlabeled receptor) is used to promote dissociation.

Materials and Methods

Reagents. Fluorescein 5-isothiocyanate (FITC) was obtained from Molecular Probes. The monovalent ligand DCT was obtained from Biosearch, Inc., and the bivalent ligand $(DCT)_2$-Cys was synthesized and characterized as described previously (Kane et al., 1986).

Monoclonal mouse anti-DNP IgE (Liu et al., 1980) was affinity purified and modified with FITC as previously described (Erickson et al., 1986). Final steps in the purification included ion-exchange chromatography on Bio-Rad AG 1-X4 at 37 °C to remove bound DNP-L-glycine (Holowka & Metzger, 1982), followed by concentration with a millipore Immersible CX-30 Filter Unit then gel filtration chromatography on a 2 cm^2 × 100 cm column of Sephacryl S-300 (Pharmacia) in 135 mM NaCl, 5 mM KCl, and 10 mM Hepes, pH 7.4, with 0.01% NaN_3 to isolate the monomeric form of IgE from any aggregates. The FITC-IgE was run on an SDS–polyacrylamide gel and no evidence of contamination was detected. Titrations of this labeled antibody preparation with DCT gave an estimate of 1.8 DNP binding sites per IgE, assuming molecular size and extinction coefficients previously described (Erickson et al., 1986).

Experimental Procedures. The proportional decrease in FITC-IgE fluorescence that accompanies Fab occupancy has been described in detail and attributed to resonance energy transfer between fluorescein and DNP (Erickson et al., 1986). When the bivalent ligand $(DCT)_2$-Cys binds to FITC-IgE the maximal quenching of fluorescein fluorescence is 26%. The monovalent ligand DCT quenches fluorescein fluorescence maximally by 17%. The difference can be explained by the monovalent ligand DCT providing a single acceptor for the fluorescence donor, whereas the bivalent ligand $(DCT)_2$-Cys provides two DNP groups to serve as acceptors. For the bivalent ligand, one DNP group is in the Fab combining site of FITC-IgE, while the second DNP group is either free or cross-linking a second FITC-IgE molecule. We have examined the fluorescence quenching per occupied Fab and determined it to be the same for low concentrations of bivalent ligand, which produces a large number of cross-links, as well as for high concentrations of ligand, which results in a minimal number of cross-links (unpublished results). Thus we conclude that the ability of the second acceptor to quench FITC fluorescence is independent of whether it is bound or free.

All fluorescence recordings were made on an SLM 8000 spectrofluorimeter operated in ratio mode with FITC excitation and emission wavelengths of 490 nM and 526 nM, respectively. Direct data acquisiton was accomplished by interfacing the spectrometer with an AST premium 286 computer.

For each dissociation experiment, 2 mL of FITC-IgE in buffered salt solution (BSS: 135 mM NaCl, 5 mM KCL, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, 0.05% gelatin (w/w), 20 mM Hepes, pH 7.4) was placed in a 10 × 10 × 48 mm acrylic cuvette and stirred continuously. Indicated amounts of $(DCT)_2$-Cys were added via microcapillary tubes to partially saturate the Fab binding sites, thereby quenching the FITC fluorescence. After the fluorescence decrease was complete, net dissociation was induced by adding either a large excess (greater than 10 μM) of DCT or varying amounts of unlabeled IgE. The rate of dissociation of $(DCT)_2$-Cys from the Fab combining sites was monitored as the consequent increase in fluorescence with time. Data points were recorded at 2.4-s intervals. All experiments were done at 15 °C.

Data Analysis. To obtain parameter estimates nonlinear least-squares data fitting was carried out by using the International Mathematics and Statistics Library (IMSL) routine ZXSSQ, which is based on a finite difference, Levenberg-Marquardt algorithm. Estimates of the standard deviation of the parameters where obtained by using the bootstrap method described by Efron and Tibshirani (1986). To obtain the estimates each experiment was simulated 100 times.

Theoretical Results

Model. We start by considering the kinetics of binding and dissociation of a bivalent ligand and a bivalent receptor when rings cannot form and neither monovalent ligand nor unlabeled receptor is present. In the Appendix we discuss how the theory is modified when rings do occur. We follow the formulation of Perelson and DeLisi (1980) and make the equivalent site approximation; i.e., we assume that all free receptor sites, no matter what size aggregate they are in, have the same binding properties. For example, we assume that the single-site forward rate constants are the same for a ligand with both sites binding to a free site on an isolated receptor or a free site on a receptor at the end of a linear chain of any length. Also the forward rate constants for a singly bound ligand to cross-link a free receptor site are the same no matter what size chains the singly bound ligand and free receptor site are on. Simiarly, only two reverse rate constants are needed to describe the dissociation of singly and doubly bound ligands.

The equivalent site approximation allows us to follow the complete kinetics simply by following the state of the ligand, rather than following all the aggregates. This can be done because in the equivalent site approximation we can fully describe a ligand by indicating whether it has both sites free, one site free and one bound, or both bound. If receptor sites were not equivalent, then, in addition, we would have to specify what size aggregate the ligand was bound to. This in turn would require writing down a rate equation for every aggregate that could form, rather than just one for singly bound and one for doubly bound ligands.

In Figure 1 we define (1) the single-site rate constants, k_{+1} and k_{-1}, for a ligand with both sites free binding reversibly to a free receptor site and (2) the single-site rate constants, k_{+2} and k_{-2}, for a ligand with one site bound and one site free binding reversibly to a free receptor site. If we call S and S_T the free and total concentrations of receptor binding sites (e.g., S_T = 2 × IgE concentration), C and C_T the free and total concentrations of bivalent ligands [C_T = (DNP site concentration)/2], Y_1 the concentration of ligands with one site free and one site bound, and Y_2 the concentration of ligands with both sites bound, then we have the following rate equations for singly and doubly bound ligand:

$$dY_1/dt = 2k_{+1}CS - k_{-1}Y_1 - k_{+2}SY_1 + 2k_{-2}Y_2 \quad (1)$$

$$dY_2/dt = k_{+2}SY_1 - 2k_{-2}Y_2 \quad (2)$$

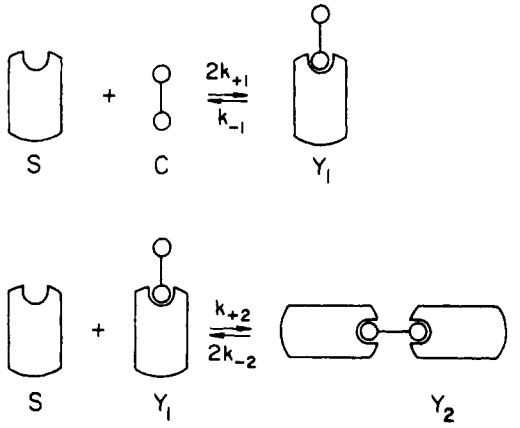

FIGURE 1: Binding and cross-linking of receptor sites by bivalent ligand. The first reaction shows the binding of a bivalent ligand with both sites free to a free receptor site. The second reaction shows the cross-linking of two receptor sites by a bivalent ligand that initially has one site free. The rate constants are defined so that they represent the interaction of a single ligand site with a single receptor site.

We also have the following conservation equations for total receptor sites and total ligand:

$$S_T = S + Y_1 + 2Y_2 \quad (3)$$

$$C_T = C + Y_1 + Y_2 \quad (4)$$

These two differential equations and two conservation laws give a complete description of the formation and dissociation of chains of all lengths (Perelson & DeLisi, 1980).

Dissociation of Bivalent Ligand in the Presence of Competing Monovalent Ligand. Let us now consider the following experiment: We allow binding to take place between bivalent ligand and IgE for some arbitrary time t_b. We then add a large excess of monovalent ligand. If the concentration of monovalent ligand is high enough, its addition will cause a rapid transition, as the free IgE site concentration S falls from its value at time t_b to essentially zero. S will then remain essentially zero, since, in the presence of excess monovalent ligand, when an IgE site that is occupied by a bivalent ligand opens up it will be immediately occupied by a monovalent ligand. Thus, under these conditions $S \simeq 0$ and eqs 1 and 2 become

$$dY_1/dt = -k_{-1}Y_1 + 2k_{-2}Y_2 \quad (5)$$

$$dY_2/dt = -2k_{-2}Y_2 \quad (6)$$

We shall take the time at which the monovalent ligand is added to be $t = 0$ and the concentrations of singly and doubly bound ligand at this time to be $Y_1(0)$ and $Y_2(0)$, respectively.[2] Equations 5 and 6 have the solution

$$Y_1 = Y_1(0)e^{-k_{-1}t} + \frac{2k_{-2}Y_2(0)}{2k_{-2} - k_{-1}}(e^{-k_{-1}t} - e^{-2k_{-2}t}) \quad (7)$$

$$Y_2 = Y_2(0)e^{-2k_{-2}t} \quad (8)$$

At $t = 0$ the concentration of IgE sites that are filled by bivalent ligands is $Y_1(0) + 2Y_2(0)$. This concentration decreases with time as bivalent ligand dissociates and competitive inhibitor (monovalent ligand) fills the vacated sites. From fluorescence measurements we can monitor any change in this concentration. It is therefore useful to define the following normalized concentration:

[2] $Y_1(0)$ and $Y_2(0)$ can be calculated from eqs 1–4 as follows: Write eqs 1 and 2 as functions of only the variables Y_1 and Y_2 by using eq 3 to eliminate S and eq 4 to eliminate C. Then integrate eqs 1 and 2 numerically for a time t_b, starting with the conditions that initially $Y_1 = Y_2 = 0$.

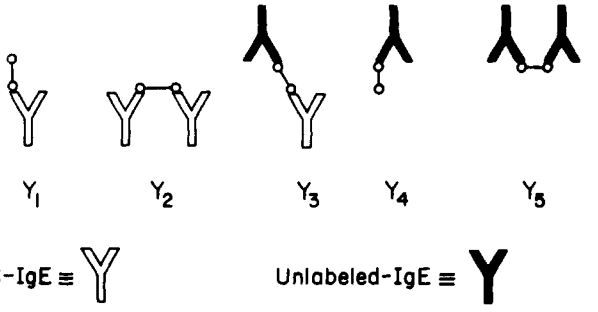

FIGURE 2: Five possible states involving bound ligand that can occur when FITC-IgE, unlabeled IgE, and bivalent ligand interact.

$$f_b \equiv \frac{Y_1 + 2Y_2}{Y_1(0) + 2Y_2(0)} = A_i e^{-k_{-1}t} + (1 - A_i)e^{-2k_{-2}t} \quad (9a)$$

$$A_i = 1 + \bar{a}\frac{k_{-1} - k_{-2}}{(2k_{-2} - k_{-1})} \quad (9b)$$

$$\bar{a} = 2Y_2(0)/(Y_1(0) + 2Y_2(0)) \quad (9c)$$

f_b is the fraction of bivalent ligand sites that remain bound to labeled receptor at time t after the addition of monovalent ligand.

Note that if $k_{-1} = k_{-2}$, i.e., if the rate constant for single-site dissociation is the same for a singly and a doubly bound bivalent ligand, then $A_i = 1$ and f_b decays as a single exponential.

An alternate model that leads to the same form for the decay of f_b is presented in the Appendix. In this model we allow, in addition to linear chains, small stable rings to form consisting of two IgEs and two ligands. We take the reverse rate constant for a bond in a linear chain to be the same whether it is on a ligand that is singly or doubly bound, i.e., $k_{-1} = k_{-2}$, but we assume that a bond in a ring dissociates with a reverse rate constant j_{-2}. Then

$$f_b \equiv \frac{Y_1 + 2Y_2 + 2R}{Y_1(0) + 2Y_2(0) + 2R(0)} = A_i e^{-k_{-1}t} + (1 - A_i)e^{-4j_{-2}t} \quad (9d)$$

$$A_i = 1 - \bar{a}\frac{(k_{-1} - j_{-2})(k_{-1} - 3j_{-2})}{(k_{-1} - 2j_{-2})(k_{-1} - 4j_{-2})} \quad (9e)$$

$$\bar{a} = 2R(0)/(Y_1(0) + 2Y_2(0) + 2R(0)) \quad (9f)$$

where $R(0)$ and R are the concentrations of doubly bound ligands in rings at time zero and at the time t after addition of excess monovalent ligand.

Dissociation of Bivalent Ligand in the Presence of Unlabeled Receptors. A second way to induce dissociation after binding between bivalent ligand and labeled receptor has occurred is by the addition of a large excess of unlabeled receptor (sink). Then, when a bivalent ligand bound to a site on a labeled receptor dissociates, an unlabeled receptor will bind to it, preventing the ligand from rebinding to a labeled receptor.

Assuming no rings can form, when both labeled and unlabeled bivalent receptors are present a bound ligand can be in one of five states as shown in Figure 2. If the rate constants are the same for ligands interacting with labeled and unlabeled receptors, then the rate equations for the bound ligands are

$$dY_1/dt = 2k_{+1}CS - (k_{-1} + k_{+2}S + k_{+2}B)Y_1 + 2k_{-2}Y_2 + k_{-2}Y_3 \quad (10a)$$

$$dY_2/dt = k_{+2}SY_1 - 2k_{-2}Y_2 \quad (10b)$$

$$dY_3/dt = k_{+2}BY_1 - 2k_{-2}Y_3 + k_{+2}SY_4 \quad (10c)$$

$$dY_4/dt = 2k_{+1}CB + k_{-2}Y_3 - (k_{-1} + k_{+2}S + k_{+2}B)Y_4 + 2k_{-2}Y_5 \quad (10d)$$

$$dY_5/dt = k_{+2}BY_4 - 2k_{-2}Y_5 \quad (10e)$$

where B and B_T are the free and total concentrations of unlabeled receptor binding sites and the Y's are the concentrations of the ligand states defined in Figure 2.

In addition to the chemical rate equations we have the conservation laws for total labeled receptor sites, total unlabeled receptor sites, and total ligand:

$$S_T = S + Y_1 + 2Y_2 + Y_3 \quad (11a)$$

$$B_T = B + Y_3 + Y_4 + 2Y_5 \quad (11b)$$

$$C_T = C + Y_1 + Y_2 + Y_3 + Y_4 + Y_5 \quad (11c)$$

If the concentration of unlabeled receptor B_T is high enough so that all rebinding of ligand to labeled receptor sites is blocked and $C = 0$, then eqs 10a–c simplify to

$$dY_1/dt = -(k_{-1} + k_{+2}B)Y_1 + 2k_{-2}Y_2 + k_{-2}Y_3 \quad (12a)$$

$$dY_2/dt = -2k_{-2}Y_2 \quad (12b)$$

$$dY_3/dt = k_{+2}BY_1 - 2k_{-2}Y_3 \quad (12c)$$

where at $t = 0$, the time the unlabeled receptor is added, $Y_1 = Y_1(0)$, $Y_2 = Y_2(0)$, and $Y_3 = 0$. In obtaining these equations we assumed, among other things, that when a site on a ligand that is bridging two labeled receptors opens it cannot rebind to any labeled receptor. From the second term in eq 10a we see this requires that $k_{+2}B \gg k_{+2}S$. This inequality can be satisfied by making $B_T \gg S_T$.[3]

Equations 12a–c have the following solution:

$$Y_1 = [Y_1(0) - \gamma_+\Gamma]e^{-\lambda_+ t} + \gamma_+\Gamma e^{-\lambda_- t} \quad (13a)$$

$$Y_2 = Y_2(0)e^{-2k_{-2}t} \quad (13b)$$

$$Y_3 = -2Y_2(0)e^{-2k_{-2}t} + [2Y_2(0) - \Gamma]e^{-\lambda_+ t} + \Gamma e^{-\lambda_- t} \quad (13c)$$

where

$$\lambda_+ = [b + [b^2 - 4k_{-2}(2k_{-1} + k_{+2}B)]^{1/2}]/2 \quad (14a)$$

$$\lambda_- = [b - [b^2 - 4k_{-2}(2k_{-1} + k_{+2}B)]^{1/2}]/2 \quad (14b)$$

$$b = k_{-1} + 2k_{-2} + k_{+2}B \quad (14c)$$

$$\gamma_+ = k_{-2}/(\lambda_+ - 2k_{-2}) \quad (14d)$$

$$\gamma_- = k_{-2}/(\lambda_- - 2k_{-2}) \quad (14e)$$

$$\Gamma = [Y_1(0) - 2\gamma_- Y_2(0)]/[\gamma_+ - \gamma_-] \quad (14f)$$

From eqs 14a,b we see that the decay constants λ_- and λ_+ satisfy the following relationships:

$$\lambda_+ + \lambda_- = k_{-1} + 2k_{-2} + k_{+2}B \quad (15a)$$

$$\lambda_+\lambda_- = 2k_{-2}k_{-1} + k_{-2}k_{+2}B \quad (15b)$$

The theory predicts that a plot of the sum or product of λ_- and λ_+ versus the sink concentration B should yield a straight line. f_b is the fraction of bivalent ligand sites that remain bound to the labeled receptor at time t after the addition of unlabeled receptor. In this case

$$f_b \equiv \frac{Y_1 + 2Y_2 + Y_3}{Y_1(0) + 2Y_2(0)} = A_s e^{-\lambda_- t} + (1 - A_s)e^{-\lambda_+ t} \quad (16a)$$

where

$$A_s = \Gamma(\gamma_+ + 1)/[Y_1(0) + 2Y_2(0)] \quad (16b)$$

or equivalently

$$A_s = \frac{\gamma_+ + 1}{\gamma_+ - \gamma_-}(1 - \bar{a} - \gamma_-\bar{a}) \quad (16c)$$

where $\bar{a}$ is given by eq 9c.

Note that if $k_{-1} = k_{-2}$, then $\gamma_- = -1$ and $A_s = 1$. Also $\lambda_- = k_{-1}$ and therefore f_b decays as a single exponential with a decay constant k_{-1} that is independent of the sink concentration. However, if $k_{-1} \neq k_{-2}$, then f_b exhibits a two-exponential decay with decay constants, eqs 14a,b, that are functions of the sink concentration. We shall use this result in the next section to experimentally test whether or not $k_{-1} = k_{-2}$.

It is useful to know how λ_+, λ_-, A_s, and f_b behave at low and high sink concentration. One can show that

$$\lim_{B\to 0} \lambda_+ = k_{-1}, \quad \lim_{B\to\infty} \lambda_+/B = k_{+2} \quad (17a)$$

$$\lim_{B\to 0} \lambda_- = 2k_{-2}, \quad \lim_{B\to\infty} \lambda_- = k_{-2} \quad (17b)$$

$$\lim_{B\to 0} A_s = \bar{a}, \quad \lim_{B\to\infty} A_s = 1 \quad (17c)$$

From the above results it follows that

$$\lim_{B\to 0} f_b = \bar{a}e^{-2k_{-2}t} + (1 - \bar{a})e^{-k_{-1}t}, \quad \lim_{B\to\infty} f_b = e^{-k_{-2}t} \quad (17d)$$

Of course the theory is only valid for $B_T \gg S_T$ so one cannot go to the $B = 0$ limit. The $B = \infty$ limit is interesting since it raises the question of whether experimentally this limit is reachable; i.e., can one observe f_b decay as a single exponential with a decay constant equal to k_{-2}? To see such a decay we must have $A_s \simeq 1$ and $\lambda_- \simeq k_{-2}$. From eqs 14e,f and 16b it follows that when $\lambda_- \simeq k_{-2}$, $\gamma_- \simeq -1$ and $A_s \simeq 1$ so such a decay can be observed if $\lambda_- \simeq k_{-2}$. Expanding eq 14b for $1/(k_{+2}B) \ll 1$ we have that

$$\lambda_- = k_{-2} + k_{-2}(k_{-1} - k_{-2})/(k_{+2}B) + \ldots \quad (18a)$$

Therefore $\lambda_- \simeq k_{-2}$ and f_B decays as a single exponential when

$$B \gg (k_{-1} - k_{-2})/k_{+2} \quad (18b)$$

In summary, when a large excess of competitive inhibitor (monovalent ligand) is added to a solution containing bivalent ligand–bivalent receptor aggregates that are in linear chains (no rings), f_b, given by eq 9, decays as two exponentials with decay constants k_{-1} and $2k_{-2}$ that are independent of the inhibitor concentration. If a large excess of sink (unlabeled receptor) is added, we see from eq 16a that again f_b decays as two exponentials, but now the decay constants, λ_+ and λ_-, given by eqs 14a,b, are functions of the sink concentration. In addition, λ_+ and λ_- depend on the forward rate constant for cross-link formation k_{+2}, as well as the two dissociation constants k_{-1} and k_{-2}. Finally, if $k_{-1} = k_{-2}$, then f_b decays in both cases as a single exponential with decay constant k_{-1}.

If small stable rings consisting of two IgEs and two ligands as well as linear chains are present, then, as discussed in the Appendix, the addition of a large excess of monovalent inhibitor will cause f_b to decay as three exponentials. However, if $k_{-1} = k_{-2}$ so that all bonds in linear chains have the same reverse rate constant, f_b will decay as two exponentials with decay constants k_{-1} and $4j_{-2}$ (see eq 9d). If, instead of inhibitor, a large excess of sink is added when some aggregates

[3] Note that if the labeled receptors were on cells, we would need $k_{+2}B \gg \bar{k}_{+2}\bar{S}$, where $\bar{S}$ is the surface concentration of sites on labeled receptors (e.g., sites/cm^2), $\bar{k}_{+2}$ is the two-dimensional cross-linking constant [e.g., cm^2/(s site)] for bridging two labeled cell surface receptors, and k_{+2} is a three-dimensional cross-linking constant (e.g., M^{-1} s^{-1}) for bridging an unlabeled solution receptor and a labeled cell surface receptor. To satisfy this inequality we need $B_T \gg \bar{k}_{+2}\bar{S}_T/k_{+2}$, a quite different condition than the one we obtained when the labeled receptors are in solution. Experiments with radioactive ligand (Erickson et al., 1991) show no detectable cross-linking between solution and surface IgE, suggesting that k_{+2} is quite small. Therefore, it may not be possible to achieve a high enough solution IgE concentration to favor the formation of a bridge between a solution and cell surface IgE when one of the sites on a ligand bridging two surface IgE sites opens up.

are in rings, the formulation we presented (eq 16d) is not applicable because we specifically did not include rings in our development of this part of the theory.

Experimental Results

Dissociation of $(DCT)_2$-Cys in the Presence of DCT (Inhibitor). We have measured the dissociation of prebound bivalent ligand [$(DCT)_2$-Cys], in the presence of DCT (competitive inhibitor), from the fluorescein-modified bivalent receptor (FITC-IgE). We are able to monitor changes in the amount of ligand bound to FITC-IgE because the fluorescein fluorescence is quenched when DNP groups bind to anti-DNP FITC-IgE combining sites. The amount of fluorescence quenching per DNP is different for $(DCT)_2$-Cys and DCT, the monovalent DNP hapten we use as an inhibitor (see under Materials and Methods). Thus, when a DNP site on a $(DCT)_2$-Cys dissociates from an FITC-IgE combining site and is replaced by a DCT, there is a net change in the fluorescence. As illustrated in Figure 3, it is this change that allows us to follow the kinetics of dissociation of DNP groups on $(DCT)_2$-Cys from FITC-IgE combining sites. In Figure 3 we plot the relative fluorescence as a function of time. Initially only FITC-IgE is present (the first plateau at $\simeq$9.4), then $(DCT)_2$-Cys is added, and the fluorescence is quenched as DNP groups bind to FITC-IgE combining sites. At approximately t = 800 s, a large excess of DCT is added, dissociation is induced, and the fluorescence partially recovers.

At a time t after the addition of DCT, the fraction of FITC-IgE sites that still have $(DCT)_2$-Cys bound is related to the relative fluorescence F by the expression

$$f_b = (F_{max} - F)/(F_{max} - F_{min}) \qquad (19)$$

where F_{max} is the value of the relative fluorescence after dissociation has gone to completion and F_{min} is the value of the relative fluorescence immediately after the addition of DCT. If we substitute into eq 19 the expression we derived for f_b when a high concentration of competitive inhibitor is present, eq 9a, we obtain the following expression for the relative fluorescence

$$F = F_{max} - (F_{max} - F_{min})(A_i e^{-k_{-1}t} + (1 - A_i)e^{-2k_{-2}t}) \qquad (20a)$$

Recall that if $k_{-1} = k_{-2}$, then $A_i = 1$ and f_b decays as a single exponential. However, from experiments using unlabeled IgE to induce dissociation (presented at the end of this section), we can directly show that $k_{-1} \neq k_{-2}$. Because of these experiments, when we analyze inhibitor-induced dissociation, we assume that f_b decays as two exponentials.

To determine k_{-1} and k_{-2} for a given experiment we perform a nonlinear least-squares fit to the data of eq 20a, taking as free parameters k_{-1}, k_{-2}, F_{max}, F_{min}, and $\bar{a}$ is given by eq 9c. (We have chosen to take $\bar{a}$ as a free parameter rather than A_i because in the absence of stable rings $0 \leq \bar{a} \leq 1$ for all values of k_{-1} and k_{-2}. Thus, if $\bar{a}$ is determined to be outside this range, it indicates something is wrong with the fit or the theory. Also this parameter is independent of k_{-1} and k_{-2}.) Figure 4 shows the fit we obtained to the data in Figure 3. The results from this and seven additional experiments are listed in Table I. The ligand and IgE concentrations for these experiments were chosen so that there would be a substantial amount of cross-linking before the addition of the competitive inhibitor. The values we obtained for $\bar{a}$, the fraction of bound sites on doubly bound ligands at the time DCT is added, confirm this. We designed the conditions such that the amplitudes of the two exponentials in eq 20a would be nonzero and more or less comparable so that we could determine k_{-1} and k_{-2} from fitting a single experiment. From a weighted average of these eight experiments we found that $k_{-1} = (2.5 \pm 1.4) \times 10^{-2}$ s^{-1} and $k_{-2} = (1.6 \pm 0.4) \times 10^{-3}$ s^{-1}.

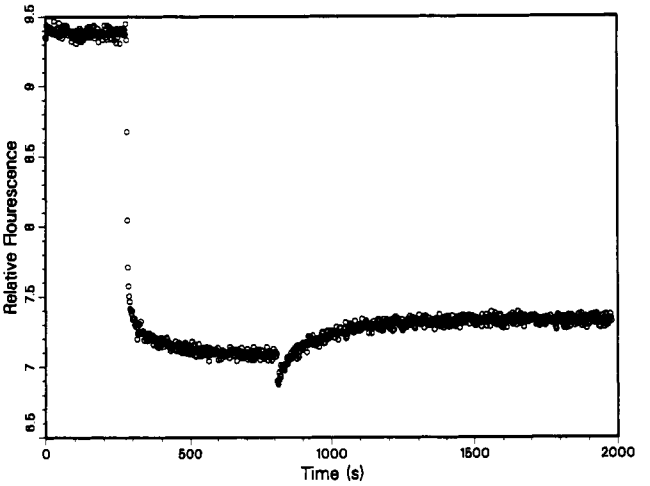

FIGURE 3: $(DCT)_2$-Cys binding to FITC-IgE and dissociation in the presence of DCT (competitor). At t = 0, FITC-IgE is present in solution. At t = 280 s, $(DCT)_2$-Cys is added and the FITC-IgE binding sites begin to fill as indicated by the quenching of the fluorescence. At t = 814 s, monovalent ligand (DCT) is added to a final concentration of 1.62×10^4 nM. There is a small, instantaneous decrease in fluorescence due to inner filter and dilution effects. As bivalent ligand dissociates, DCT fills the vacated FITC-IgE binding sites and a partial recovery of the fluoresecence is observed due to the difference in fluorescence quenching per DNP group for $(DCT)_2$-Cys and DCT. (After the addition of DCT the final concentrations of $(DCT)_2$-Cys and FITC-IgE were 31.6 and 14.8 nM, respectively.)

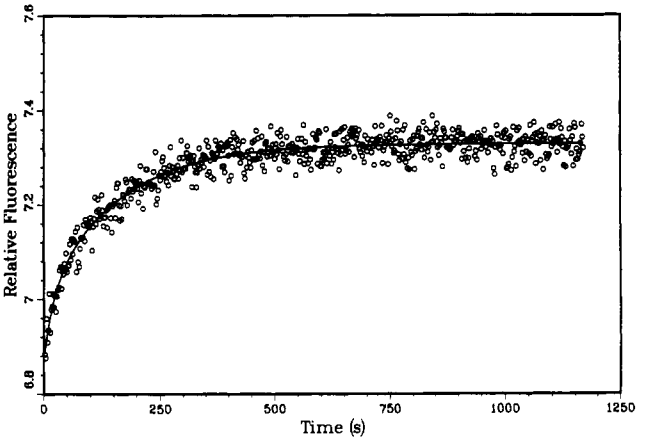

FIGURE 4: Theoretical fit to the DCT-promoted dissociation of Figure 3. Shown is the fluorescence recovery portion of the curve in Figure 3 with t = 0 corresponding to the time when DCT is added. The solid curve was obtained from a nonlinear least-squares fit of eq 20a (with A_i given by eq 9b), to the recovery portion of the curve in Figure 3. The best-fit values of the five parameters that were allowed to vary are $k_{-1} = 4.94 \times 10^{-2}$ s^{-1}, $2k_{-2} = 6.44 \times 10^{-3}$ s^{-1}, $\bar{a} = 3.23 \times 10^{-1}$, $F_{max} = 7.33$, and $F_{min} = 6.87$.

When ligand and IgE concentrations are chosen so as to minimize cross-lining ($\bar{a}$ = 0, hence A_i = 1), eq 20a becomes

$$F = F_{max} - (F_{max} - F_{min})(e^{-k_{-1}t}) \qquad (20b)$$

We have observed that under such conditions the recovery is described by a single exponential and reasonable values for k_{-1} are obtained (data not shown).

In using eq 20a to analyze our data, we have assumed that all bound sites on singly bound ligands have the same reverse rate constant k_{-1} and all bound sites on doubly bound ligands have the same reverse rate constant k_{-2}, i.e., a doubly bound ligand in a chain has the same dissociation properties as a doubly bound ligand in a ring. If this is so, then the results listed in Table I show that the rate at which the first site on a doubly bound ligand dissociates is approximately 15 times slower than the rate at which a singly bound ligand dissociates. Previously we studied the dissociation of DCT from the same

Table I: The Results of Fitting Equation 20, with A_i Given by Equation 9b, to Eight Competitive Inhibitor Dissociation Experiments[a]

	$(DCT)_2$-Cys (nM)	FITC-IgE (nM)	DCT (10^3 nM)	k_{-1} (10^{-2} s^{-1})	$2k_{-2}$ (10^{-3} s^{-1})	$\bar{a}$ (10^{-1})
1	62.6	76.0	10.7	10.08 ± 1.16	6.30 ± 0.36	3.3 ± 0.2
2	178.0	76.0	25.3	4.35 ± 1.05	6.92 ± 0.26	7.1 ± 0.4
3	178.0	72.0	25.3	4.55 ± 0.84	7.28 ± 0.23	6.6 ± 0.3
4	12.8	15.0	5.5	9.03 ± 2.65	7.48 ± 0.75	4.5 ± 0.5
5	31.6	14.8	16.2	4.94 ± 2.12	6.44 ± 0.38	6.8 ± 0.5
6	62.2	14.6	26.5	1.14 ± 0.56	4.95 ± 1.71	2.5 ± 1.7
7	109.0	78.0	21.3	2.73 ± 0.07	2.87 ± 0.02	5.9 ± 0.1
8	109.0	78.0	21.3	1.89 ± 0.04	2.56 ± 0.02	5.2 ± 0.1

[a] Bivalent ligand, $(DCT)_2$-Cys, was allowed to bind with labeled bivalent receptor, FITC-IgE, for approximately 300–500 s, and then a high concentration of monovalent inhibitor (DCT) was added to promote dissociation. Equation 20 was fit to the recovery portion of the curve as illustrated in Figure 4. The five parameters k_{-1}, $2k_{-2}$, $\bar{a}$, F_{max}, and F_{min} were varied simultaneously and determined from the fit. The standard errors we report are bootstrap estimates. The standard error in the values for F_{max} and F_{min} (not given in the table) were always less than 0.5% of the parameter value. From the above data, the following weighted averages were obtained: $k_{-1} = (2.5 \pm 1.4) \times 10^{-2}$ s^{-1} and $k_{-2} = (1.6 \pm 0.4) \times 10^{-3}$ s^{-1}. These averages are dominated by experiments 7 and 8.

monoclonal FITC-IgE. For FITC-IgE in solution, we found $k_{-1} = (2.6 \pm 0.1) \times 10^{-2}$ s^{-1} (Goldstein et al., 1989), which is in agreement with the value determined for dissociation of singly bound $(DCT)_2$-Cys.

Although the origin of the fast decay is clear, the origin of the slow decay is not. As we pointed out, a second way in which the fluorescent decay can have a slow and fast component is if there are small stable rings present. In this model bonds in linear chains, whether on singly or doubly bound ligands, decay with the same rate constant k_{-1}, but bonds in rings composed of two ligands and two IgEs (the only rings that are present in this model) decay with a slower rate constant j_{-2}. The only changes this model leads to in eq 20a are that the exponent in the second exponential becomes $-4j_{-2}$ and that the interpretation of A_i changes and is given by eq 9e. For this model $j_{-2} = (8.2 \pm 1.8) \times 10^{-4}$ s^{-1}.

Dissociation of $(DCT)_2$-Cys in the Presence of Unlabeled IgE (Sink). We have also studied the dissociation of prebound $(DCT)_2$-Cys and FITC-IgE induced by the addition of high concentrations of unlabeled IgE. In Figure 5 we show a typical experiment. Initially only FITC-IgE is present. Next $(DCT)_2$-Cys is added, FITC-IgE combining sites are occupied, and the fluoresence is quenched. Lastly, at approximately t = 700 s, a high concentration of unlabeled IgE (sink) is added and the fluorescence recovers as the $(DCT)_2$-Cys dissociates from the combining sites on the labeled IgE.

Our theory predicts that if $k_{-1} = k_{-2}$ and stable rings are not present, the fluorescence should recover as a single exponential with decay constant k_{-1}. We therefore performed a series of experiments similar to that shown in Figure 5 using identical conditions except that different concentrations of unlabeled IgE were added to induce dissociation. To the recovery portion of these curves we fit the following single exponential function:

$$F = F_{max} - (F_{max} - F_{min})e^{-\lambda t} \qquad (21)$$

Figure 6 shows the results from one such series of experiments. Plotted are the values of the exponent λ we determined from these fits as a function of the sink concentration used in each experiment to induce dissociation. The systematic increase in λ as a function of the sink concentration indicates that $k_{-1} \neq k_{-2}$ or, alternatively, that stable rings are present in the aggregates before dissociation. It is also interesting that λ is a monotonically increasing function of the sink concentration up to at least $B = 1$ μM. One of the theoretical results we obtained was that in the limit that $B \to \infty$, F recovers as a single exponential with decay constant k_{-2} (see eq 17d). This

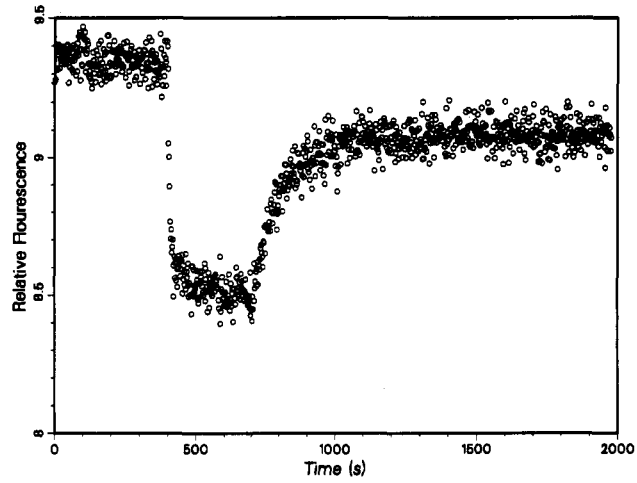

FIGURE 5: $(DCT)_2$-Cys binding to FITC-IgE and dissociation in the presence of unlabeled IgE (sink). At t = 0, FITC-IgE is present in solution. At t = 402 s, $(DCT)_2$-Cys is added and the FITC-IgE binding sites begin to fill as indicated by the quenching of the fluorescence. At t = 706 s, unlabeled IgE is added to a final concentration of 130 nM. The final concentrations of $(DCT)_2$-Cys and FITC-IgE respectively are 1.61 and 2.56 nM. Because unlabeled IgE is in 50-fold excess of FITC-IgE, bivalent ligand that dissociates from FITC-IgE tends to bind to unlabeled IgE. This frees FITC-IgE binding sites, leading to the observed fluorescence recovery. The apparent incomplete recovery of initial fluorescence at $t > 1500$ s is due to a combination of dilution and a small amount nonspecific quenching that is also observed when excess unlabeled IgE is added to FITC-IgE in the absence of ligand.

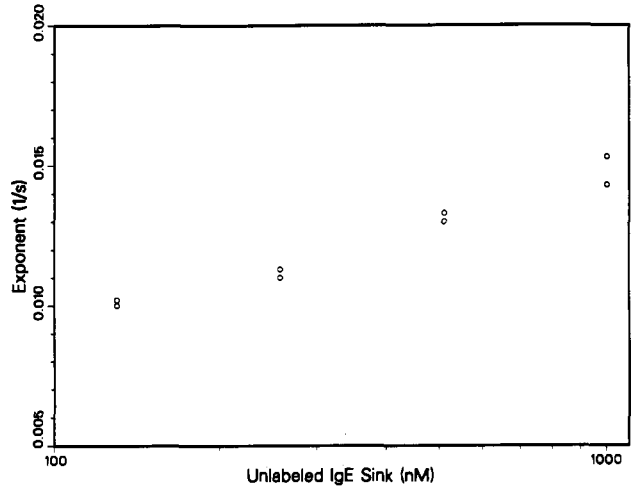

FIGURE 6: Exponent λ as a function of the unlabeled IgE concentration used to promote dissociation. Each value of λ was determined by fitting eq 21 to a sink-induced dissociation experiment such as that shown in Figure 5. If $k_{-1} = k_{-2}$ and no stable rings form, theory predicts that λ should be independent of the unlabeled IgE concentration used to promote dissociation. The systematic increase of λ that is observed suggests that either $k_{-1} \neq k_{-2}$ or stable rings are present at the start of the experiment.

means that at sufficiently high sink concentrations eq 21 should become exact and λ should approach k_{-2}. Since we have determined that $k_{-2} = (1.6 \pm 0.4) \times 10^{-3}$ s^{-1}, we expect for B high enough the plot of λ versus B should go through a maximum and then decrease until $\lambda = k_{-2}$. For the high B limit to hold, $B \gg (k_{-1} - k_{-2})/k_{+2}$ (see the discussion proceeding eq 18b). Since we see in Figure 6 that λ does not appear to be approaching k_{-2}, this inequality does not hold even for $B = 1$ μM. This tells us that k_{+2} must be quite small. In particular k_{+2} must not be much greater than $(k_{-1} - k_{-2})/B = 2 \times 10^{-5}$ nM^{-1} s^{-1}.

Figure 6 shows that if there are no stable rings present, then $k_{-1} \neq k_{-2}$ and we expect f_b to be given by eq 16a. Therefore, from eq 19, the relative fluorescence is

$$F = F_{max} - (F_{max} - F_{min})(A_s e^{-\lambda_- t} + (1 - A_s)e^{-\lambda_+ t}) \qquad (22)$$

Table II: The Results of Fitting Equation 22, with A_s Given by Equation 16c, to 15 Sink Dissociation Experiments[a]

	$(DCT)_2$-Cys (nM)	FITC-IgE (nM)	IgE (nM)	λ_+ (10^{-2} s^{-1})	λ_- (10^{-3} s^{-1})	$\bar{a}$ (10^{-1})
1	2.56	1.61	130	1.5 (+1.7, −0.3)	6.7 ± 2.3	5.7 ± 2.7
2	2.56	1.61	130	1.6 (+0.9, −0.4)	6.8 ± 2.1	5.5 ± 2.4
3	2.57	1.49	130	5.2 (+4.4, −2.0)	9.5 ± 1.3	2.0 ± 1.5
4	2.56	1.83	194	4.2 (+5.3, −1.8)	9.8 ± 1.4	1.3 ± 1.8
5	2.55	1.60	258	1.6 (+0.9, −0.2)	6.3 ± 2.5	7.1 ± 2.3
6	2.55	1.60	258	4.1 (+8.1, −2.7)	10.7 ± 2.1	4.7 ± 2.9
7	2.55	1.48	258	1.8 (+0.7, −0.2)	6.5 ± 2.0	6.8 ± 1.9
8	2.54	1.83	322	5.8 (+6.5, −2.5)	12.7 ± 1.7	1.6 ± 1.8
9	2.53	1.59	511	3.2 (+5.1, −1.1)	10.8 ± 2.6	2.8 ± 2.4
10	2.53	1.59	511	2.2 (+1.0, −0.5)	8.7 ± 2.8	5.7 ± 2.2
11	2.53	1.47	511	2.8 (+1.4, −0.8)	9.5 ± 2.5	4.7 ± 2.1
12	2.51	1.80	636	5.9 (+8.0, −2.9)	14.9 ± 2.6	1.9 ± 2.3
13	2.53	1.56	1002	1.9 (+0.5, −0.2)	5.2 ± 3.5	8.7 ± 1.9
14	2.48	1.56	1002	2.3 (+2.3, −0.5)	9.5 ± 3.5	5.7 ± 2.8
15	2.48	1.44	1002	5.3 (+3.1, −2.2)	12.5 ± 2.1	3.2 ± 1.8

[a] Bivalent ligand, $(DCT)_2$-Cys, was allowed to bind with labeled bivalent receptor, FITC-IgE, for 300–500 s, and then a high concentration of unlabeled IgE was added to promote dissociation. Equation 22 was fit to the recovery portion of the curve. The five parameters k_{-1}, $2k_{-2}$, $\bar{a}$, F_{max}, and F_{min} were varied simultaneously and determined from the fit. The error estimates we report are bootstrap estimates. The standard error values of F_{max} and F_{min} (not given in the table) were always less than 0.5% of the parameter value. The error bounds for λ_+ correspond to 68% confidence intervals. The distribution of estimates for λ_+ is highly nonsymmetric. This is also true for $\bar{a}$, although, for this parameter, we only report the bootstrap estimates of the standard error.

The theory suggests a way to determine k_{+2}, k_{-1}, and k_{-2} from these sink experiments: Fit eq 22 to each experiment to determine λ_+ and λ_- at different sink concentrations; plot ($\lambda_+ + \lambda_-$) versus B and ($\lambda_+\lambda_-$) versus B and fit straight lines through these plots. From eqs 15a,b we see that the slopes and intercepts determined from these fits can be used to detemine k_{+2}, k_{-1}, and k_{-2}. In Table II the results of fitting eq 22 to 15 experiments are given. The results are disappointing because of the large asymmetrically distributed errors in the parameter estimates of λ_+. These errors are too large to use plots of ($\lambda_+ + \lambda_-$) and ($\lambda_+\lambda_-$) to determine slopes and intercepts with sufficient accuracy to allow reasonable determinations of the rate constants. However, we will use the data to get rough estimates of k_{-1}, k_{-2}, and k_{+2} and, for example, see if the estimates of k_{-1} and k_{-2} are consistent with the results we obtained using DCT as a competitive inhibitor. To estimate k_{-1} and k_{+2} we note that when $k_{-1} \gg k_{-2}$, which is the case here, λ_+ can be approximated as follows:

$$\lambda_+ \approx k_{-1} + k_{+2}B \qquad (23)$$

Equation 23 is exact in both limits $B \to 0$ and $B \to \infty$ as well as in the limit $k_{-2} \to 0$. Shown in Figure 7 is the weighted straight line fit of eq 23 to the data with best fit values of the parameters: $k_{-1} = 3.6 \times 10^{-2}$ s^{-1} and $k_{+2} = 2.0 \times 10^{-5}$ nM^{-1} s^{-1}. This estimate of k_{-1} agrees with the estimate $k_{-1} = (2.5 \pm 1.4) \times 10^{-2}$ s^{-1} obtained by using DCT. The parameter k_{+2} only arises when unlabeled IgE is used to promote dissociation. It cannot be determined by using DCT in high concentrations as a competitive inhibitor. The errors in the data are such that we cannot determine k_{+2} with any certainty. From our previous determination of the equilibrium cross-linking constant, $K_2 = (6.9 \pm 2.5) \times 10^7$ M^{-1} (Erickson et al., 1986), and our present determination of $k_{-2} = (1.6 \pm 0.4) \times 10^{-3}$ s^{-1}, we calculate that $k_{+2} = 1.1 \times 10^{-4}$ nM^{-1} s^{-1}.

To get an estimate of k_{-2} from the data in Table II we note that for all values of the sink concentration $2k_{-2} \geq \lambda_- \geq k_{-2}$ (see eq 17b). The weighted average of the values in Table II gives $\lambda_- = (9.5 \pm 2.4) \times 10^{-3}$ s^{-1}. This seems somewhat high since from our DCT experiments we found that $2k_{-2} = (3.3 \pm 0.70) \times 10^{-3}$ s^{-1}.

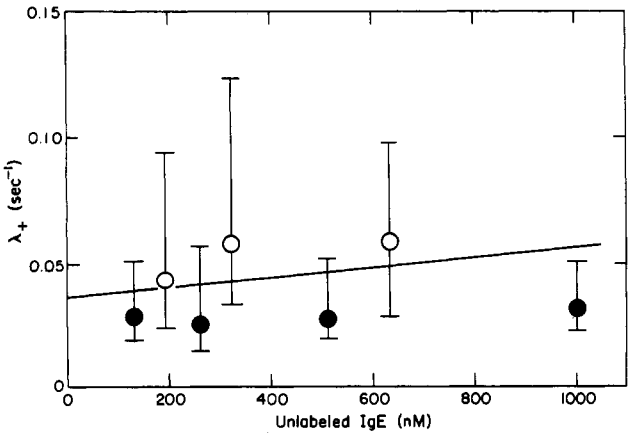

FIGURE 7: Plot of λ_+ versus the sink (unlabeled IgE) concentration. For such a plot theory predicts (see eq 23) that the slope $\approx k_{+2}$ and the intercept $\approx k_{-1}$ when $1 \gg k_{-2}/k_{-1}$. From our DCT experiments we have that $k_{-2}/k_{-1} = 0.07$. The values of λ_+, as well as the 68% confidence intervals, were calculated from Table II. The solid circles correspond to averages of three experiments with the error bars being averages of the 68% confidence intervals. The open circles correspond to a single experiment. A weighted straight line fit to all the data (solid line) gives a slope = 2.0×10^{-5} nM^{-1} s^{-1} and an intercept = 3.6×10^{-2} s^{-1}.

Discussion

We have shown previously that $(DCT)_2$-Cys binds to and cross-links anti-DNP IgE in solution (Erickson et al., 1986). By use of gel chromatography it has been demonstrated that a distribution of aggregates up to at least trimers of IgE is created upon $(DCT)_2$-Cys binding (Kane et al., 1986). We have used two different methods to study the dissociation of aggregates composed of symmetric bivalent ligands, $(DCT)_2$-Cys and symmetric bivalent receptors, monoclonal anti-DNP FITC-IgE. In one method a high concentration of monovalent ligand, DCT, was added to a solution containing aggregates of $(DCT)_2$-Cys and FITC-IgE. When we promoted dissociation in this way we found that the fraction of IgE binding sites occupied by bivalent ligand decayed as a sum of two exponentials with half-lives of 28 and 213 s. The faster decay corresponds to the dissociation of singly bound ligand. Its value is approximately the same as that for the dissociation of DCT (Goldstein et al., 1989). The origin of the slow decay is more difficult to identify and may be a combination of several possibilities.

If the concentration of monovalent ligand we used to promote dissociation is sufficiently high to fill all free sites as soon as they open up, theory predicts the following: (1) If, independent of the size or type (chain or ring) of aggregate, all bonds on singly bound and doubly bound ligands dissociate with the same rate constant k_{-1}, then the fraction of IgE sites occupied by bivalent ligands should decay as a single exponential with exponent $-k_{-1}$. (2) If, independent of the size or

type (chain or ring) of aggregate, all singly bound ligands dissociate with the same rate constant, k_{-1}, and all doubly bound ligands open up to form singly bound ligands with the same rate constant, $2k_{-2}$, then the fraction of IgE sites occupied by bivalent ligands should decay as two exponentials with exponents $-k_{-1}$ and $-2k_{-2}$. (3) If the only aggregates that form are chains and small stable rings composed of two IgEs and two ligands and if all bonds in chains on singly and doubly bound ligands have the same reverse rate constant k_{-1} and all bonds on doubly bound ligands in rings have a reverse rate constant j_{-2}, then the fraction of IgE sites occupied by bivalent ligands should decay as two exponentials with exponents $-k_{-1}$ and $-4j_{-2}$. (4) If the only aggregates that form are linear chains and small stable rings composed of two IgEs and two ligands and all singly bound ligands dissociate with the same rate constant, k_{-1}, all doubly bound ligands in chains open up to form singly bound ligands with the same rate constant, $2k_{-2}$, and all doubly bound ligands in rings open up with rate constant, $2j_{-2}$, then the fraction of IgE sites occupied by bivalent ligands should decay as a sum of three exponentials with exponents $-k_{-1}$, $-2k_{-2}$, and $-4j_{-2}$ (see the Appendix).

In the above list, kinetic scheme 1 is inconsistent with the observed dissociation kinetics. Thus, either all ligand–IgE bonds do not have the same reverse rate constant or we have not achieved a high enough monovalent concentration to block all sites. We will return to this point, but for the moment let us assume the DCT concentrations we used were high enough. Kinetic scheme 4 also seems inconsitent with experiment since we observed a two- rather than a three-exponential decay. However, if the half-life of the slowest decay was comparable to, or longer than, the time of our experiments ($\geq 2 \times 10^3$ s) or if the amplitude of one of the exponents was very small, one of the decays might go undetected. Kinetic schemes 2 and 3 are most consistent with experiment and we will discuss them in more detail.

If kinetic scheme 2 is correct, a bond on a doubly bound ligand is considerably more stable than a bond on a singly bound ligand (k_{-2} is about 15 times smaller than k_{-1}). This might occur if there were nonspecific attractive interactions between antibodies. However, previous equilibrium measurements showed little difference between the equilibrium constants for cross-linking of IgEs and Fab fragments (Erickson et al., 1986). Further, as we show in the following paper, k_{-2} has a very similar value when the IgE is anchored to Fc_ϵ receptors on RBL cells (Erickson et al., 1991). One difference between the two rate constants is that k_{-1} characterizes the dissociation of a small ligand from an antibody while k_{-2} characterizes the dissociation of a ligand–antibody complex from an antibody. The relative rates of separation due to diffusion are quite different in these two cases. The relative diffusion coefficient when a small ligand dissociates from an aggregate is approximately equal to the diffusion coefficient of the ligand itself, $\approx 10^{-5}$ cm^2/s. If two antibodies separate then the relative diffusion coefficient is $2D$, where $D \approx 4 \times 10^{-7}$ cm^2/s (Newman et al., 1977) is the diffusion coefficient of a single IgE. If a large aggregate breaks up into two smaller aggregates the relative diffusion coefficient of the separating aggregates will be even smaller. These considerations are only relevant if the cross-linking reaction is diffusion limited, but simple estimates, which we present in the Appendix, suggest the reaction is far from the diffusion limit. Also, as we pointed out, we obtain similar results for IgE anchored to Fc_ϵ receptors on RBL cells. It is hard to see why the reduction in k_{-2} compared to k_{-1} should be approximately the same in solution and on cell surfaces if diffusion is the explanation, since the diffusion properties of proteins are so different in solution and on cell surfaces.

If kinetic scheme 3 is correct, small stable rings must form during aggregate formation so that a bond on a doubly bound ligand in a ring has a considerably longer lifetime than a bond in a linear chain. Evidence for the existence of small rings composed of bivalent DNP ligands and anti-DNP IgE has been presented (Schweitzer-Stenner et al., 1987). The question this scheme raises is why the reverse rate constant is different for a bond on a doubly bound ligand in a ring and in a chain. One possibility is that the intrinsic reverse rate constants are the same but the forward rate constant for ring closure (j_{+2}) is so great that as soon as a ring opens it almost always recloses. This would cause the ring state to have an apparent reverse rate constant, j_{-2}, that is much slower than the intrinsic reverse rate constant, k_{-1}. If this is the explanation for the slow decay we observe, then we have not achieved a sufficiently high monovalent ligand concentration to block the reclosing of rings. This would occur if when one end of a doubly bound ligand in a ring opens up, the effective local concentration of the free ligand binding site as seen by the free antibody binding site is higher than the monovalent ligand concentration we are using to block free sites. From our experiments this means that the effective concentration must be much greater than the maximum DCT concentration we used, which was approximately 20 μM = 1.2×10^{16} particles/cm^3. We can crudely estimate the effective concentration as follows: In the open configuration the maximum the distance can be between the free antibody site and free ligand site is the span of two antibodies plus the span of two ligands, one of which has both sites bound and one of which has one site bound. For our system this is about 350 Å. The minimum the distance can be between the sites is zero, just before it closes. If, on average, the distance between the free antibody site and free ligand site is about the span of an antibody, $\approx$150 Å, then the effective concentration would correspond to having one site in a cube with sides of approximate length 150 Å. This gives an effective concentration of 3.0×10^{18}, which is about 2 orders of magnitude higher than the DCT concentration we used. Of course, detailed calculations using the real three-dimensional configuration of the complex could dramatically change our estimate of the effective concentration, but this simple argument shows that it is plausable we have not been able to achieve a sufficiently high DCT concentration to block the reformation of rings. We believe that the most likely explanation of our data is the rapid reclosure of cyclic complexes that inhibits the binding of monovalent competitor.

We also studied dissociation by adding unlabeled IgE to preformed aggregates. Again the decay of bound sites on labeled ligand was observed to have a slow and fast component. The rates of these decays were of the same order of magnitude as that seen when excess DCT was used to induced dissociation. Theory shows that in the absence of rings, at sufficiently high unlabeled IgE concentrations the fraction of ligand sites bound to FITC-IgE will decay as two exponentials, but not with the same exponents that one predicts when excess monovalent ligand is used to induced dissociation. When unlabeled IgE is used the exponents are predicted to depend on the forward rate constant for cross-linking, k_{+2}, the unlabeled IgE concentration, B, and the two dissociation rates, k_{-1} and k_{-2}. Unfortunately, we were unable to test these predictions because the variation in the estimates of the exponents for duplicate experiments was too high. Despite this, the experiments are instructive as we can use them to guide experiments where the FITC-IgE is bound to its receptor on cell surfaces. Whether or not rings form, it is clear that to see significant dissociation upon the addition of unlabeled IgE, the unlabeled

IgE concentration has to be much larger than that of FITC-IgE. This was easy to achieve in our experiments. In the following paper we study the dissociation of bivalent ligand bound to cell surface IgE. Here unlabeled solution IgE must compete with surface FITC-IgE; i.e., a three-dimensional concentration must compete with a two-dimensional concentration. In addition, cellular components may interact with and thereby affect the stability of the cross-linked IgE–receptor complexes. This very different situation leads to very different kinetics of dissociation.

ACKNOWLEDGMENTS

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APPENDIX

The Effect of Diffusion on Aggregate Dissociation. We consider the cross-linking in solution of two antibodies by a bivalent ligand. We want to estimate the diffusion-limited forward rate constant k_+ for the cross-linking reaction, where a ligand with one site free and one bound to an antibody binds to a free site on another antibody. We assume that a binding site on an arm of an IgE can be modeled as a circle of radius $s = 5$ Å. If rotational diffusion is very slow, as is the case for large molecules such as an IgE (Slattery et al., 1985), $k_+ \approx 4\pi Ds$, where D is the sum of the diffusion coefficients of the two antibodies (Shoup et al., 1981). The diffusion coefficient of IgE is 3.5×10^{-7} cm^2/s (Newman et al., 1977). Thus $k_+ \approx 5 \times 10^{13}$ cm^3/s $= 3 \times 10^{-1}$ nM^{-1} s^{-1}. If the observed forward rate constant k_{+2} is comparable to k_+, then diffusion will be important; otherwise cross-linking is reaction limited and diffusion does not effect the observed rate constants. We have previously determined that the equilibrium cross-linking constant for cross-linking Fab fragments is $K_2 = 3.5 \times 10^{-2}$ nM^{-1} (Erickson et al., 1986). Recall that if all doubly bound ligands, whether in chains or rings, have the same reverse rate constant, then we found that $k_{-2} = 1.6 \times 10^{-3}$ s^{-1}. Then $k_{+2} = k_{-2}K_2 = 6 \times 10^{-5}$ nM^{-1} s^{-1}, which is 4 orders of magnitude below the diffusion limit.

Dissociation Kinetics in the Presence of Small Rings. We wish to understand the kinetics of dissociation that occurs when an excess concentration of monovalent ligand is added to aggregates in solution containing chains and rings. We assume that the monovalent ligand concentration is high enough that we can assume the free IgE site concentration, S, instantly drops to zero and remains so for the duration of the experiment. In this case we can write the following set of chemical rate equations to describe the changes in the concentration of ligands in chains with one site bound, Y_1, in chains with two sites bound, Y_2, and in rings of length n (containing n IgEs) with two sites bound (Perelson & DeLisi, 1980). In the equations below, k_{-1} is the reverse rate constant for a singly bound ligand in a chain, k_{-2} is the reverse rate constant for a doubly bound ligand in a chain, and j_{-2} is the reverse rate constant for a doubly bound ligand in a ring of length n. The

$$dY_1/dt = 2k_{+1}CS - k_{-1}Y_1 - k_{+2}SY_1 + 2k_{-2}Y_2 + 2\sum_{n=1}^{\infty} j_n\bar{R}_n \quad \text{(A1)}$$

$$dY_2/dt = k_{+2}SY_1 - 2k_{-2}Y_2 + 2\sum_{n=1}^{\infty}(n-1)j_n\bar{R}_n \quad \text{(A2)}$$

$$d\bar{R}_n/dt = -2nj_n\bar{R}_n \qquad \text{for} \quad n = 1, 2, \ldots \quad \text{(A3)}$$

factor $2n$ in eq A3 is there because a ring of n IgEs can open in $2n$ places. The sums in eqs A2 and A3 arise because when a ring opens up there is a gain of one singly bound antibody and $n - 1$ doubly bound antibodies.

We consider the special case when only rings of size two can occur. Our bivalent ligand is too small to span both arms of the same antibody, so rings of size one are ruled out. If rings of size two are very favorable, then the concentration of rings of size greater than two may be negligible. A second model that will give the identical kinetics is if rings of size three or more form, but a double bond in these larger rings has the same reverse rate constants as a double bond in a linear chain. In our experiments this could arise if the DCT concentration we used was sufficiently high to block reclosure of rings of size three or greater, but not of size two.

The fluorescence we measure is related to the IgE sites that are bound by bivalent ligand. Calling this concentration S_b we have that

$$S_b = Y_1 + 2Y_2 + 2\bar{R}_2 \quad \text{(A4)}$$

It is straightforward to solve eqs A1–A3 for Y_1, Y_2, and $\bar{R}_2$. When this is done we find

$$S_b = A_1e^{-k_{-1}t} + A_2e^{-2k_{-2}t} + A_3e^{-4j_{-2}t} \quad \text{(A5)}$$

where

$$A_1 = Y_1(0) + \frac{2k_{-2}}{2k_{-2} - k_{-1}}Y_2(0) + \frac{2j_2\bar{R}_2(0)}{2j_2 - k_{-2}}\left[\frac{k_{-2}}{2k_{-2} - k_{-1}} + \frac{k_{-2} - j_2}{k_{-1} - 4j_2}\right] \quad \text{(A6)}$$

$$A_2 = \left[2Y_2(0) - \frac{2j_2\bar{R}_2(0)}{k_{-2} - 2j_2}\right]\frac{k_{-2} - k_{-1}}{2k_{-2} - k_{-1}} \quad \text{(A7)}$$

$$A_3 = \frac{2\bar{R}_2(0)(k_{-2} - j_2)(k_{-1} - 3j_2)}{(k_{-2} - 2j_2)(k_{-1} - 4j_{-2})} \quad \text{(A8)}$$

Note that when $k_{-1} = k_{-2} = j_2$, $A_2 = A_3 = 0$ and eq A4 reduces to a single exponential, and when $k_{-2} = j_2$, $A_3 = 0$ and eq A4 reduces to a sum of two exponentials. The special cases discussed in the text, eqs 9a–c and eqs 9d–f, can be obtained from eqs A5–A8.

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