Structural Mapping of Membrane-Bound Immunoglobulin E–Receptor Complexes: Use of Monoclonal Anti-IgE Antibodies To Probe the Conformation of Receptor-Bound IgE†

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ABSTRACT: Previous resonance energy-transfer measurements have suggested that immunoglobulin E (IgE) may bend near the junction of its Fc and Fab segments in order to bind to its high-affinity receptor on rat basophilic leukemia cells. In order to test this possibility, two monoclonal antibodies were employed that bind specifically to rat IgE (IgE_R) when IgE_R is in solution and when it is bound to receptors on the plasma membrane. The $F(ab')_2$ fragment of one monoclonal (B5) that is specific for the Fab region of IgE_R was labeled with donor probes and bound to IgE_R, and the quenching of the fluorescence of these donors due to simultaneous binding of the Fab' fragment of an anti-Fc monoclonal (A2) that was labeled with an acceptor probe at its interchain disulfide bond was measured. Significantly less energy transfer between these probes was observed when IgE_R was bound to its receptor on membrane vesicles than when it was free in solution, and this result is interpreted in light of other energy-transfer measurements using A2 and B5 that were preferentially labeled near their combining sites with donors and acceptors, respectively, as well as measurements of the distances of closest approach between these sites and the membrane surface. These results along with previous energy-transfer measurements and other biochemical information form the basis for a working model of the conformation and orientation of receptor-bound IgE. This study demonstrates the use of fluorescently labeled monoclonal antibodies as highly selective energy-transfer probes in assessing structures of macromolecular complexes on the plasma membrane.

Resonance energy transfer has proven to be a useful method for elucidating the three-dimensional structure and orientation of receptor-bound IgE.[1] In previous studies, fluorescent donor probes have been placed at specific sites on IgE, and the distance of closest approach between these probes on receptor bound IgE and amphipathic acceptor probes present at the outer membrane bilayer surface has been measured (Holowka & Baird, 1983a,b; Baird & Holowka, 1985). These results have placed some limits on the possible orientations and positions of IgE when it is bound to its receptor and, in particular, have indicated that the antibody combining sites must be quite far from the membrane surface (≥100 Å; Baird & Holowka, 1985), while the interchain disulfide bonds in the $C_\epsilon 2$ "hinge domain" are as close as 45 Å (Holowka & Baird, 1983b). These results together with biochemical and immunochemical information (Perez-Monfort & Metzger, 1982; Conrad et al., 1983) have led us to consider a model in which IgE must bend somewhere between the $C_\epsilon 1$ domain of the Fab segments and the $C_\epsilon 3$ domain in the Fc segment in order to bind to its receptor (Holowka & Baird, 1983b). This model implies that the IgE molecule takes on a different average conformation when it is receptor-bound than when it is in solution, such that spatial relationships between the Fc segment and the two Fab arms of IgE are altered.

In an effort to test this "bent-IgE" model directly, monoclonal antibodies specific for the Fab segments (B5) and the Fc segment (A2) of IgE_R were employed as a means of very selectively labeling these two regions simultaneously. As summarized in Table I, both of these antibodies bind tightly to IgE in solution and to IgE that is receptor-bound (Conrad et al., 1983). In the present study, A2 and B5 or their enzymatic fragments were labeled separately with donor or acceptor probes (Table I), and the average distances between pairs of probes were compared for the antibody derivatives bound simultaneously to IgE in solution and to IgE that was bound to receptors on membrane vesicles. In one situation, that is, when donor was present on an $F(ab')_2$ fragment of B5 and acceptor was located at the C-terminal end of an Fab' fragment of A2, a significantly greater amount of energy transfer occurred in solution than when IgE was receptor-bound. This result is interpreted in light of measurements made between other derivatives of A2 and B5 and between donor probes on these antibodies and acceptors at the membrane bilayer surface. A refined model for the structure of receptor-bound IgE is proposed.

Experimental Procedures

Chemicals. Fluoresceinyl 5-isothiocyanate (FITC) and 5-(hexadecanoylamino)eosin (HAE) were obtained from Molecular Probes, Inc., and tetramethylrhodaminyl isothiocyanate (RITC) and (iodoacetamido)tetramethylrhodamine (IAR) were from Research Organics, Inc.

Preparation of IgE and Monoclonal Anti-IgE Derivatives. Monoclonal rat IgE (IgE_R, myeloma IR162), purified as de-

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[1] Abbreviations: IgE, immunoglobulin E; IgE_R, rat IgE; RBL, rat basophilic leukemia; FITC, fluoresceinyl 5-isothiocyanate; RITC, tetramethylrhodaminyl isothiocyanate; IAR, (iodoacetyl)tetramethylrhodamine; HAE, 5-(hexadecanoylamino)eosin; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HAF, 5-(hexadecanoylamino)fluorescein; ORB, octadecylrhodamine B; $DiOC_n$, 3,3'-dialkyloxacarbocyanine.

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Table I: Properties of Labeled Monoclonal Anti-IgE Antibodies and Fragments

row	derivative[a]	specificity on IgE[b]	stoichiometry with IgE at saturation[b]	K_a (M^{-1})[b]	location of fluorescent probes
1	FITC-B5 $F(ab')_2$ (1.2)	Fab ($C_\epsilon1$)	1:1	5.1×10^8	random (?)
2	FITC-B5 (2.3)	Fab ($C_\epsilon1$)	1:1	5.1×10^8	preferentially N-terminal α-amino groups
3	RITC-B5 (1.5)	Fab ($C_\epsilon1$)	1:1	5.1×10^8	preferentially N-terminal α-amino groups
4	IAR-A2 Fab' (2.3)	Fc ($C_\epsilon3$–$C_\epsilon4$)	1:1[c]	3.4×10^7	C-terminal interchain cysteines
5	FITC-A2 Fab' (2.3)	Fc ($C_\epsilon3$–$C_\epsilon4$)	1:1[c]	3.4×10^7	preferentially N-terminal α-amino groups
6	FITC-A2 (2.9)	Fc ($C_\epsilon3$–$C_\epsilon4$)	1:1 (solution) 0.4:1 (membrane bound)	4.2×10^8 2.8×10^9	preferentially N-terminal α-amino groups

[a] Preparation of these IgE derivatives is described under Experimental Procedures. The molar ratio of label to protein is indicated in parentheses. [b] From the data of Conrad et al. (1983). [c] A second, much lower affinity site is occupied at high concentrating of A2 Fab' (see Results).

scribed (Kulczycki et al., 1974), was a gift from Dr. Henry Metzger (NIH). Monoclonal mouse IgE (H1 26.82; Liu et al., 1980) was purified and labeled with ^{125}I as previously described (Holowka & Baird, 1983a).

Murine monoclonal anti-IgE_R antibodies B5 and A2 were purified and iodinated with ^{125}I as previously described (Conrad et al., 1983). For one experiment, fluorescently labeled $F(ab')_2$ and Fab' fragments of these antibodies were prepared by pepsin digestion (Conrad et al., 1983), followed by gel filtration on a 2 $cm^2 \times 100$ cm column of Sephacryl S-300 (Pharmacia) in 135 mM NaCl, 5 mM KCl, and 10 mM Hepes, pH 7.4. Gel filtration analysis provides a characteristic partition coefficient corresponding to each peak, $K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the peak elution volume, V_0 is the void volume, and V_t is the total (bed) volume of the column. The pepsin-treated B5 preparation showed a single symmetrical peak with $K_{av} = 0.37$, and pooled fractions from the center portion of this profile showed a single band with an apparent M_r of 100 000 when overloaded on an nonreduced sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel as expected for an $F(ab')_2$ fragment of this antibody. Modification of this fragment (11 μM) with FITC (200 μM) at pH 9.1 in 0.1 M sodium borate and 0.08 M NaCl for 18 h at 4 °C resulted in the incorporation of 1.2 mol of FITC/mol of $F(ab')_2$ (Table I, row 1).

Sephacryl S-300 gel filtration of ^{125}I-labeled, pepsin-digested A2 antibody yielded a broad major peak with $K_{av} = 0.30$, which contained both undigested IgG and $F(ab')_2$ fragments as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. Fractions from this band that were enriched in $F(ab')_2$ fragments were pooled and subjected to reduction with 2 mM dithiothreitol followed by alkylation with 4.5 mM (iodoacetamido)tetramethylrhodamine (IAR). Fractionation of this sample on Sephacryl S-300 yielded a major peak with $K_{av} = 0.50$ and two lesser peaks at $K_{av} = 0.32$ and $K_{av} = 0.38$. The major peak was pooled and concentrated to yield a fragment that showed three fluorescent bands on a nonreduced $NaDodSO_4$ gel with apparent M_r 46 000, 28 000, and 25 000 and no detectable amount of intact IgG or $F(ab')_2$. Reduction of the fragment prior to electrophoresis yielded just two fluorescent bands of apparent M_r 30 000 and 28 000, which correspond to the Fd portion and light chain, respectively, of the Fab fragment as judged by the brighter fluorescence of the M_r 30 000 band and the comigration of the M_r 28 000 band with light chain from undigested A2. ^{125}I quantification showed that greater than 95% of the A2 fragments in this fraction are contained in these two bands. These results taken together indicate that the nonreduced Fab' fragments are composed of a mixture of disulfide-linked and noncovalently interacting Fd segments and light chains.

For other energy-transfer experiments, intact A2 and B5 were modified with 300–600 μM FITC or RITC in 50 mM sodium phosphate (pH 7.0), 0.1 M NaCl, and 1 mM EDTA for 24–48 h at ambient temperature and then quenched with 50 mM glycine, and free dye was removed by passage through a centrifuge column (Penefsky, 1977) containing Sephadex G-50 fine in the same buffer at pH 8.0. Under these conditions of modification (pH 7.0), isothiocyanates remain preferentially reactive with amino groups (Stark, 1970), and the N-terminal α-amino groups of mouse immunoglobulin heavy and light chains are more reactive than most ϵ-amino groups of the lysine residues (Kaplan et al., 1980). For one experiment, ^{125}I-labeled A2 $F(ab')_2$ was modified by FITC under similar conditions, except that the concentration of A2 $F(ab')_2$ was 2 μM and 80 μM FITC was reacted for 50 h, followed by quenching with glycine and extensive dialysis. ^{125}I–Labeled FITC A2 Fab' was subsequently produced by reduction with 2 mM dithiothreitol followed by alkylation with 5 mM *N*-ethylmaleimide as described above for reduction and alkylation with IAR. $NaDodSO_4$ gel analysis of fluorescently labeled derivatives of A2, B5, and their fragments was carried out and analyzed as previously described (Holowka & Baird, 1983b).

Attachment of A2 and B5 Donors to IgE on Vesicles and in Solution. Plasma membrane vesicles were prepared from RBL cells, and their receptors were saturated with IgE_R as described in the preceding paper (Baird & Holowka, 1985). In most experiments, vesicles resuspended after sedimentation were incubated with a 2–4-fold molar excess over IgE of donor-labeled A2, B5, or their fragments. After 30 min at ambient temperature, the vesicles were repelleted at 25000*g* for 20 min following ≤3-fold dilution of samples, then supernatants containing nonbound donors were carefully removed, and the vesicles were resuspended in 10 mM sodium phosphate (pH 7.5), 0.15 NaCl, 1 mM phenylmethanesulfonyl fluoride, and 0.01% NaN_3, in the same volume in which they had been incubated. A sample to control for nonspecific binding of donors contained vesicles without IgE_R bound to receptors. To prepare samples with donor derivatives bound to IgE_R in solution, receptors on the vesicles were blocked with excess mouse IgE, and these were pelleted and resuspended in parallel with the other samples. Then, extra mouse IgE was added followed by addition of IgE_R and the donor derivatives at concentrations similar to those in the samples containing receptor-bound IgE_R.

Binding Assays. Binding of ^{125}I and donor-labeled A2 and B5 derivatives to vesicles was assessed by layering a 50–100-μL aliquot onto 300 μL of 10% (V/V) glycerol in phosphate-buffered saline, followed by centrifugation at 90000*g* for 5 min in a Beckman airfuge. Binding was quantified either by counting ^{125}I in the supernatant and pellet in a Beckman Gamma 4000 or by analysis of the fluorescence intensity of the solution before and after pelleting the vesicles. Binding of ^{125}I derivatives of A2 and B5 to IgE_R on RBL cells was assessed following incubation for 30–60 min at 37 °C in Eagle's minimal essential medium with 10% (v/v) calf serum and 20 mM Hepes. Then, samples were layered in duplicate over

a mixture of phthalate oils (Matthysens et al., 1979) and centrifuged in a Beckman microfuge B for 1.5 min to separate cells from nonbound soluble ligand. Nonspecific binding of A2 or B5 derivatives was assessed with cells without receptor-bound IgE_R. The amount of IgE_R bound to the cells at saturation was determined with ^{125}I-labeled IgE and separate aliquots of cells.

Spectroscopic Measurements. Absorption spectra were recorded on a Cary 118 spectrophotometer. The stoichiometry of FITC conjugation to IgG or its fragments was calculated by assuming $\epsilon_{FITC}^{495nm} = 7.2 \times 10^4\ M^{-1}\ cm^{-1}$ (Garel, 1976), and the protein concentration was determined either from ^{125}I quantification if the specific activity of the labeled derivative was known or by assuming $\epsilon_{IgG}^{280nm} = 1.4\ mL\ mg^{-1}\ cm^{-1}$ (Rodwell et al., 1983) and correcting for the contribution by FITC at that wavelength with $\epsilon_{FITC}^{280nm} = 2.4 \times 10^4\ M^{-1}\ cm^{-1}$. The latter extinction coefficient was determined from an FITC derivative of mouse ^{125}I-labeled IgE by using the ^{125}I specific activity and $\epsilon_{IgE_M}^{280nm} = 2.98 \times 10^5\ M^{-1}\ cm^{-1}$ (Liu et al., 1980) to determine the contribution of protein to the OD at 280 nm. Molecular weights were assumed to be 150000 for intact IgG, 100000 for $F(ab')_2$ of IgG, and 50000 for Fab' of IgG (Davies et al., 1975). Because of partial dimer formation with exciton splitting of absorption bands, the molar concentrations of tetramethylrhodamine were calculated by solving simultaneous equations with extinction coefficients reported by Selwyn & Steinfeld (1972). Molar ratios of tetramethylrhodamine to protein were determined by ^{125}I quantification of protein concentration as described above.

Fluorescence measurements were carried out with an SLM 8000 fluorescence spectrophotometer in which the sample (150 μL) in microcuvettes was maintained at a constant temperature set between 20–25 °C. Excitation of the donor probe (FITC) was generally at 480 nm, while emission was monitored at 515 nm for experiments in which RITC and IAR derivatives served as acceptors and 505–510 nm for experiments with HAE as acceptor. The general procedures for making energy-transfer measurements is described in the preceding paper (Baird & Holowka, 1985), and specific details for each experiment reported are given under Results and in the figure legends.

The efficiency of energy transfer (E) is defined as

$$E = (Q_D - Q_{DA})/Q_D \quad (1)$$

where Q_D and Q_{DA} are the quantum yields of donor in the absence of presence of acceptor. In our experiments, the quantum yield is assumed to be proportional to the fluorescence intensity, F, at a single wavelength, so that

$$E = (F_0 - F_A)/(F_0 x) \quad (2)$$

where F_0 is the initial donor fluorescence intensity before titration with acceptors, F_A is the donor fluorescence intensity in the presence of acceptor, and x is the fraction of total donor that is bound to IgE_R. For the measurements of energy-transfer distances between an FITC donor and HAE acceptors at the membrane surface, the R_0 (critical transfer distance) was determined independently, and four different samples were used to calculate the Q_D/Q_{DA} and L (the distance of closest approach between donors and the acceptor plane) as described in the preceding paper (Baird & Holowka, 1985).

The Q_D values of the various FITC donors were determined in a solution of 10 mM sodium phosphate (pH 7.5), 0.15 M NaCl, and 0.05% (w/w) gelatin (Sigma), with fluorescein in 0.1 N NaOH as a standard as previously described (Holowka & Baird, 1983a). The Q_D value for both FITC-B5 and FITC-B5 $F(ab')_2$ was found to be 8% larger when these derivatives were bound to excess IgE_R, while neither A2 derivative (FITC-A2 and FITC-A2 Fab') showed a significant change in Q_D due to binding.

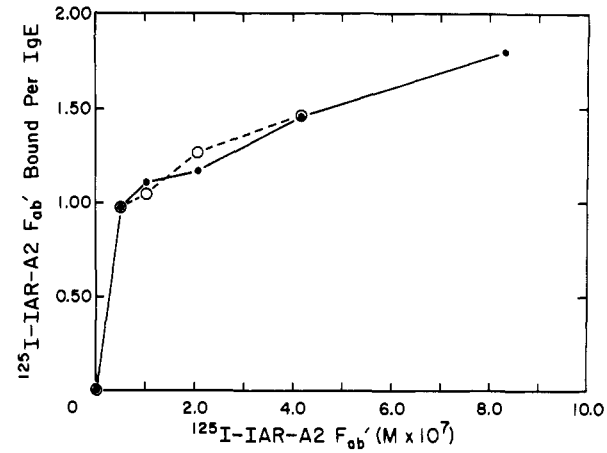

FIGURE 1: Binding of ^{125}I-IAR-A2 Fab' to cell-bound IgE_R in the presence (●) and absence (○) of 116 nM B5 antibody. The concentration of cell-bound IgE_R was 8 nM, and the binding incubation was carried out for 30 min at 37 °C. Nonspecific binding to cells in the absence of IgE_R (<15% of specifically bound) has been subtracted.

RESULTS

Characterization of FITC-B5 $F(ab')_2$ and IAR-A2 Fab'. For use as donor and acceptor probes in one set of energy-transfer measurements, monoclonal antibodies A2 (specific for the Fc region of IgE_R) and B5 (specific for the Fab region of IgE_R) were proteolytically fragmented and labeled with ^{125}I and fluorophores as summarized in Table I. The donor in these measurements, ^{125}I-FITC-B5 $F(ab')_2$, binds to cell-bound IgE_R, saturating at a ratio of 0.9 mol/mol of IgE_R (data not shown), and this agrees within experimental error with the 1:1 stoichiometry obtained in similar studies with intact ^{125}I-B5 (Table I, row 1). The acceptor probe was ^{125}I-A2 Fab' that had been labeled at its C-terminal end by alkylation of reduced interchain disulfides with IAR (Table I, row 4). The binding of ^{125}I-IAR-A2 Fab' to IgE_R on cells is shown in Figure 1. As expected from previous results (Conrad et al., 1983), binding is unaffected by the presence of the B5 antibody at saturating concentrations. Shown in Figure 1 and observed consistently with different preparations (A. K. Menon, D. Holowka, W. W. Webb, and B. Baird, submitted for publication), binding of A2 Fab' to IgE_R on cells appears to be biphasic, indicating a single high-affinity antigenic site per IgE molecule and a second weaker site that is not saturated at 100-fold molar excess A2 Fab'. It is likely that the antigenic determinants are at the same location on the two polypeptides, and it may be that binding of the A2 Fab' to one of these determinants interferes sterically with binding to the second.

Energy Transfer between FITC-B5 $(Fab')_2$ and IAR-A2 Fab'. Measurements of energy transfer between donor (FITC) and acceptor (IAR) when the B5 and A2 derivatives were simultaneously bound to IgE_R are shown in Figure 2. IAR-A2 Fab' was titrated into one sample containing FITC-B5 $F(ab')_2$ bound to excess IgE_R in solution in the presence of membrane vesicles that had receptors blocked with mouse IgE, and the quenching of donor fluorescence [Figure 2 (△, ▲)] was observed to follow the binding curve for ^{125}I-IAR-A2 Fab' and cell-bound IgE_R (Figure 1). The maximal quenching of FITC-B5 $F(ab')_2$ fluorescence was 10–12% in this sample. A similar titration was carried out for a second sample under identical conditions except that the FITC-B5 $F(ab')_2$ were attached to IgE_R bound to vesicle receptors. No significant

Table II: Summary of Energy-Transfer Measurements

row	donor	Q_D[a]	A[b]	acceptor	R_0 (Å)	E_{max}[c]	L (Å)
1	FITC-B5 $F(ab')_2$	0.75	0.160	IAR-A2 Fab'		0.14[d]	
2	FITC-B5 $F(ab')_2$	0.75	0.160	IAR-A2 Fab'		0.01[e]	
3	FITC-B5 $F(ab')_2$	0.75	0.160	HAE	58.2		87
4	FITC-B5	0.53	0.185	HAE	55.0		78
5	FITC-A2	0.72	0.140	RITC-B5		0.082[d,f]	
6	FITC-A2	0.72	0.140	RITC-B5		0.11[e]	
7	FITC-A2	0.72	0.140	HAE	57.9		73
8	FITC-A2 Fab'	0.18	0.105	HAE	46.0		54

[a] Quantum yield of donor bound to IgE_R in the absence of acceptor. [b] Steady-state anisotropy in solution measured at 480-nm excitation and 520-nm emission. [c] Maximum transfer efficiency observed at saturating concentrations of acceptor and corrected for donor binding under experimental conditions used. [d] Maximum transfer efficiency observed for IgE_R in solution. [e] Maximum transfer efficiency observed for IgE bound in receptor on membrane. [f] Average value for two experiments in Figure 3 (error is ±0.008).

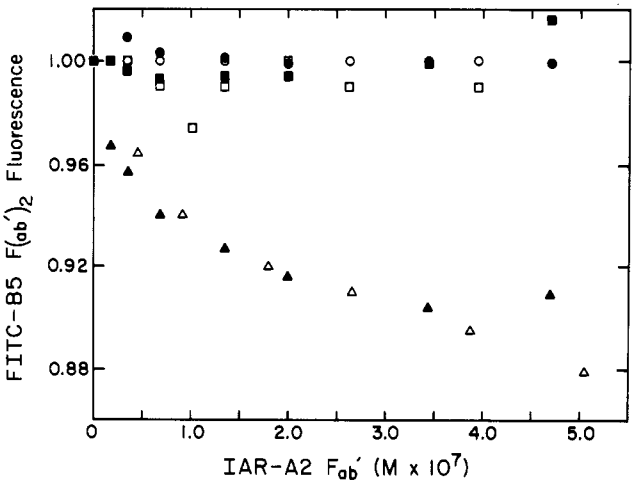

FIGURE 2: Quenching of donor fluorescence of FITC-B5 $F(ab')_2$ due to binding of the acceptor probe IAR-A2 Fab' to IgE_R. Donors bound to IgE_R in solution in the presence of membrane vesicles (△, ▲); donors bound to IgE_R on vesicle-associated receptors (□, ■); donors and acceptors in solution in the absence of IgE_R but in the presence of membrane vesicles (○, ●). Open and closed symbols represent data from separate experiments with different preparations of vesicles; the concentration of receptor-bound IgE_R was 14–19 nM, and IgE in solution was 63–71 nM.

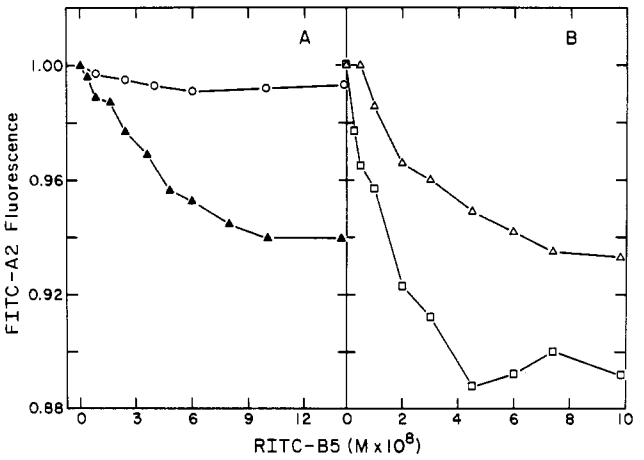

FIGURE 3: Quenching of donor fluorescence of FITC-A2 due to the binding of the acceptor probe RITC-B5 to IgE_R. (A) FITC-A2 (12.4 nM) in phosphate-buffered saline, pH 7.5, with 0.05% gelatin in the presence (▲) and absence (○) of IgE_R (56 nM). (B) FITC-A2 bound to either IgE_R (4.2 nM) on cell-surface receptors (□) or IgE_R (20 nM) in solution in the presence of cells containing blocked receptors (△). (Nonspecific A2 binding, <5%, has been subtracted.)

quenching was observed [Figure 2 (○, ●)]. Using the ^{125}I label, we could determine that about 88% of the ^{125}I-FITC-B5 $F(ab')_2$ was specifically attached to vesicle-bound IgE_R in the second sample, and we estimated that about 80% of this derivative was bound to excess IgE_R in the first sample by measuring its ability to bind to excess vesicle-bound IgE_R in a separate experiment. Therefore, we conclude that the amount of the donor probe bound to IgE_R on the vesicles is at least as great as that for IgE_R in solution in the titrations shown in Figure 2. The fluorescence quenching observed for the solution sample appears to be due to resonance energy transfer because (i) it required the presence of IgE as shown with a third sample [Figure 2 (□, ■)] and (ii) there was no quenching if unlabeled A2 antibody was bound to the IgE instead of IAR-A2 Fab' (data not shown). The maximal energy-transfer efficiencies (E_{max}) were calculated from the data in Figure 2, and these values are listed in Table II, rows 1 and 2.

The lack of fluorescence quenching that was observed for the receptor-bound IgE_R sample cannot be explained by an inability of IAR-A2 Fab' to bind to IgE under these conditions because tight binding with and without B5 bound was demonstrated in the experiments of Figure 1, and cosedimentation of ^{125}I counts with the vesicles at the end of the titration showed that binding of IAR-A2 Fab' does not cause dissociation of bound ^{125}I-FITC-B5 $F(ab')_2$. We also tested the possibility that incubation of the vesicle-bound IgE_R with an excess of FITC-B5 F(ab')2 might have selected for a subpopulation of donors, which could account for the differences obtained. Titrations with IAR-A2 Fab' were carried out on samples of vesicles with bound IgE_r and substoichiometric FITC-B5 $F(ab')_2$ and also on samples containing vesicles with a 2-fold excess of FITC-B5 $F(ab')_2$ over IgE_R. The data in these experiments (not shown) were subject to larger error, but the results are consistent with those of Figure 2: in all cases the observed maximal amount of donor fluorescence quenching due to binding of acceptor when both were bound to IgE in solution was at least twice as much as when they were attached to IgE bound to receptor on the vesicles.

Energy Transfer between FITC-A2 and RITC-B5. For a second measurement, we employed the monoclonal anti-IgE_R antibodies labeled in an alternative manner with donor and acceptor probes. The intact A2 was reacted with the donor probe FITC at pH 7.0 in order to obtain preferential modification of the N-terminal α-amino groups on heavy and light chains (Table I, row 6). These amino groups are located near the antibody combining sites (<20 Å) but about 80 Å away from the interchain disulfides that we labeled for IAR-A2-Fab' (Amzel & Poljak, 1979). Similarly, the B5 antibody was modified with the acceptor probe RITC at pH 7.0 (Table I, row 3). When FITC-A2 was bound to excess IgE_R in solution and titrated with RITC-B5, the FITC fluorescence was quenched to a maximal extent of 6% [Figure 3A (▲)], and when corrected for the amount of A2 determined to be bound under these conditions, the maximal quenching was 7.5%. Less than 1% quenching was observed in the absence of IgE_R

Table III: Summary of Distances to the Membrane Surface (L) Measured by Resonance Energy Transfer

row	donor	location[a]	acceptor[b]	L (Å)	reference
1	dansyllysine	anti-dansyl IgE combining sites	HAE, ORB	100–120	Baird & Holowka (1985)
2	FITC	Fab	HAE, ORB	86–91	Holowka & Baird (1983b)
3	CPM(−)	Fab ($C_\epsilon1$ domain)	HAF, $DiOC_6$	75–87	Baird & Holowka (1985)
4	FITC	B5 or B5 $F(ab')_2$ bound to Fab	HAE	78–87	this paper
5	FITC	A2 Fab′ bound to Fc	HAE	54	this paper
6	CPM(+)	$C_\epsilon2$ domain	HAF, $DiOC_{10}$	43–44	Holowka & Baird (1983b)

[a] Sites on receptor-bound IgE on membrane vesicles. [b] Acceptors located at the bilayer surface.

[Figure 3A (○)], and no quenching was detected if the titration was carried out with unlabeled B5 (data not shown).

We attempted to measure energy transfer between FITC-A2 and RITC-B5 that were attached to IgE_R bound to receptors on the vesicles, but these experiments were complicated by the fact that the vesicles become highly agglutinated by A2 antibody in such a manner that fluorescently labeled IgE becomes aggregated in the region of contact between the vesicles. It is noteworthy that this effect is not seen with the B5 antibody, which binds to fluorescent IgE_R on the vesicles without altering the uniform surface fluorescence (Baird et al., 1984; unpublished observations).

In order to avoid complications due to agglutination by A2, the energy-transfer experiments were carried out with intact cells. The acceptor RITC-B5 was titrated into one sample containing FITC-A2 bound to an excess soluble rat IgE in the presence of cells that had receptors blocked with mouse IgE [Figure 3B (△)], and the same titration was carried out on a second sample containing the same amount of FITC-A2 that was attached to IgE bound to the cellular receptors [Figure 3B (□)]. Unlike the situation seen in Figure 2 for fragments of these antibodies labeled at different sites, some quenching of the donor fluorescence is seen for both samples. The concentration of RITC-B5 corresponding to the half-maximal quenching appears to be slightly less for the sample containing receptor-bound IgE_R, which may be attributed to a slightly higher affinity constant for B5 binding to IgE_R on cells compared to binding in solution (unpublished observations). The absolute values for the maximal quenching and therefore the maximal energy-transfer efficiency may be compared for the two if they are corrected for the amount of FITC-A2 bound in each case. For the receptor-bound IgE_R sample, all of the fluorescence seen in Figure 3B (□) was found to be due to bound A2, and the maximal-transfer efficiency is 0.11. The amount of FITC-A2 binding to IgE_R in solution [Figure 3B (△)] was determined to be 73% on the basis of the bindable fraction of A2 and its affinity constant (Conrad et al., 1983). The maximal transfer efficiency for the FITC-A2 in this sample was calculated to be $E = 0.090$, which is similar to the value obtained from the corresponding measurement with IgE_R in solution in the absence of cells [Figure 3A (▲); $E = 0.075$]. Thus, unlike the situation in Figure 2, the average value for E is very little different from the value of $E = 0.11$ determined for cell-bound IgE_R. These values are given in Table II, rows 5 and 6.

Distances between the A2 and B5 Antigenic Sites and the Membrane Surface. Energy transfer was also measured between acceptor probes at the membrane surface and donor probes on A2 and B5 when these antibodies or their fragments were attached to IgE_R that was bound to receptors on the vesicles. Two different derivatives of B5 were used in separate experiments: (1) FITC-B5 $F(ab')_2$ (Table I, row 1), which is the same derivative employed in the experiments of Figure 2, and (2) FITC-B5 for which the FITC modification was carried out at pH 7.0 to preferentially label N-terminal amino groups (Table I, row 2). In these experiments, 85% of the

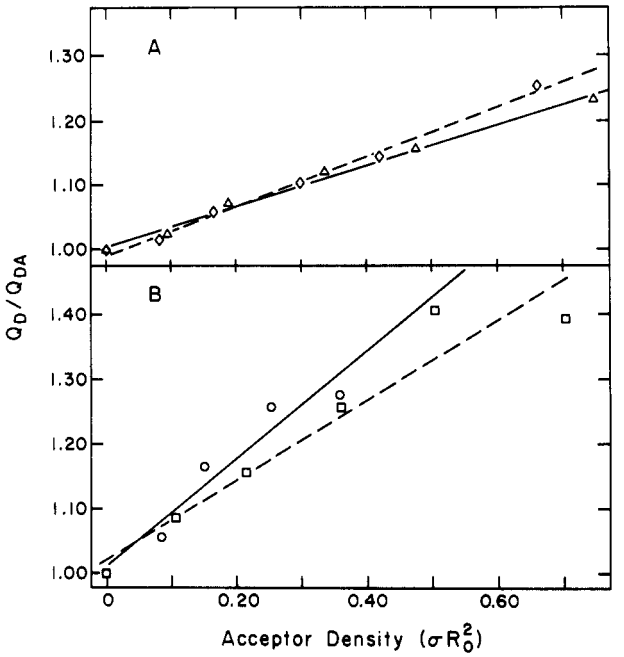

FIGURE 4: (A) Energy transfer between FITC-B5 (◇) or FITC-B5 $F(ab')_2$ (△) bound to IgE_R on membrane vesicles and the amphipathic acceptor HAE titrated into the bilayer surface. (B) Energy transfer between FITC-A2 (□) or FTIC-A2 Fab′ (○) bound to IgE_R on membrane vesicles and the amphipathic acceptor HAE titrated into the bilayer surface. The ratio of donor quantum yield in the absence (Q_D) and the presence (Q_{DA}) of acceptors is plotted as a function of acceptor density (acceptors per R_0^2), and the best linear fits are shown.

FITC-B5 $F(ab')_2$ present and 94% of the FITC-B5 present were bound specifically to the IgE_R on the vesicles. The amphipathic acceptor probe HAE was titrated into the samples while the FITC fluorescence was monitored to detect energy transfer. The ratio of specifically bound FITC fluorescence in the absence of acceptor (Q_D) to this fluorescence in the presence of acceptor (Q_{DA}) is plotted as a function of the surface density of HAE for both of these experiments in Figure 4A. Linear least-squares fit of the data yields minimal distances to the membrane of 78 ± 1 Å for FITC-B5 and 87 ± 2 Å for FITC-B5 $F(ab')_2$, where the error reflects random scatter of points about the best line (Baird & Holowka, 1985). We estimate a more realistic error in these values for L is ±20% on the basis of sources of systematic uncertainty in characterizing donors and acceptors in the samples and fitting the measurements with particular energy-transfer models as discussed previously (Holowka & Baird, 1983b). On this basis these two distances for the B5 binding site on IgE are probably not significantly different.

Measurement of energy transfer between FITC-A2 (Table II, row 6) and HAE was carried out on vesicles in the same manner, and the results are shown in Figure 4B (□). The best linear fit to the data corresponds to a minimal distance of 73 ± 3 Å between the FITC donors and the membrane surface (Table III, row 7). We considered that the interpretation of this result may be complicated since the A2 antibody causes

extensive clustering of IgE–receptor complexes as noted above. Therefore, we modified an Fab′ fragment of A2 with FITC at pH 7.0 (Table I, row 5) in order to carry out a similar measurement. Microscopy showed that this derivative binds to IgE_R on the vesicles, and the resulting fluorescence is uniformly distributed over the surface as expected for a monovalent antibody fragment. The Fab′ binds more weakly to vesicle-bound IgE than the intact A2 antibody (Table I, rows 5 and 6), and we determined that 27% of ^{125}I-FITC-A2 Fab′ present was attached during the energy-transfer measurement. The extent of binding was taken into account in calculating the Q_D/Q_{DA} ratio as a function of HAE surface density, and the results are shown in Figure 4B (○). The linear fit to these data corresponds to a minimal distance of the membrane surface of 54 ± 2 Å (Table II, row 8). The discrepancy between these two distances for the A2 binding site on IgE may be due in part to complicating factors with FITC-A2 including location of some donors in the Fc region and/or exclusion of HAE in the region of aggregated receptors on the vesicles.

Discussion

Does IgE Undergo a Conformational Change upon Binding to Its Receptor? In the present study we have attempted to address this question by employing monoclonal antibodies or their fragments that are specific for the Fab and Fc segments of IgE. Our previous results had suggested that the potential region of IgE bending might be between these segments (Holowka & Baird, 1983b), and the use of fluorescently-labeled anti-Fab (B5) and anti-Fc (A2) allows the highly specific placement of donor and acceptor probes on either side of this conformationally sensitive region in a manner that does not interfere with the binding of IgE to its receptor. A major drawback to the use of randomly labeled antibodies or even Fab fragments as energy-transfer probes is that their effective sizes are comparable to the distances being measured. This problem has been partially overcome in the present study by selective chemical modification of the monoclonal anti-IgE antibodies, in one case by limited reduction and alkylation of the interchain disulfide bonds of A2 $F(ab')_2$ to yield IAR-A2 Fab′ (Table I, row 4), and also by preferential labeling of the N-terminal α-amino acids of A2 and B5 with FITC or RITC (Table I, rows 2, 3, 5, and 6). For this latter case, a similar modification of IgE had indicated that a large fraction of FITC is located close to the anti-DNP IgE combining sites (Holowka & Baird, 1983b; unpublished observations), and preliminary analysis by Edman degradation indicates that at least 40% of the FITC in A2 Fab′ or B5 derivatives is located at the N-terminal residues (unpublished observations). For experiments designed to detect conformational changes in the present study, precise placement of the donor and acceptor probes on the monoclonal anti-IgE antibodies is not essential, since changes in average distances are of primary interest rather than absolute spatial relationships.

Two separate experiments to assess the possible conformational effects of IgE binding to membrane-bound receptors have been carried out. In one experiment (Figure 2), FITC-B5 $F(ab')_2$ was employed as the donor probe, and IAR-A2 Fab′ labeled at the C-terminal disulfides was employed as the acceptor (see Table I, rows 1 and 4, and Figure 5). The results indicated that the average distance between these probes is greater when IgE is bound to receptors on membrane vesicles than when it is in solution. Several trivial explanations based on differential sample preparation were tested and ruled out in independent experiments. It is also unlikely that the change in the apparent distance between FITC donor and IAR acceptor probes is due simply to a change in their relative dipole orientation since this would be averaged over all dipole positions and both FITC and IAR probably react with more than one residue in the antibody fragments. Furthermore, the steady-state anisotropies of FITC-B5 $F(ab')_2$ indicate that these donors are not rigidly attached but are depolarized significantly during their fluorescence lifetime (Table II). One possible explanation for the differences in the efficiency of energy transfer shown in Figure 2 is that both forms of IgE may have an identical average conformation but that receptor-bound IgE is less flexible than IgE in solution, and since energy transfer is much more heavily weighted to shorter distances, the more flexible form would have more possible configurations for which donors attached to the Fab segment are closer to acceptors attached to the Fc segment. This explanation appears to be unlikely since recent fluorescence polarization studies in our laboratory have indicated that receptor-bound and free IgE have a similar degree of segmental flexibility (Slattery et al., 1985). We conclude that this experiment reveals some alteration in the average spatial relationships between the donor and acceptor probes bound to IgE that occurs as the result of the interaction with receptors on the membrane vesicles. The structural nature or the extent of this change is uncertain and may well involve some change in the possible modes of motion of IgE without substantially altering the observed fluorescence depolarization.

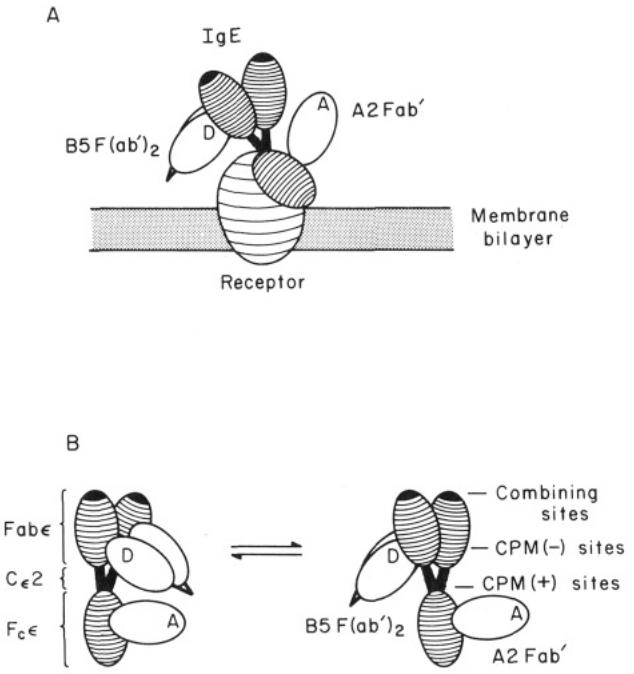

FIGURE 5: Models for the structures of A2 Fab′ and B5 $F(ab')_2$ bound to (A) IgE–receptor complex on the plasma membrane and (B) IgE in solution. D and A represent the approximate locations of the donor and acceptor probes, respectively. See text for explanation.

In the second experiment that was designed to investigate a possible conformational change in IgE, the A2 antibody was labeled with donor (FITC) and B5 was labeled with acceptor (RITC), both under conditions chosen to maximize the amount of fluorescent probes that is localized at N-terminal residues near their respective antibody combining sites (Table I, rows 3 and 6). This experiment was carried out with IgE in solution or bound to receptors on cells in order to avoid problems due to membrane vesicle agglutination by A2 and also to test the feasibility of carrying out such measurements on intact cells. Energy transfer between donor-labeled A2 and acceptor-labeled B5 on IgE was readily measurable but did not show any

significant difference for IgE bound to its receptors compared with that in solution (Figure 3 and Table II, rows 5 and 6). Under the conditions of this experiment, FITC-A2 probably cross-links pairs of IgE_R–receptor complexes on the cell surface, and in solution the excess of IgE_R over FITC-A2 used is likely to result in a similar situation in which pairs of IgE molecules are cross-linked (Conrad et al., 1983; unpublished experiments). If these binding configurations are correct, then the results obtained indicate that the average distance between the *binding sites* for A2 and B5 on IgE is probably not significantly different for IgE in solution vs. receptor bound.

How can this latter conclusion be rationalized in light of the results obtained in Figure 2 that provide evidence for some structural alteration in the A2–IgE–B5 complex when it binds to its receptor? The simplest explanation is that the change detected with donor-labeled B5 $F(ab')_2$ and acceptor-labeled A2 Fab′ (Figure 2) is fairly subtle, such that the position of the donor and acceptor labels on the anti-IgE antibodies or their fragments is crucial for the detection of this alteration in IgE conformation. The models presented in Figure 5 and discussed below provide a possible structural solution that is consistent with this explanation. It appears that the disparate results of Figures 2 and 3 come from our use of different derivatives of A2 and B5 in the two experiments, and we cannot rule out the possibility that the orientation, conformation, and/or flexibility of these derivatives binding to IgE may not be constant, making any particular one potentially unsuitable as a probe for the conformation of IgE. Further energy-transfer measurements will be necessary to resolve this issue. For example, it should be possible to measure the distances from the highly localized CPM(−) or CPM(+) sites (Figure 5B) to fluorescein at either the N-terminal or C-terminal ends of A2 Fab′ and compare the attachment for free and receptor-bound IgE_R.

Despite some remaining uncertainties of these energy-transfer experiments, it is worth noting several additional pieces of information that are consistent with a conformational change occurring upon IgE binding to its high-affinity receptor. During the course of the energy-transfer experiments described in the preceding paper (Baird & Holowka, 1985), we observed selective binding by a particular amphipathic acceptor probe to sites near the donor probes located at the inter ϵ-disulfide bonds of IgE but only when reduced and alkylated IgE was receptor-bound. This suggests that there is a region in or near the $C_\epsilon2$ domains of this IgE that is normally unexposed in solution but that becomes accessible when IgE binds to receptor. Chemical cross-linking experiments in our laboratory suggest a similar conclusion: the homobifunctional reagent bis(sulfosuccinimidyl) suberate can react at one end with a region in mouse IgE such that the other end is sequestered and protected from reaction with small water-soluble primary amines, but it becomes available to cross-link IgE to receptor when these two proteins are associated (T. Chung, B. Baird, and D. Holowka, unpublished results). Also consistent with the view that a conformational change in IgE is required for tight binding to receptor is the forward rate constant for this association on cells and membranes, which is known to be small [$k_f = (1\text{–}2) \times 10^5\ M^{-1}\ s^{-1}$; Kulczycki & Metzger, 1974; Metzger et al., 1976] and not diffusion-limited (Wank et al., 1983). The k_f for solubilized receptor is about 30 times larger than that for membrane-bound receptor (Wank et al., 1983), suggesting that the conformational change in IgE may not occur upon binding to receptor in detergent solution. This prediction can be tested with energy-transfer measurements, since these experiments can now be carried out before and after solubilization of membrane vesicles (unpublished observations).

Distances to the Membrane Surface. We measured energy transfer between the membrane surface and FITC-B5 $F(ab')_2$, FITC-B5, FITC-A2 Fab′, and FITC-A2 (Table I, rows 1, 2, 5, and 6) attached individually to receptor-bound IgE in order to localize these probes with respect to other sites on receptor-bound IgE that have been similarly mapped (Holowka & Baird, 1983b; Baird & Holowka, 1985). For both FITC-B5 $F(ab')_2$ and FITC-B5, the value of L calculated is the same within experimental error (Figure 4A, Tables II and III) when HAE is used as the amphipathic energy-transfer acceptor. This agreement is not surprising since the FITC modification of intact B5 at pH 7.0 should be confined primarily to the N-terminal amino acids near the antibody combining sites while the FITC label on the B5 $F(ab')_2$ must be $\leq$80 Å (the length of an Fab) from these combining sites and may also be located in large part at the N-terminal residues. The value of L obtained for FITC-A2 was significantly greater than that for FITC-A2 Fab′ (Table II, rows 7 and 8), but since the former measurement was complicated by the occurrence of receptor aggregation, it is likely that the value obtained for FITC-A2 Fab′ is more accurate.

Table III is a summary of the distances we have measured between sites on receptor-bound IgE and the membrane surface. Although there may be as much as 20% error in any individual measurement as discussed previously (Holowka & Baird 1983b), the composite provides a rather detailed structural map. The sites on IgE that are farthest away from the membrane surface are the antibody combining sites at the N-terminal ends of the Fab segments (row 1), and the average distance from the FITC sites to the surface is only slightly less (row 2), consistent with the expectation that at least a portion of these probes is located at the N-terminal α-amino groups. The range of distances measured for the FITC-B5 derivatives (row 4) is similar to that determined for CPM(−) located in the $C_\epsilon1$ domain of the Fab segments (row 3), and this is reasonable since the observed cross-reactivity profile for B5 (Conrad et al., 1983) suggests that its specificity is for the $C_\epsilon1$ domain. Furthermore, we have observed that energy transfer between CPM(−) and FITC-B5 is much more efficient than that between CPM(−) and FITC-A2 (unpublished results). The distance for FITC-A2 Fab′ (row 5) is similar to, but slightly greater than, that previously determined for CPM(+) at the inter heavy chain disulfide bonds in the $C_\epsilon2$ domains (row 6). Although subject to some uncertainty, this result clearly indicates that the antigenic site for A2 on the $C_\epsilon3$–$C_\epsilon4$ domains of receptor-bound IgE is not likely to be significantly closer to the membrane surface than the inter heavy chain disulfide bonds in the $C_\epsilon2$ domain, thus supporting the hypothesis of a bent conformation for IgE.

Structural Model of Receptor-Bound IgE. The model depicted in Figure 5A is consistent with the results obtained in this study as well as all of the energy-transfer distance measurements listed in Table III and other biochemical information that is available. In this model, the Fc region of IgE is bent out of the 2-fold axis of symmetry, which extends lengthwise through the center of this molecule such that one face of the Fc region interacts with the receptor while the other face is exposed. This view is consistent with the *limited* protection of the Fc region when IgE is receptor-bound (Perez & Metzger, 1982) and with the presence of a single tight binding site for the the Fab fragment A2 to IgE in solution or bound to receptor (Conrad et al., 1983). The model agrees with our fluorescence microscopy observations in that the bound Fab fragment of A2 is oriented in a manner that would

allow agglutination of vesicles by the intact A2 antibody, while the $F(ab')_2$ fragment of B5 is shown as binding to one or two Fab segments in a manner that would not permit IgE–receptor complexes to be cross-linked readily on the same or different vesicles. The distances from the A2 and B5 antigenic sites on IgE to the membrane surface indicated in Figure 5A are consistent with the measured values listed in Tables II and III. Details of the subunit structure of the receptor are not shown in Figure 5A, but the transmembrane orientation indicated is consistent with our recent observations (Holowka & Baird, 1984).

In order to explain the results shown in Figure 2 of this paper, we propose that IgE bound to its receptor has a conformation such that the A2 and B5 molecules or their respective fragments cannot bind simultaneously to the same side of the IgE molecule (Figure 5A). For IgE in solution, we suggest that A2 and B5 can bind simultaneously to the same side or to opposite sides (Figure 5B), and this is reasonable from the evidence for segmental flexibility in IgE (Slattery et al., 1985; Cathou, 1978) and the probable presence of an antigenic site for B5 on each IgE Fab segment. If the distance between donor and acceptor probes is significantly greater when FITC-B5 $F(ab')_2$ and IAR-A2 Fab′ are on opposite sides of the IgE molecule than when they are on the same side, then the *average* distance measured for IgE in solution (Figure 5B) would be less than that for receptor-bound IgE (Figure 5A). The models of Figure 5 are also consistent with the results shown in Figure 3 since the distance between donor and acceptor probes located near the antibody combining sites of A2 and B5, respectively, may not be significantly different when these derivatives are attached to IgE in solution than when the IgE is receptor-bound.

Conclusions

The results described in this and the preceding paper (Baird & Holowka, 1985), together with our previous measurements (Holowka & Baird, 1983b), have led us to propose a working model for the conformation of receptor-bound IgE and its orientation with respect to the membrane surface. The bending of receptor-bound IgE at the junction of its $C_\epsilon 2$ and $C_\epsilon 3$ segments suggested by our results has some precedence in the "staple" configuration of pentameric IgM bound to an antigen-coated surface (Feinstein et al., 1971) and in the asymmetric bend in the structure of the Mcg Bence-Jones light-chain dimer (Schiffer et al., 1973), but further experiments will be necessary to test our model more conclusively. The functional significance of this proposed asymmetric interaction of IgE with its receptor is unknown, but one possibility is that an orientation effect can be exerted on the interactions of receptors with each other or with other cellular components that may be necessary for cell triggering.

Of perhaps more general interest is our successful use of selectively labeled monoclonal antibodies as a means of placing donor and acceptor probes for resonance energy-transfer measurements, and this approach should be widely applicable to other cell surface proteins where selective modification of a particular component with fluorescent reagents is often difficult to achieve. Measurement of distances between different molecules on the cell surface should also be feasible with this approach, and possible interactions between these components may be investigated. In principle, monoclonal antibodies can be obtained for any surface component of interest, and further developments in selective chemical modifications of these antibodies will permit even more precise analyses of distances than those demonstrated in this study.

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