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Structural Mapping of Fc Receptor Bound Immunoglobulin E: Proximity to the Membrane Surface of the Antibody Combining Site and Another Site in the Fab Segments†

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ABSTRACT: Resonance energy-transfer methods have been used to investigate the structure of immunoglobulin E (IgE) bound to its high-affinity receptor on plasma membrane vesicles derived from rat basophilic leukemia cells. The structural mapping of receptor-bound IgE was initiated in an earlier study [Holowka, D., & Baird, B. (1983) *Biochemistry 22*, 3475], and it is based on measuring the minimal distance from IgE sites that are selectively labeled with donor probes to a plane of amphipathic acceptors at the membrane surface. This paper describes the use of monoclonal IgE specific for 5-(dimethylamino)naphthalene-1-sulfonyl (DNS) to place a donor probe, DNS-L-Lys, in the antibody combining sites. The distance from these sites to the membrane surface was determined to be greater than 100 Å with two different amphipathic acceptor probes. Another site in the Fab segments of monoclonal IgE (anti-dinitrophenyl) could be labeled selectively with *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide (CPM) in the absence of reducing agents [CPM(−)], and the reaction could not be blocked by prereaction with *N*-ethylmaleimide. The pattern of CPM(−)-labeled proteolytic fragments and the lack of fluorescence quenching by (trinitrophenyl)lysine in the antibody combining sites suggested the CPM(−)-labeled site to be in the $C_\epsilon1$ domain of IgE. The distance between this site on receptor-bound IgE and the membrane surface was determined to be 75-87 Å with two different amphipathic acceptors. When IgE was labeled with CPM at the inter heavy chain disulfides in $C_\epsilon2$ in the presence of reducing agents [CPM(+)] and employed in further energy-transfer experiments, we observed an apparent interaction between this probe and one particular amphipathic acceptor but only when the IgE was bound to receptor. This suggests a conformational change in IgE accompanies receptor binding. The results from the experiments reported here support our previous findings and provide additional information about the structural orientation of receptor-bound IgE.

Immunoglobulin E (IgE)[1] binds very tightly to its receptor on mast cells and basophils and mediates the triggering of cellular degranulation by multivalent antigen. As shown schematically in Figure 1, IgE is similar to other classes of antibodies and is composed of two heavy (ϵ) and two light polypeptide chains that are connected by disulfide bonds as well as by nonconvalent interactions. Amino acid sequencing of human ϵ (Bennich & von Bahr-Lindstrom, 1974) and DNA sequencing of the rodent ϵ gene (Ishida et al., 1982; Liu et al., 1982) have shown IgE to have a domain structure that is quite similar to immunoglobulin G except that IgE has an extra domain, $C_\epsilon2$, in the region corresponding to the hinge of IgG.

Some information has been obtained regarding the manner in which IgE associates with its receptor. The binding region apparently is contained within the $(C_\epsilon2\text{-}C_\epsilon3\text{-}C_\epsilon4)_2$ domains (Ishizaka et al., 1970; Dorrington & Bennich, 1978), and more specific localization of this region has been suggested by proteolytic digestion studies with rodent IgE (Perez-Montfort & Metzger, 1982). These studies revealed a site of trypsin cleavage between the $C_\epsilon2$ and $C_\epsilon3$ domains on free rat IgE that

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[1] Abbreviations: IgE, immunoglobulin E; RBL, rat basophilic leukemia; HAF, 5-(hexadecanoylamino)fluorescein; ORB, octadecylrhodamine B; HAE, 5-(hexadecanoylamino)eosin; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DNP, dinitrophenyl; TNP, trinitrophenyl; DNS, 5-(dimethylamino)naphthalene-1-sulfonyl; DNP-Lys, TNP-Lys, and DNS-Lys, L-lysine modified as indicated at the ϵ-amino group; IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; CPM, *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]maleimide; CPM(+)IgE, IgE that has been first reduced with dithiothreitol and then alkylated with CPM; CPM(−)IgE, IgE that has been reacted with CPM without prior reduction; $NaDodSO_4$, sodium dodecyl sulfate.

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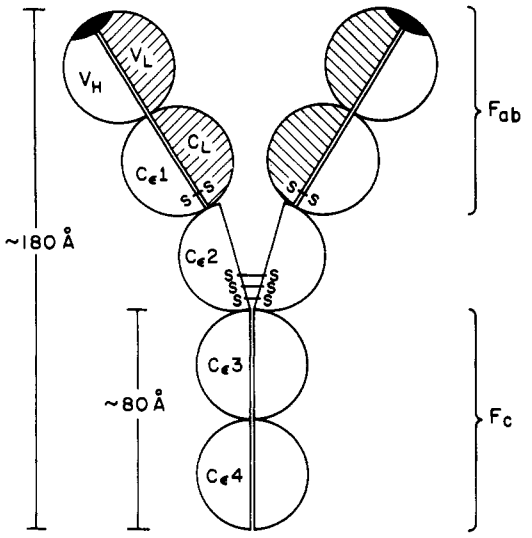

FIGURE 1: A schematic drawing of IgE. The light chains (hatched) and ϵ chains are arranged in domains, and the segments designated Fab and Fc are shown. The antibody combining sites at the top of the Fab segments (solid) and approximate locations of the interchain disulfide bonds are indicated. The length of the whole IgE molecule and the length of two domains are estimated from X-ray crystallographic studies of immunoglobulin G (Amsel & Poljak, 1979).

is markedly protected when this IgE is bound to its high-affinity receptor (α subunit) purified from rat basophilic leukemia (RBL) cells, while another cleavage site located within the $C_\epsilon 3$ domains is only marginally protected by receptor binding. Additional information has come from the use of two monoclonal antibodies generated against rat IgE, one apparently specific for a site in the Fab (V_H–$C_\epsilon 1$) domains and one apparently specific for a site in the F_c ($C_\epsilon 3$–$C_\epsilon 4$) domains (Conrad et al., 1983). Both of these antibodies appear to bind to the same extent whether the IgE is soluble or bound to receptors on RBL cells, indicating that these sites are not obscured by the receptor or other components of the plasma membrane.

In order to obtain more detailed information about the structure of receptor-bound IgE, we have applied resonance energy-transfer methods to measure distances between selected sites on IgE and the membrane surface. These measurements are carried out with large, unilamellar, and correctly oriented plasma membrane vesicles prepared from RBL cells (Holowka & Baird, 1983a). IgE that is selectively labeled with fluorescent donor probes binds with characteristic affinity to receptors on these vesicles, and the fluorescence of the donors is monitored as amphipathic acceptor probes are titrated into the sample and spontaneously insert into the vesicle bilayer in a random, noninteracting manner. Thus, the fluorescence quenching of donor observed as a function of the surface density of acceptor can be quantitatively interpreted in terms of a minimal distance between donor and plane of acceptors. A series of distances between different parts of receptor-bound IgE and the membrane surface can provide evidence concerning the level, orientation, and conformation of the IgE. In a previous paper (Holowka & Baird, 1983b), we described measurements of this type for two regions of IgE. The distance to the membrane from (coumarinylphenyl)maleimide (CPM) at sulfhydryls in the $C_\epsilon 2$ domains (Figure 1) was found to be about 45 Å, and the distance from fluoresceinyl isothiocyanate labeled groups in the Fab domains was found to be about 90 Å. These results put some constraints on the possible structure of receptor-bound IgE. In particular, they indicated that the Fab domains extend away from the membrane surface but that the whole IgE cannot be extended upward perpendicularly from its C-terminal end at the membrane surface since the distance to the membrane from the $C_\epsilon 2$ domains is substantially less than the length of two immunoglobulin domains corresponding to $C_\epsilon 3$ and $C_\epsilon 4$ (~80 Å). We suggested the possibility that bound IgE is bent near its point of attachment to the receptor.

In this and the following paper (Holowka et al., 1985), we describe further energy-transfer experiments that extend our structural mapping of IgE bound to receptors on plasma membrane vesicles. In this paper we report the measurement of distances to the membrane from two additional sites on IgE (see Figure 1): (1) the antibody combining site of a monoclonal anti-dansyl (DNS) IgE and (2) an unusually reactive site in the Fab region, in the $C_\epsilon 1$ domain, that is labeled by CPM in the absence of reducing agents. These measurements fully support our earlier results and interpretations. During the course of these experiments, we observed an apparent interaction between CPM at the $C_\epsilon 2$ sulfhydryls of IgE and an added amphipathic acceptor, but this occurred only when the IgE was bound to receptor, which suggested that receptor binding is accompanied by a conformation change. As described in the following paper (Holowka et al., 1985), we have directly investigated the conformation of receptor-bound IgE by placing donor and acceptor probes on monoclonal antibodies against the Fab and Fc portions of IgE (Conrad et al., 1983) and measuring the distance between these when they are bound to free IgE in solution and to IgE that is bound to the plasma membrane receptor.

Experimental Procedures

Chemicals. 5-(Hexadecanoylamino)fluorescein (HAF), 5-(hexadecanoylamino)eosin (HAE), 3-hexadecanoyl-7-hydroxycoumarin (HHC), octadecylrhodamine B (ORB), *N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]-maleimide (CPM), and 5-[[(iodoacetyl)amino]ethyl]-amino]naphthalene-1-sulfonic acid (IAEDANS) were obtained from Molecular Probes, Inc. 3,3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) was synthesized in the laboratory of A. Waggoner (Sims et al., 1974) and was a gift from W. W. Webb (Cornell University). Trinitrophenyl-L-lysine (TNP-Lys) 7-chloro-4-nitro-2,1,3-benzoxadiazole were from Research Organics. *N*-Ethylmaleimide and dansyl-L-lysine (DNS-Lys) were from Sigma Chemical Co. Dithiothreitol was from Bio-Rad. *N*-Succinimidyl propionate was a gift from J. Inman (NIH), and methyl acetamidate was from Pierce Chemical Co.

Preparation of IgE Derivatives. Murine monoclonal anti-2,4-dinitrophenyl-IgE from the tumor H1-DNP-ϵ-26.82 (Liu et al., 1980) was purified and labeled with ^{125}I as previously described (Holowka & Baird, 1983a). Purified murine monoclonal anti-DNS IgE (Oi et al. 1984) was the generous gift of Drs. Jeffry Reidler and Vernon Oi (Stanford University). It was further purified on a Sephacryl 300 column before use for most experiments.

The anti-DNP-IgE was alkylated with CPM either with [CPM(+)IgE] or without [CPM(−)IgE] prior reduction with dithiothreitol as previously described (Holowka & Baird, 1983b) with minor modifications. Briefly, IgE was treated with or without 1 mM dithiothreitol at pH 8.0 in a phosphate-buffered saline solution for 1–2 h, and then the pH was reduced to 7.5, and CPM was added. After 1–2 h at ambient temperature, the unreacted CPM was removed by centrifugation to separate insoluble dye followed by 0.45-μm membrane filtration and a centrifuge column of Sephadex G-50. For some experiments, CPM(−)IgE was prepared without lowering the pH for the CPM reaction because this increased

the stoichiometry of labeling from about 0.25 to 0.8 without changing the quantum yield, steady-state anisotropy, or papain digestion pattern of this derivative. In some cases, IgE was prereacted with 3 mM *N*-ethylmaleimide at pH 7.5 or 8.0 prior to reaction with CPM, but this resulted in little or no difference in the stoichiometry of CPM labeling (see Results). The concentration of IgE-bound CPM was determined from the OD at 395 nm, assuming $\epsilon = 3.0 \times 10^4$ M^{-1} cm^{-1} (Sippel, 1981). The IgE concentration was determined by using $\epsilon =$ 1.62 mL mg^{-1} cm^{-1} with M_r of 1.84×10^5 (Liu et al., 1980) and assuming no significant contribution from CPM at that wavelength (Holowka & Baird, 1983b). AEDANS(−)IgE was prepared with 5 mM IAEDANS at pH 7.0 and the same procedure as for CPM(−)IgE except that particular care was taken to keep the IAEDANS reagent in the dark prior to and during the reaction with IgE. A stoichiometry for IAEDANS labeling of 0.4 was determined from the OD at 337 nm, assuming $\epsilon = 6.5 \times 10^3$ M^{-1} cm^{-1} (Hudson & Weber, 1973). In some cases, IgE was prereacted with 10 mM *N*-ethylmaleimide, 10 mM *N*-succinimidyl propionate, or 0.5 mM 7-chloro-4-nitro-2,1,3-benzoxadiazole at pH 7.0 or 1 mM methyl acetamidate at pH 8.5 prior to reaction with IAEDANS, but this resulted in little or no difference in the stoichiometry of AEDANS modification. Derivatives of IgE were digested with papain and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as previously described (Holowka & Baird, 1983b).

RBL Cell Membrane Vesicle Preparation. The RBL cell subline 2H3 (Barsumian et al., 1981) was maintained in stationary cell culture (Taurog et al., 1979). Plasma membrane vesicles that contain the receptor for IgE and are largely right side out and sealed were prepared by chemically inducing adherent cells to vesiculate as described previously (Holowka & Baird 1983a) except that the vesiculating solution contained 25 mM formaldehyde and 2 mM DTT. The purified, dialyzed (phosphate-buffered saline, pH 7.4, 0.01% NaN_3, 1 mM phenylmethanesulfonyl fluoride) vesicles had receptors saturated with IgE derivatives for energy-transfer measurements as described (Holowka & Baird 1983b) by incubation of aliquots with a 3–5-fold excess over receptors of bindable IgE at 4 °C for several hours and then 10-fold dilution in the same buffer, centrifugation at 25000*g* for 30 min, and resuspension of the vesicles in the pellet. Energy-transfer samples generally contained about 10^7 cell equivalents of vesicles (as determined by relative ^{125}I-labeled IgE binding) in a volume of about 200 μL. This corresponds to an IgE receptor concentration of 17 nM.

Spectroscopic Measurements. Absorption spectra were recorded with a Cary 118 spectrophotometer. Fluorescence measurements were carried out at controlled temperature set at 20–25 °C in a Perkin-Elmer MPF-44B or an SLM 8000 fluorescence spectrophotometer. General procedures used for measuring energy transfer and probes in the membrane vesicle bilayer have been described (Holowka & Baird 1983a,b). For each distance determination, four separate samples, a–d, containing equivalent amounts of vesicles with receptor-bound and nonbound IgE derivatives were prepared, and the fluorescence of these was monitored as the acceptor probe partitioned into the membrane bilayer at increasing density. For experiments with CPM(−)IgE and CPM(+)IgE, sample a contained vesicles having receptors saturated with the donor-labeled IgE. Sample b vesicle receptors were saturated with excess unlabeled IgE, and donor-labeled IgE (non bound) was added such that the initial fluorescence was nearly the same as that of sample a. Sample c contained no donor-labeled IgE. For experiments with DNS in the antibody combining site, sample a vesicle receptors were saturated with anti-DNS IgE, and this IgE was titrated with DNS-Lys until the enhanced fluorescence signal (due to bound DNS) leveled off. Samples b and c were saturated with nonmodified anti-DNP IgE, and they were titrated with DNS-Lys in parallel to sample a to correct for any contributions of free DNS-Lys. Sample b was then titrated with anti-DNS (which became bound to DNS-Lys in solution) such that the fluorescence was nearly the same as that of sample a.

The energy transfer occurring from receptor-bound, donor-labeled IgE to acceptors at the membrane surface was calculated in terms of the ratio of the quantum yields of the donor in the absence (Q_D) and presence (Q_{DA}) of acceptor at each addition of the acceptor probe to the samples according to

$$Q_D/Q_{DA} = (I^b - I^c)/(I^a - I^c) \quad (1)$$

where I^a, I^b, and I^c are the intensities for the corresponding samples at the optimal excitation and emission wavelengths of the donor probe (see figure legends). In order to compare the anti-DNS IgE experiments, which had rather noisy data, the Q_D/Q_{DA} plots were slightly shifted (<5%) such that the best straight line through the data points of each experiment had an intercept at (0, 1), but the slope of the line was not affected. This small correction was not required for comparison of the CPM(−)IgE experiments.

Sample d was used to measure the surface density of the amphipathic acceptor being used at each point of the titration. This sample contained no donor-labeled IgE, but the vesicles had an appropriate amphipathic donor probe inserted in the bilayer: HAF was used as donor for acceptors HAE and ORB, and HHC was the donor for $DiOC_6$ and HAF. For calculation of acceptor density, the fluorescence of the donor probe was monitored at optimal excitation and emission wavelengths, and Q_D was taken as the fluorescence before any addition of acceptor. Aliquots of acceptor were added, and the donor fluorescence was measured to obtain the Q_{DA} for that point. As described previously (Holowka & Baird, 1983a), these data were fit to the two exponential curves presented by Wolber & Hudson (1979) for the situation of energy transfer in two dimensions, and in this way a given molar concentration of the amphipathic acceptor probe could be directly related to the surface density ($Å^{-2}$) of acceptors in the vesicles.

The time required for partitioning into the vesicle bilayer was predetermined for each amphipathic acceptor probe by monitoring fluorescence enhancement at the optimal wavelengths for that fluorophore (Holowka & Baird, 1983a). Since the acceptor ORB requires several hours to equilibrate at ambient temperature, an alternative titration scheme to that described above was employed. A single addition of ORB was made to each sample a–d, and the fluorescence of the donor in each was monitored at several time points such that increasing time corresponded to increasing density of acceptor in the membrane. Each sample was timed separately and then normalized. The ratio Q_D/Q_{DA} was calculated as in eq 1 for several time points at regular intervals by using the I^a, I^b, and I^c corresponding to those times, and these were determined by interpolating between the bracketing data points. The surface densities of acceptor at those points were determined from sample d in the same manner.

Energy-Transfer Analysis. Distances of closest approach (L) between donors located at sites on the receptor-bound IgE molecule and acceptors randomly distributed along the membrane surface were obtained by fitting the experimental quenching profile [Q_D/Q_{DA} vs. acceptor density ($Å^{-2}$)] to a

straight line with a linear least-squares algorithm. The slope of the best fit was related to L according to equation derived previously (Shaklai et al, 1977; Hammes, 1981):

$$\text{slope} = (\pi R_0^6/2)(1/L^4) \quad (2)$$

For the distances measured in the experiments reported here, where $L \geq 1.5\ R_0$, determination of L by a linear fit and eq 2 yields the same result within 2% as that obtained by fitting the experimental quenching profile with theoretical curves derived from Monte Carlo calculations of energy transfer between a plane of donors and a plane of acceptors (Snyder & Friere, 1982; Holowka & Baird, 1983b). The scatter of points about the best straight line was assessed in terms of a standard deviation of its slope (Bevington, 1969), and the range of distances L corresponding to this uncertainty was calculated from eq 2. The critical transfer distance, R_0, was calculated from (Forster, 1959)

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_D \eta^{-4})^{1/6}\ \text{Å} \quad (3)$$

Overlap integrals (J) were calculated as previously described (Cantley & Hammes, 1975); the orientation factor (κ^2) was taken as $^2/_3$; the refractive index of the medium (η) was taken as 1.4. The values for Q_D and the steady-state anisotropy ($\bar{A}$) used in this calculation were determined for each of the donors as described previously (Holowka & Baird, 1983a) with sodium fluorescein in 0.1 N NaOH as a standard for HAF and quinine sulfate in 0.1 N H_2SO_4 as a standard for DNS, IAEDANS, CPM, and HHC.

The energy-transfer distance R between the site on IgE labeled by CPM(−) or IAEDANS(−) in the absence of reduction (donors) and TNP-Lys in the antibody combining site (acceptor) was determined by monitoring the quenching of the donor fluorescence as the TNP-Lys was added in aliquots to a solution of labeled anti-DNP IgE. The data were analyzed according to (Forster, 1959)

$$Q_{DA}/Q_A = [1 + (R_0/R)^6]^{-1} \quad (4)$$

In eq 4, Q_D is the fluorescence before addition of TNP-Lys, and Q_{DA} is the fluorescence at a concentration where all antibody combining sites are filled as determined by monitoring the simultaneous quenching of IgE endogenous tryptophan (Eisen, 1964).

Results

Distance from IgE Antibody Combining Site to Membrane Surface. An IgE specific for the DNS moiety allows specific placement of a donor fluorescent probe in the antibody combining site. As described previously (Oi et al., 1984), DNS-Lys binds tightly to the monoclonal IgE used in our experiments with $K_d = 17 \pm 1$ nM. Furthermore, the fluorescence of the DNS group is enhanced >15-fold when it binds to the antibody combining site (Reidler et al., 1982) such that the relative fluorescence of free DNS-Lys is small under our experimental conditions. The four samples used routinely to measure energy transfer and to determine the distance from bound DNS-Lys to acceptor probes at the membrane surface allow correction for nonrelevant fluorescence changes as well as calculation of the acceptor surface density (see Experimental Procedures). Figure 2 shows the Q_D/Q_{DA} ratio as a function of surface density when the amphipathic acceptor HAE was titrated into the samples in two separate experiments. The fluorescence of the DNS-Lys was monitored at 505 nm with an excitation wavelength of either 340 (direct excitation) or 285 nm (indirect excitation via tryptophans present in IgE), and similar results were obtained. The data are scattered due to the low concentration of IgE in the samples (~17 nM), the low extinction

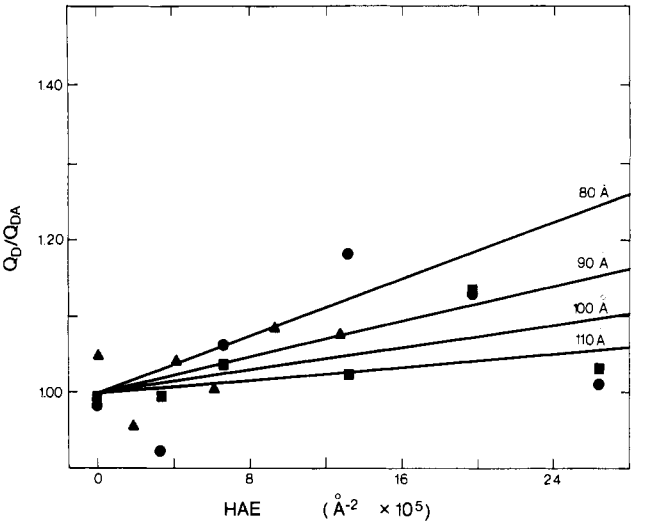

FIGURE 2: Energy transfer between DNS-Lys in the antibody combining sites of receptor-bound anti-DNS IgE and HAE acceptors at the membrane surface. The ratio of donor quantum yield in the absence of acceptors (Q_D) to that in their presence (Q_{DA}) is plotted as a function of acceptor density (acceptor molecules per angstrom squared). The (●) and (▲) points were calculated from separate experiments in which DNS fluorescence was monitored with excitation at 340 nm and emission at 505 nm. The (■) points were calculated from the same experiment and titration as the (●) points, but the excitation was at 285 nm. The solid lines were drawn from eq 2 with $R_0 = 53.7$ Å (Table I) and different possible values of L.

Table I: Summary of Energy-Transfer Measurements from Receptor-Bound IgE to the Vesicle Membrane Surface

donor	Q_D	$\bar{A}^a$	acceptor	R_0 (Å)	L (Å)
DNS-Lys bound to anti-DNS IgE	0.54	0.16	HAE	53.7	102[b]
DNS-Lys bound to anti-DNS IgE	0.54	0.16	ORB	57.2	117[c]
CPM(−)IgE	0.58	0.29	$DiOC_6$	55.7	87[d]
CPM(−)IgE	0.58	0.29	HAF	50.5	74[e]

[a] Steady-state anisotropy measured for donor at the wavelengths of maximum excitation and emission. [b] Average from three experiments shown in Figure 2. [c] Average from two experiments shown in Figure 4. [d] Average from three experiments shown in Figure 7A. [e] Taken from experiment shown in Figure 7C.

coefficient of DNS, and the resulting weakness of the fluorescence signal, but it is clear that only a small amount of energy transfer occurs between DNS-Lys in the antibody combining sites and HAE acceptors at the membrane surface when the donor probes are in the antibody combining sites. The best linear least-squares fit of all of the points has a slope that corresponds to a distance of 102 Å according to eq 2. The standard deviation of the slope corresponds to distances in the range of 92–119 Å (see Experimental Procedures). For visual comparison, Figure 2 shows theoretical lines for distances ranging from 80 to 110 Å. The best distance estimate of 102 Å for these data is included in the summary of energy-transfer parameters for all of the experiments in Table I.

To obtain a second energy-transfer measurement of the distance between DNS in the antibody combining site and the membrane surface, a different amphipathic acceptor, ORB, was used. Several hours are required for ORB to partition into the membrane bilayer at 20 °C such that monitoring sequential small additions of acceptor is impractical. Therefore, the experiment was carried out by making a single large addition of ORB to the vesicle samples and following appropriate fluorescence signal changes with time as this probe inserted into the bilayer. The uncorrected data from such a time course are shown in Figure 3 for the different experi-

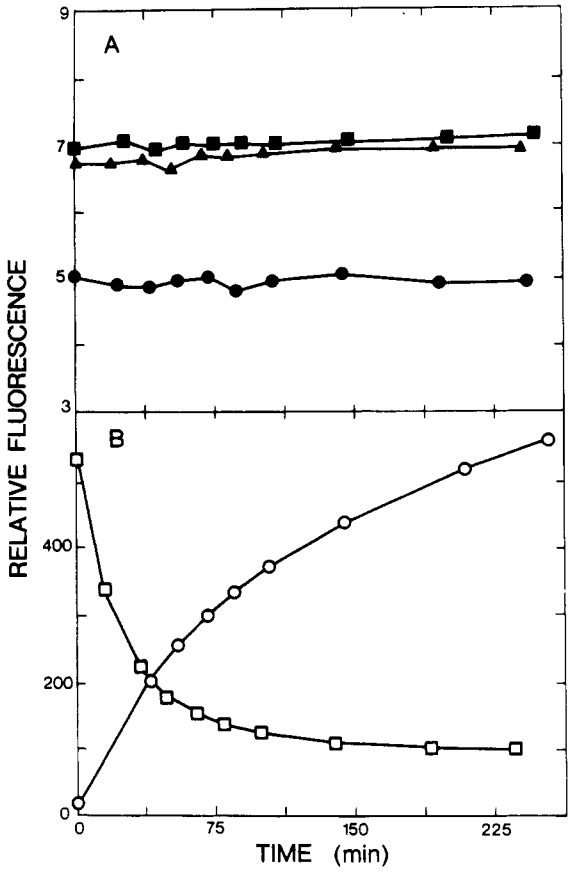

FIGURE 3: (A and B) Fluorescence changes of probes accompanying the time-dependent partitioning of ORB into vesicle membranes of different samples measured in parallel. A single addition of ORB (20 μM) was made to each sample at time = 0, and its fluorescence became enhanced as insertion into the membrane bilayer occurred [(○) excitation 340 nm, emission 580 nm]. Sample a [(■) excitation 340 nm, emission 505 nm] contained vesicles with receptor-bound anti-DNS IgE that had DNS-Lys in the antibody combining sites. Sample b [(▲) excitation 340 nm, emission 505 nm] contained vesicles with receptors saturated by anti-DNP IgE, and DNS-Lys bound to anti-DNS IgE was present in solution. Sample c [(●) excitation 340 nm, emission 505 nm] contained vesicles with receptors saturated by anti-DNP IgE, and DNS-Lys was present in solution, but no anti-DNS IgE was present. Sample d [(□) excitation 470 nm, emission 505 nm] contained vesicles that had the amphipathic donor HAF (4 μM) inserted into the bilayer. See Experimental Procedures for more detailed description of samples a–d.

mental samples. There is very little change in the fluorescence signal of DNS-Lys in the combining sites of receptor-bound IgE [sample a; Figure 3A (■)] as ORB partitions into the vesicle bilayer [Figure 3B (○)] and quenches the fluorescence of the amphipathic donor HAF [sample d; Figure 3B (□)]. Plots of Q_D/Q_{DA} vs. ORB surface density are shown in Figure 4 for two experiments. The best linear least-squares fit of the data yields a distance of 117 Å according to eq 2, and the standard deviation in the estimate of the slope corresponds to distances in the range of 107–135 Å.

Distance from the CPM(−) Site on IgE to the Membrane Surface. In our previous investigation (Holowka & Baird, 1983b) monoclonal anti-DNP IgE was specifically labeled in the $C_\epsilon 2$ domain by selective reduction of the disulfide bonds located there (Ishida et al., 1982; Liu et al., 1982), followed by alkylation with CPM. This site has been designated CPM(+). As we noted earlier (Holowka & Baird, 1983b), limited labeling of IgE by CPM occurs in the absence of reduction, and this site is designated CPM(−). The site is similarly labeled with IAEDANS or (iodoacetamido)-fluorescein, but it is not blocked by prereaction with *N*-

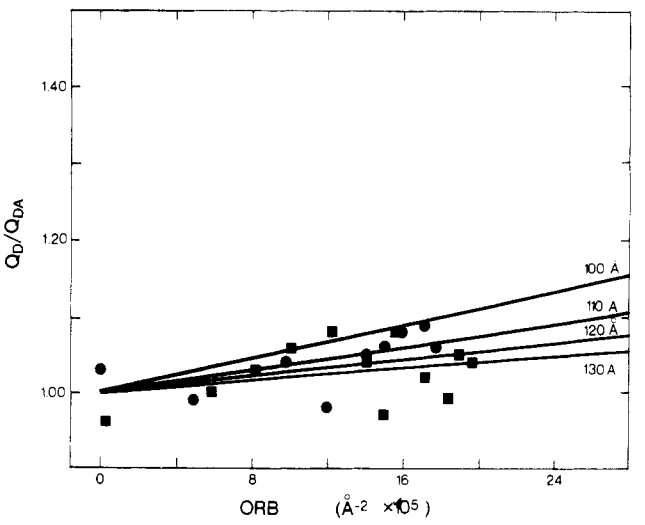

FIGURE 4: Energy transfer between DNS-Lys in the antibody combining site of receptor-bound anti-DNS IgE and ORB acceptors at the membrane surface. The ratio of donor quantum yield in the absence of acceptors (Q_D) to that in their presence (Q_{DA}) is plotted as a function of acceptor density (acceptor molecules per angstrom squared). The (●) points were calculated from the experiment shown in Figure 2, and the (■) points were calculated from a separate experiment carried out in the same manner. The solid lines are drawn from eq 2 with R_0 = 57.2 Å (Table I) and different possible values of L.

ethylmaleimide (Holowka & Baird, 1983b), iodoacetamide, methyl acetimidate, *N*-succinimidyl propionate, or 7-chloro-4-nitro-2,1,3-benzoxadiazole, suggesting the importance of the hydrophobicity provided by the ring structures of the fluorophores. This site is restricted to the ϵ chain as shown by the appearance of a fluorescent band in that molecular weight region after the modified protein has been run on NaDodSO_4–polyacrylamide gels in the presence of reducing agents [data for CPM(−) given in Holowka & Baird (1983b)]. Further localization of the site is provided by papain digestion of CPM(−)IgE followed by electrophoresis on NaDodSO_4–polyacrylamide gels in the presence of reducing agents. These gels show fluorescence in bands at 50 and 40 kDa that appear to correspond to the V_H–$C_\epsilon 1$–$C_\epsilon 2$ and V_H–$C_\epsilon 1$ fragments, respectively, as previously described (Holowka & Baird, 1983b). No fluorescence was observed in a band at 34 kDa, which may be composed of the $C_\epsilon 3$ and $C_\epsilon 4$ domains on the basis of the observations that this papain fragment also is not fluorescent in CPM(+)IgE (with label in $C_\epsilon 2$ domains) or fluoresceinyl isothiocyanate labeled IgE (label in V_H–$C_\epsilon 1$ domains) (Holowka & Baird, 1983b). Since the site labeled in CPM(−)IgE appeared to be at a selected location in the Fab region on the ϵ chain, it was of interest to measure the distance between it and the antibody combining site. TNP-Lys binds tightly to the anti-DNP IgE, and this chromophore has an absorption spectrum that partially overlaps the fluorescence spectrum of CPM(−)IgE such that energy transfer may occur over a limited distance range. Figure 5 shows a titration of CPM(−)IgE with TNP-Lys in which the fluorescence of both CPM and endogenous tryptophans are monitored for quenching due to TNP binding in the antibody combining site. Under conditions where the tryptophan fluorescence is maximally quenched (≤16 nM TNP-Lys) there is less than 1% quenching of the CPM fluorescence. The R_0 value for CPM(−)IgE and TNP-Lys was determined to be 31.5 Å, and by use of Q_{DA}/Q_D = 0.97 as a lower limit to account for some error in measurement, the distance between the CPM(−) site on IgE and TNP in the combining site was calculated to be ≥56 Å with eq 4. The same experiment was carried out with

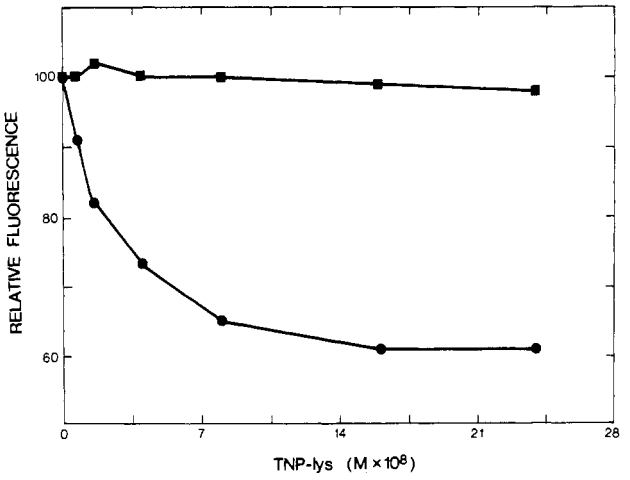

FIGURE 5: Fluorescence changes of CPM(−)IgE (anti-DNP) accompanying the addition of TNP-Lys to the antibody combining sites: (■) CPM fluorescence (excitation 395 nm, emission 470 nm); (●) fluorescence of tryptophans that are endogenous to the IgE (excitation 280 nm, emission 350 nm).

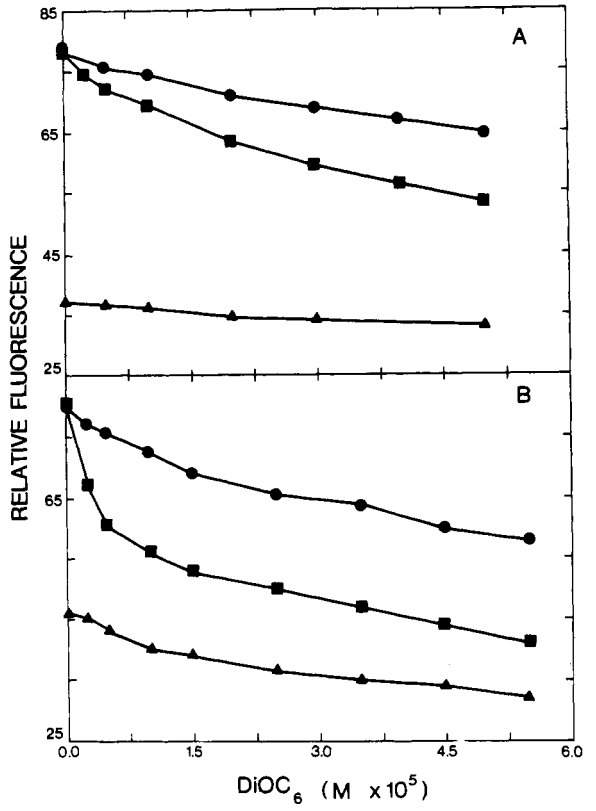

FIGURE 6: CPM fluorescence changes with addition of amphipathic acceptor $DiOC_6$ to vesicle samples measured in parallel for experiments with (A) CPM(−)IgE (excitation 400 nm, emission 450 nm) and (B) CPM(+)IgE (excitation 390 nm, emission 460 nm). Sample a (■) contained vesicles with receptor-bound CPM(+ or −)IgE. Sample b (●) contained vesicles with receptors saturated by unlabeled IgE, and CPM(+ or −)IgE was present in solution. For the CPM(−)IgE experiment, sample c (A, ▲) contained vesicles with receptor-bound unlabeled IgE. For the CPM(+)IgE experiment, sample c (B, ▲) contained vesicles with receptor-bound CPM(−)IgE (prepared under exactly identical conditions as the CPM(+)IgE but without dithiothreitol) in order to correct for nonrelevant CPM contributions to the fluorescence.

anti-DNP IgE that had been labeled in the absence of reduction with IAEDANS, which is probably going to the same site on the basis of stoichiometry, lack of blocking by *N*-ethylmaleimide, and migration on $NaDodSO_4$ gels. Little or no quenching of the IAEDANS(−) on IgE due to TNP-Lys

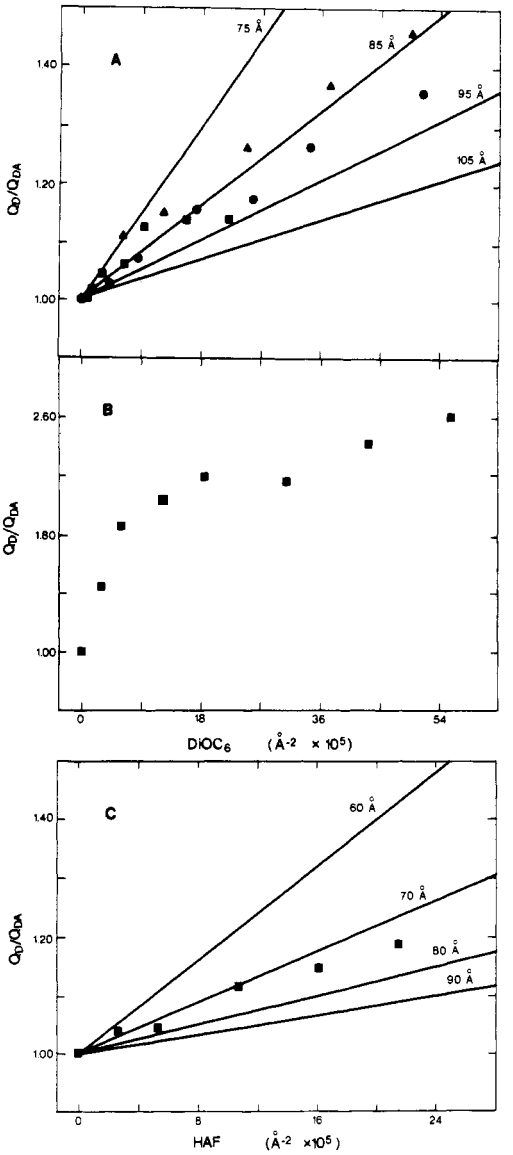

FIGURE 7: Energy transfer between receptor-bound CPM(−)IgE (A and C) or CPM(+)IgE (B) and the amphipathic acceptor $DiOC_6$ (A and B) or HAF (C). The ratio of donor quantum yield in the absence of acceptors (Q_D) to that in their presence (Q_{DA}) is plotted as a function of acceptor density (acceptor molecules per angstrom squared). In (A) the (▲) points were calculated from the experiment shown in Figure 6A; the (●) and (■) points were calculated from separate experments carried out in the same manner. In (B) the points were calculated from the experiment shown in Figure 6B. The solid lines in (A) and (C) were drawn from eq 2 with the R_0 value given in Table I and different possible values of L.

in the antibody combining site was observed, and a minimal distance ≥41 Å between this donor and acceptor was calculated from eq 4. Since the length of a single immunoglobulin domain is about 40 Å (Amsel & Poljak, 1979), these energy-transfer measurements together with the papain digestion studies described above indicate that the site labeled in CPM(−)IgE is probably located in the $C_\epsilon 1$ domain.

The distance between CPM(−) on vesicle receptor bound IgE and the membrane surface was measured with $DiOC_6$ as an amphipathic acceptor probe. Figure 6A shows fluorescence quenching data from a representative experiment, and Figure 7A shows a plot of Q_D/Q_{DA} vs. $DiOC_6$ surface density from three different experiments. The best linear least-squares fit of the data in Figure 7A yields a distance of 87 Å, and the standard deviation in the estimate of the slope corresponds to distances in the range of 82–94 Å.

A second amphipathic acceptor, HAF, was used to measure energy transfer between CPM(−) and the membrane surface, and a plot of Q_D/Q_{DA} vs. acceptor density for this case is shown in Figure 7C. The best linear least-squares fit of these data correspond to a distance of 74 Å with an uncertainty in the range of 72–77 Å.

Evidence for a Conformational Change When CPM(+)IgE Binds to Receptor. We previously used fluorescence quenching to measure the distance between CPM(+) at the sulfhydryl sites in the $C_\epsilon 2$ domain of receptor-bound IgE and the vesicle bilayer surface with titrations of both HAF and 3,3′-didecyloxacarbocyanine ($DiOC_{10}$) as amphipathic acceptor probes (Holowka & Baird, 1983b). When attempting to make this measurement with a similar titration of $DiOC_6$ as acceptor, we consistently observed unusual biphasic quenching of receptor-bound CPM(+)IgE. In particular, there appeared to be a binding interaction between CPM(+) and $DiOC_6$, which occurred only when the IgE was bound to receptor. As compared to free CPM(+)IgE (●) in Figure 6B, the fluorescence of CPM(+)IgE that is vesicle receptor bound (■) decreases steeply with initial additions of $DiOC_6$ followed by a much more gradual quenching. Correspondingly, the plot of Q_D/Q_{DA} vs. $DiOC_6$ surface density is biphasic (Figure 7B). The high degree of quenching observed with CPM(+)IgE at low concentrations of $DiOC_6$ is not obvious when CPM(−)IgE bound to vesicle receptors is titrated with the same acceptor, although a small amount of extra quenching in the initial points may be present and not discernible from the experimental error (Figures 6A and 7A). A possible explanation for these observations is that there is a saturable high-affinity site for $DiOC_6$ on the receptor, but more quenching of receptor-bound CPM(−)IgE and an underestimated value for the corresponding L would be expected in this case. The data are more suggestive of a unique site for $DiOC_6$ present only on CPM(+)IgE near the CPM(+)-labeled sulfhydryls in the $C_\epsilon 2$ domains, and this site becomes exposed only after the reduced and alkylated IgE binds to receptor.

Discussion

The energy-transfer distances summarized in Table I support our previous measurements (Holowka & Baird, 1983b) and provide a more detailed structural map of IgE bound to its high-affinity receptor on the plasma membrane. Elucidation of this structure has been aided substantially by our ability to measure the distance between the membrane surface and the antibody combining sites at the tips of the Fab segments of IgE (see Figure 1). This was made possible by the availability of a monoclonal IgE that is a member of a family of monoclonal immunoglobulins with identical specificty for DNS that has been extensively characterized (Reidler et al., 1982; Oi et al., 1984). DNS-Lys was very useful as a donor probe because of its tight binding to this site and its spectral overlap with the amphipathic acceptor probes HAE and ORB, resulting in R_0 values of about 55 Å (Table I). There was very little quenching of DNS-Lys bound to IgE by either of these acceptors inserted into the membrane (Figures 2–4), and the small increase of quenching observed with increasing surface density of acceptor corresponded to distances greater than 100 Å according to eq 2.

Although the CPM(−) site on IgE that is labeled in the absence of reducing agents is not so well-defined as the antibody combining site, we have partially characterized its location. The site can be labeled to about the same extent (about 0.4 mol/mol of IgE) by the fluorophores CPM, IAEDANS, and (iodoacetamido)fluorescein, which are generally selective for sulfhydryl groups, but the modification cannot be blocked by prereaction with excess *N*-ethylmaleimide or iodoacetamide. The modification also cannot be blocked by methyl acetimidate or *N*-succinimidyl proprionate, which react with amino groups (Wofsy & Singer, 1963; Lomant & Fairbanks, 1976), or by 7-chloro-4-nitro-2,1,3-benzoxadiazole, which reacts with the amino, sulfhydryl, and phenol hydroxyl groups (Ferguson et al., 1974). It may be that the unusual reactivity of this site is due to its microenvironment in the IgE polypeptide, and the hydrophobic rings of the fluorophores enhance its accessibility. Digestion of CPM(−)IgE with papain yielded a pattern of fluorescently labeled fragments that suggested the site is located in either the V_H or the $C_\epsilon 1$ domain (see Figure 1). Location in the $C_\epsilon 1$ domain is consistent with the distance to TNP-Lys in the antibody combining site that we determined to be >56 Å (Figure 5), since the length of one immunoglobulin domain corresponding to V_H is about 40 Å (Amsel & Poljak, 1979). The distance between the CPM(−) site on receptor-bound IgE and the membrane surface was measured to be in the range of 75–85 Å $DiOC_6$ and HAF as amphipathic acceptor probes (Figure 7A,C).

We have discussed previously the features of our experimental samples and procedures that facilitate the accurate measurement of distances in the structure of IgE bound to receptor on plasma membrane vesicles (Holowka & Baird, 1983a,b). The generally good reproducibility obtained with a given donor and the same acceptor in separate experiments is evident in Figures 2, 4, and 7. In each set of experiments, the data could be fit with a straight line according to eq 2, and the degree of scatter (random error) could be assessed in terms of the standard deviation of the slope and the resulting range of distances [<20% for the DNS-Lys experiments; <10% for the CPM(−) experiments]. Systematic errors in these experiments have been pointed out (Holowka & Baird, 1983a,b), including those introduced by subtracting the fluorescence signals of two samples (eq 1) and in the determination of acceptor density. The use of $\kappa^2 = {}^2/_3$ in eq 3 should not introduce a substantial error since the steady-state anisotropy values (Table I) show the CPM(−) and DNS-Lys emission dipoles to have some angular rotation, and a distribution of acceptor dipoles also is expected (Holowka & Baird, 1983a). A simple analysis of propagation of the major sources of systematic error in the data gives limits of ±20% to the distances determined for a given donor–acceptor pair. This range is larger than the variation in the measured distances between the same donor and the two different acceptors listed in Table I, which indicates that any conformational or other unusual effects imposed by a particular acceptor are relatively minor.

The determined distances between CPM(−) and DNS-Lys bound to IgE and the membrane surface should be interpreted in terms of a distributional average position of the donor probe. For the case of DNS-Lys in the antibody combining sites, the limits of the possible positions would be set by the flexibility of the Fab segments in receptor-bound IgE. For the case of CPM(−), the average would be over the position allowed by IgE flexibility as well as by any heterogeneity of the amino acids labeled within the polypeptide. In both cases, all of the possible distances would be averaged according to an equation analogous to eq 2:

$$\text{slope} = \frac{\pi R_0^6}{2n} \sum_{j=1}^{n} \frac{a_j}{L_j^4} \qquad (5)$$

where n is the number of possible positions, L_j is the minimal distance from the membrane to the jth position, and a_j is the

relative amount of probe in that position (Holowka & Baird, 1983b). Thus, eq 5 shows how the probes closer to the membrane are more heavily weighted in our distance determinations.

The distances obtained in these experments fit into the structural map of IgE bound to receptor on the plasma membrane that was initiated by experiments described in our earlier paper (Holowka & Baird, 1983b) (see introduction). At an average distance of about 80 Å, the CPM(−) sites that are probably located in the $C_\epsilon1$ domains appear to be further away from the membrane surface than the CPM(+) sites, which are at the two or three pairs of sulfhydryls in the $C_\epsilon2$ domains at an average distance of about 45 Å (see Figure 1). The average distance from the membrane surface of the fluoresceinyl isothiocyanate labeled sites was previously determined to be about 90 Å. These sites are also located in the Fab segments and on the basis of energy transfer to TNP-Lys appear to be closer to the antibody combining sites than are the CPM(−) sites (Holowka & Baird, 1983b). Our current experiments indicate that a large fraction of the sites reacting with fluoresceinyl isothiocyanate under the conditions used are the N-terminal amino groups (Holowka et al., 1985). DNS-Lys in the antibody combining sites appears to be farthest away from the membrane surface at greater than 100 Å. Considering the distances of CPM(+) and DNS-Lys sites from the membrane together with the dimensions of the IgE polypeptide (Figure 1), these results can be interpreted in terms of, but do not distinguish between, the two models proposed previously (Holowka & Baird, 1983b). The first model has the C-terminal end of receptor-bound IgE near the membrane surface, and the length of the polypeptide is rather rigidly disposed at an angle to the surface. The second model suggests that binding to receptor imposes a conformational change on IgE such that the polypeptide is bent in the region of the $C_\epsilon2$–$C_\epsilon3$ interface near its point of attachment to the receptor subunit. Recent studies in our laboratory indicate that IgE in solution has some segmental flexibility and that this is largely retained when IgE becomes bound to receptor on plasma membrane vesicles (Slattery et al., 1985), and so the first model may be less likely considering the average distances determined in the present study.

In this paper we presented some new data regarding the conformation of receptor-bound IgE. These were in the energy-transfer experiment using the reduced and alkylated derivative CPM(+)IgE and $DiOC_6$. We reproducibly observed that $DiOC_6$ at low concentrations quenched the CPM fluorescence significantly more for bound CPM(+)IgE than for free CPM(+)IgE (Figures 6B and 7B) or for free or bound CPM(−)IgE (Figures 6A and 7A), indicating a site for direct $DiOC_6$ interaction on CPM(+)IgE that becomes exposed only upon binding to the receptor. This site appears to saturate, and the more gradual quenching of CPM(+) that occurs with higher concentrations of $DiOC_6$ is similar in nature to the other titrations of donor-labeled IgE with amphipathic acceptors that are inserting into the membrane bilayer, i.e., Q_D/Q_{DA} is roughly linear with the surface density of acceptor (Figure 7B). Although this evidence is circumstantial, a conformational change in reduced and alkylated IgE upon binding to receptor is suggested. A more systematic investigation of the conformation of receptor-bound IgE is presented in the following paper.

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