Biochemistry

 Volume 30, Number 38 September 24, 1991

Accelerated Publications

Two Distinct Subunits of Hemerythrin from the Brachiopod *Lingula reevii*: An Apparent Requirement for Cooperativity in O_2 Binding†

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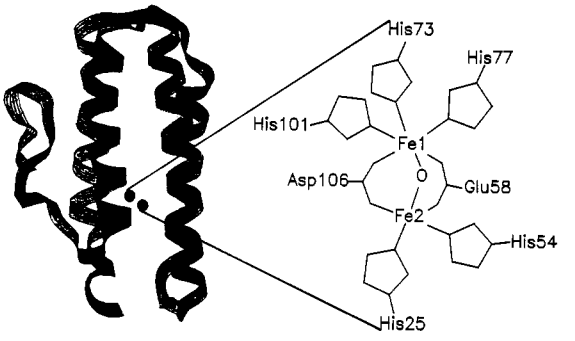
Received May 8, 1991; Revised Manuscript Received July 12, 1991

ABSTRACT: Reported are results on the subunit composition of octameric hemerythrin (Hr) from the brachiopod *Lingula reevii*. Unlike most other Hrs, *L. reevii* Hr shows cooperativity in O_2 binding. Purified *L. reevii* Hr was found to consist of two different subunits in approximately equimolar proportions. These two subunits differ in molecular weight by approximately 1000. Amino acid sequence data for the first 24 residues of the two subunits, labeled α and β, show 70% identity with each other. Comparisons to amino acid sequences of other Hrs show approximately 50% identity in the first 24 residues and that both the α and β subunits of *L. reevii* Hr have one residue deleted at their amino termini. Very recently, one other Hr, that from the brachiopod *Lingula unguis*, was also shown to contain equimolar proportions of two different subunits [Satake, K., Yugi, M., Kamo, M., Kihara, H., & Tsugita, A. (1990) *Protein Seq. Data Anal. 3*, 1–5], and this Hr also shows cooperativity in O_2 binding. An $\alpha_4\beta_4$ octamer is, therefore, proposed to be a common feature of those Hrs that show such cooperativity. Likely arrangments of α and β subunits within an $\alpha_4\beta_4$ octamer having the same configuration of subunits as that in other octameric Hrs are proposed. The most probable arrangements can be readily derived from physically resonable restrictions on the types of intersubunit interactions and on transmission of allosteric effects.

Hemerythrin (Hr)[1] is an O_2-carrying protein found in a few phyla of marine invertebrates, including sipunculids and brachiopods. Despite its name, Hr contains no heme group but rather a non-heme diiron site that reversibly binds one molecule of O_2. Hr thus poses interesting contrasts in evolution, physiology, and molecular structure to the more widespread heme oxygen carriers. Several reviews have summarized the available data on spectroscopy, reactivity, and molecular structure/function relationships of Hr (Wilkins & Harrington, 1983; Kurtz & Klotz, 1984; Sanders-Loehr, 1989; Kurtz, 1991). Both sipunculid and brachiopod Hrs usually occur as octamers with $M_r \sim 108\,000$ and subunit $M_r \sim 13\,500$. MyoHr is an analogous monomeric O_2-carrying protein found in muscle tissues of sipunculids, having a molecular weight and structure very similar to those of a subunit of Hr (Holmes et al., 1991; Sheriff et al,. 1987). The secondary, tertiary, and diiron site structures of both Hr and myoHr are shown schematically in Chart I.

Chart I



Hrs from coelomic hemerythrocytes of sipunculids do not bind O_2 in a cooperative manner, whereas Hrs from coelomic hemerythrocytes of two brachiopod species, *Lingula unguis*

† This research was supported by a grant from the National Institutes of Health (D.M.K., GM 40388). D.M.K. is an NIH Research Career Development Awardee during 1988–1993 (HL 02207).

[1] Abbreviations: Hr, hemerythrin; myoHr, myohemerythrin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid.

0006-2960/91/0430-9121$02.50/0

and *Lingula reevii*, do show such cooperativity (Manwell et al., 1960; Richardson et al., 1983, 1987). For *L. reevii* Hr the Hill coefficient, n, varies from ~ 1 at pH ≤ 6.8 to $n_{max} \sim 2$ at pH 7.7 in phosphate buffer. Brief reports have appeared claiming that vascular Hrs of some sipunculids show cooperativity in O_2 binding (Sanders-Loehr, 1989; Mangum & Burnett, 1987). However, these vascular Hrs have not been characterized in any detail. Here we report results on the subunit composition of *L. reevii* Hr which suggest that an $\alpha_4\beta_4$ octamer is a common feature of those Hrs that show cooperativity in O_2 binding.

Materials and Methods

Hr from coelomic hemerythrocytes of the sipunculid *Phascolopsis gouldii* was isolated and purified as described previously (Zhang et al., 1991). Live specimens of *L. reevii* were obtained from The Deep Blue C Company, Honolulu, Hawaii. OxyHr was isolated and purified from coelomic hemerythrocytes by the previously described procedure (Richardson et al., 1987) except that a 2.6 × 100 cm water-jacketed Superose 12 column (from Pharmacia LKB) maintained at 4 °C was used for the gel filtration. Eluted fractions with an absorbance ratio A_{280}/A_{330} between 4.2 and 4.7 were collected and frozen at −80 °C. *L. reevii* Hr was further purified for amino acid sequencing by reverse-phase HPLC on a Beckman Model 332 instrument using a 4.6 × 250 mm ultrasphere octyl column, which had been preequilibrated with 0.1 vol % TFA in water prior to sample loading. After injection of protein, a linear 0–80 vol % water/acetonitrile gradient with both solvents containing 0.1 vol % TFA was run in 2.5 h at 0.35 mL/min while the eluate was monitored at 280 nm. Two major bands appeared on the chromatogram. The fractions comprising each band were lyophilized, redissolved in 0.1 vol % TFA in water, and rerun on the HPLC column as described above. After lyophilization, the major protein-containing fractions were subjected to N-terminal amino acid sequencing by automated Edman degradation on an Applied Biosystems Model 477A sequencer. In one experiment *L. reevii* Hr was subjected to anion-exchange chromatography between the gel filtration and reverse-phase HPLC steps. The anion-exchange chromatography was performed on a Pharmacia LKB FPLC system using a 5 × 50 mm Mono Q column equilibrated with 10 mM TAPS buffer, pH 8.9. Approximately 1 mg of Hr was applied to the column. The protein was eluted with a linear gradient containing from 0 to 100% 0.25 M Na_2SO_4 in 10 mM TAPS buffer pH 8.9, over 140 min. Absorbance of the eluate was monitored at 280 nm by using an ISCO V^4 absorbance detector. SDS–PAGE was performed according to the procedure of Laemmli (1970) in a 16.5% acrylamide gel with a Bio-Rad cell and Model 3000xi power supply. Gels were stained for proteins with Coomassie Blue R-250. Myoglobin fragments as molecular weight standards were purchased from LKB Bromma.

Results and Discussion

The native *L. reevii* oxyHr eluted from the 2.6 × 100 cm Superose 12 gel-filtration column as a single band, and the combined major fractions comprising this band were subjected to both SDS–PAGE and reverse-phase HPLC. Two bands with approximately equal intensity were resolved upon SDS–PAGE, as shown in Figure 1. We label the higher molecular weight band as α and the lower molecular weight band as β. Molecular weights of approximately 14000 for α and 13000 for β were estimated by comparison to molecular weights of

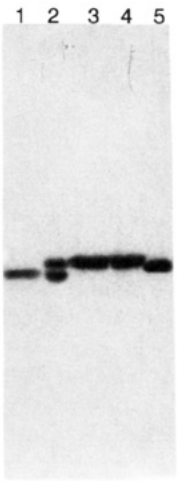


FIGURE 1: SDS–PAGE of *L. reevii* Hr (lanes 1–4) and *P. gouldii* Hr (lane 5). Lane 1, second eluting major band from reverse-phase HPLC (cf. Figure 2); lane 2, Hr purified only by gel filtration; lanes 3 and 4, first eluting band from reverse-phase HPLC (cf. Figure 2).

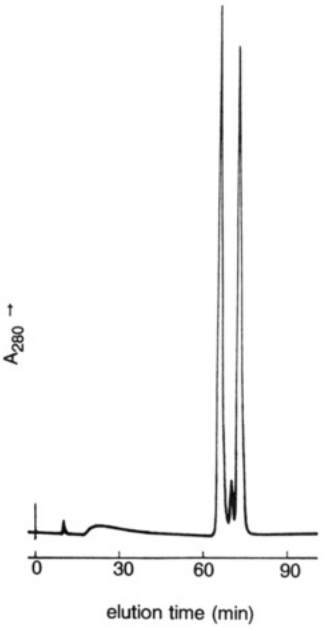


FIGURE 2: Reverse-phase HPLC chromatogram of *L. reevii* Hr, as monitored by absorbance of the eluate at 280 nm. The minor peak between the two major bands, which could be a contaminating protein or a minor Hr component, was not further analyzed.

the *P. gouldii* Hr subunit (13500), myoglobin fragment I (8160), and myoglobin fragments I and II (14400) (the latter two standards were run with *L. reevii* Hr on a different gel and are not shown in Figure 1.) Figure 2 shows that passage of the *L. reevii* Hr over a reverse-phase HPLC column also resolves two major bands of approximately equal intensity, when monitored by absorbance at 280 nm. By using the relatively shallow gradient of water/acetonitrile described under Materials and Methods, the two bands were well resolved and eluted from the column at about 45–55 vol % acetonitrile. *L. reevii* Hr that had been purified by gel filtration eluted as a single sharp band from an anion-exchange column at ~23% of 0.25 M Na_2SO_4 in 10 mM TAPS buffer, pH 8.9. Reverse-phase HPLC of this anion-exchange purified Hr showed two bands of approximately equal intensity, similar to that shown in Figure 2. The proteins in the two major HPLC bands shown in Figure 2 were further purified separately by the same HPLC procedure. SDS–PAGE of these

	1							10											20						
L. reevii α	V	K	V	P	A	P	F	A	W	N	E	D	F	A	T	S	Y	K	F	I	D	L	E	H
L. reevii β	M	K	V	P	A	P	Y	A	W	N	S	D	F	A	T	T	Y	E	N	I	D	S	E	H
L. unguis α	V	K	V	P	E	P	F	A	W	N	E	S	F	A	T	S	Y	K	N	I	D	L	E	H
L. unguis β	M	K	I	P	V	P	Y	A	W	T	P	D	F	K	T	T	Y	E	N	I	D	S	E	H
P. gouldii	G	F	P	I	P	D	P	Y	V	W	D	P	S	F	R	T	F	Y	S	I	V	D	D	E	H
T. dyscritum	G	F	P	I	P	D	P	Y	C	W	D	I	S	F	R	T	F	Y	T	I	V	D	D	E	H
S. cumanense	G	F	E	V	P	D	P	F	I	W	D	A	S	F	K	T	F	Y	D	D	L	D	N	Q	H
T. zoster. myo	G	W	E	I	P	E	P	Y	V	W	D	E	S	F	R	V	F	Y	E	Q	L	D	E	E	H
P. gouldii myo	P	F	D	I	P	E	P	Y	V	W	D	E	S	F	R	V	F	Y	D	N	L	D	D	E	H

FIGURE 3: N-terminal amino acid sequences of Hrs and myoHrs (the latter labeled as myo). *L. reevii* Hr α and β sequences are from this work. *L. unguis* Hr α and β sequences are from Satake et al. (1990). *P. gouldii*, *T. dyscritum*, and *Siphonosoma cumanense* Hr sequences are from Klippenstein et al. (1968), Loehr et al. (1978), and Uchida et al. (1990), respectively. *T. zostericola* and *P. gouldii* myoHr iso I sequences are from Klippenstein et al. (1976) and R. C. Long, D. M. Kurtz, Jr., and J.-H. Zhang (unpublished results), respectively. The *Lingula* sequences are aligned to start at position 2 of the other Hr and myoHr sequences.

purified fractions indicates that the first eluting HPLC band consists of α subunit and the second eluting major band consists of β subunit (Figure 1). The N-terminal amino acid sequence data shown in Figure 3 confirm that α and β are indeed distinct subunits with 70% identity found in the first 24 residues.

Previous reports (Richardson et al., 1983, 1987) indicated that *L. reevii* Hr is octameric with M_r of ~105 000 and a subunit M_r of ~13 500, which agrees with the average molecular weight for a 1:1 mol ratio of α (~14 000) and β (~13 000) subunits found in the present work. However, these previous reports contained no evidence for more than one type of subunit. In fact, prior to this work, all Hrs were reported to contain either one type of subunit or a set of very closely related subunits, differing in no more than 5 residues out of 113 (Klippenstein, 1972, 1980). In no previous case were only two different subunits obtained in a ~1:1 mole ratio. After completion of this work we became aware of a brief report by Satake et al. (1990), which contains results very similar to ours on another Hr known to bind O_2 cooperatively, namely, that from the brachiopod *L. unguis*. We have included their N-terminal amino acid sequence data in Figure 3. A comparison of N-terminal sequences of the two α subunits (i.e., the heavier subunits) or the two β subunits (i.e., the lighter subunits) of *L. reevii* vs *L. unguis* Hr shows that 21 out of the first 24 residues are conserved in the α subunits and 19 out of the first 24 residues are conserved in the β subunits. Comparisons with sequences of other Hrs and myoHrs (Figure 3) indicate that both α and β subunits in *L. reevii* Hr have one residue deleted at their amino termini, as is also observed for *L. unguis* Hr subunits (Satake et al., 1990). Although half of the first 24 residues are conserved in the four different subunits of *L. reevii* and *L. unguis* Hrs, only seven of the *Lingula* Hr residues, namely, Pro5, Pro7, Trp10, Phe14, Tyr18, Asp22, and His25, are conserved in the three other Hrs and two myoHrs (cf. Figure 3), none of which show cooperativity in O_2 binding. This comparison suggests that these seven conserved residues by themselves are not essential for transmitting allosteric interactions between subunits. His25 is almost certainly an iron ligand residue in the *Lingula* Hrs, as has been proven by X-ray crystallography of *Themiste dyscritum* Hr and *Themiste zostericola* myoHr (cf. Chart I) (Stenkamp et al., 1984; Sheriff et al., 1987). Iron ligand residues could, of course, be involved

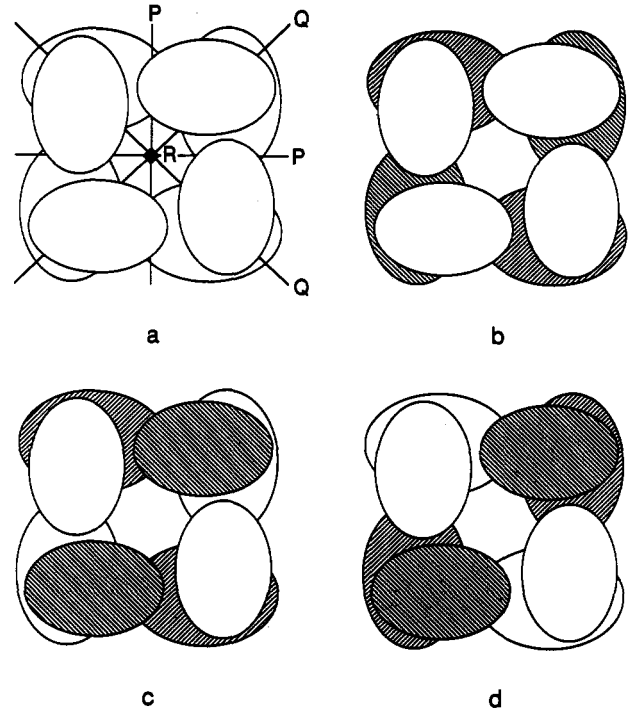


FIGURE 4: Schematic quaternary structures of Hr for octamers having (a) identical subunits, and (b–d) $\alpha_4\beta_4$ composition. Subunits are represented as ellipses. In (a), *P* and *Q* label 2-fold rotation axes through the faces and corners, respectively, and *R* labels a 4-fold rotation axis through the center of the octamer (Ward et al., 1975; Stenkamp & Jensen, 1981). Shaded and open ellipses in (b–d) show probable arrangements of α and β subunits in an $\alpha_4\beta_4$ octamer having the same configuration as in (a).

in the transmission of allosteric effects to and from the surface of each subunit (Kurtz, 1991). We defer a more detailed analysis until complete sequences of the *Lingula* Hrs become available.

Both the peak areas in the reverse-phase HPLC chromatogram (Figure 2) and the intensities of the resolved bands on SDS–PAGE (Figure 1) indicate that the α and β subunits of *L. reevii* Hr are present in approximately equal amounts, leading to an average $\alpha_4\beta_4$ composition of the protein. The homogeneity of native *L. reevii* oxyHr on gel filtration and anion-exchange chromatography and the approximately 1-kDa mass difference between the two subunits indicate that a mixture containing only α_8 and β_8 octamers is unlikely and is consistent with an $\alpha_4\beta_4$ subunit composition for the octamer. Satake et al. (1990) reported evidence that the α and β subunits of the *L. unguis* Hr octamer could be chemically cross-linked. The very similar results for *L. reevii* and *L. unguis* Hrs raise the distinct possibility that an $\alpha_4\beta_4$ subunit composition is a common feature of those Hrs showing cooperativity in O_2 binding.

If one assumes that the quaternary structure of *L. reevii* Hr is the same as that for other octameric Hrs (cf. Figure 4a), then the most probable arrangements of subunits in an $\alpha_4\beta_4$ octamer can be deduced as follows. The intersubunit interactions can be divided into three types, on the basis of the D_4 point group symmetry of the octamer. Two distinct types of intersubunit interactions can occur across the interfaces related by the 2-fold rotation axes labeled *P* and *Q*, respectively, in Figure 4a, and a third type can occur across the remaining interfaces, which are related by the 4-fold rotation axis, *R* (Stenkamp & Jensen, 1981; Ward et al., 1975). There are 13 possible unique arrangements of subunits in an $\alpha_4\beta_4$ octamer, assuming the same configuration as in Figure 4a.[2] The

implicit requirement that four α and four β subunits always assemble into an $\alpha_4\beta_4$ octamer as opposed to a statistical mixture of $\alpha_n\beta_{8-n}$ (n = 0–8) octamers makes a subset of the 13 possible arrangements more probable according to the following criterion. All four subunits of a given type, α or β, in an $\alpha_4\beta_4$ octamer must be in an identical environment. That is, a given type of subunit, α or β, in the octamer can have only one type of intersubunit contact, namely, α/α, β/β, or α/β, across a given interface, *P*, *Q*, or *R*. The three arrangements shown in Figure 4b–d constitute the subset that satisfies this criterion.[3] Starting from these three arrangements, an additional constraint can be imposed: because Hrs with identical subunits show no cooperativity, α/β intersubunit contacts are required for allosteric behavior and those arrangements having the largest number of interactions across such contacts are favored. The numbers of close intersubunit interactions across the three interfaces in *T. dyscritum* and *P. gouldii* Hrs vary in the order $Q > R \gg P$ (Stenkamp & Jensen, 1981; Ward et al., 1975). Either very few or no intersubunit interactions are found across the *P* interfaces, which are, therefore, ignored in the following discussion. For each α and β subunit in the octamers shown in Figure 4, the α/β contacts across a given interface can be summarized as follows: *Q* in (b), *Q* and *R* in (c), and *R* in (d). Thus, if α/β contacts across the *Q* interface, i.e., the interface with the largest number of intersubunit interactions, are the most important for allosteric behavior, then the arrangements in (b) and (c) would be favored over that in (d).

Richardson et al. (1987) proposed a three-state model to interpret their O_2 binding data for *L. reevii* Hr. This model gave a better fit to the data than did a two-state model. Their three-state model consists of a high-affinity "relaxed" conformation, R_8, a low-affinity "tense" conformation, T_8, and a state of intermediate "tension", R_4T_4, with the three states related by the equilibrium

$$R_8 + T_8 \rightleftharpoons 2R_4T_4 \qquad (1)$$

According to this model, the observed cooperativity can be described by a deviation of the equilibrium constant from the statistical value of 4. The deviation was proposed to result from distinct allosteric interactions within vs between "upper" and "lower" tetrameric halves of the octamer. Such distinct interactions would be present in any of the structures shown in Figure 4. Addition of two more intermediate states, R_2T_6 and R_6T_2, to this model could readily accommodate distinct allosteric interactions within vs between $\alpha\beta$ pairs across the *Q* vs *R* interfaces, respectively, for the arrangements in Figure 4b,c (or within vs between α_2 and β_2 pairs for the arrangement in Figure 4d). Thus, while this five-state allosteric model could possibly improve fits of the O_2 binding data compared to the three-state model (due to the large number of adjustable parameters), neither model can distinguish between the possible subunit arrangements.[4]

It has long been known that the O_2-binding cooperativity in hemoglobin, which has a tetrameric $\alpha_2\beta_2$ subunit composition, requires both α and β subunits (Rossi-Fanelli et al., 1964). Thus, two different subunits may be a common strategy for generating allosteric interactions in both the heme and non-heme iron O_2 carriers. Complete amino acid sequences of the *L. reevii* Hr subunits will provide more detailed information about intersubunit interactions, possible allosteric mechanisms, and the reason for the pH dependence of the cooperativity. Moreover, our recently successful diiron site reconstitution (Zhang et al., 1991) and metal substitutions (J.-H. Zhang and D. M. Kurtz, Jr., unpublished results) in Hrs should allow preparation of separate α_n and β_n oligomers or hybrid metal $\alpha(M)_4\beta(M')_4$ forms of *L. reevii* Hr. Such studies are currently in progress in our laboratory.

[2] Mirror image arrangements are not included in this total. An illustration of the 13 unique arrangements is included as supplementary material.

[3] This criterion turns out to define the arrangements having the two highest point group symmetries, namely, C_4 in (b) and D_2 in (c) and (d).

[4] A further constraint on the possible subunit arrangements can be added, on the basis of the assumption that the α and β subunits have different net charges and/or charge distributions. The arrangement in Figure 4c would be favored, because it distributes the charges more uniformly than that in 4b.

ACKNOWLEDGMENTS

We thank Russell C. Long for assistance in isolating *L. reevii* Hr and Ronald E. Stenkamp for helpful discussions.

SUPPLEMENTARY MATERIAL AVAILABLE

One figure depicting the unique subunit arrangements in an $\alpha_4\beta_4$ octamer (1 page). Ordering information is given on any current masthead page.

Registry No. O_2, 7782-44-7; hemerythrin (*Lingula reevii* α subunit N-terminal fragment), 135339-92-3; hemerythrin (*Lingula reevvi* β subunit N-terminal fragment), 135339-93-4.

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Articles

Conformations of IgE Bound to Its Receptor $F_c\epsilon RI$ and in Solution†

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Received January 18, 1991; Revised Manuscript Received May 24, 1991

ABSTRACT: Previous resonance energy transfer studies suggested that murine immunoglobulin E (IgE) is bent near the junction of its F_c and F_{ab} segments when bound to its high-affinity receptor ($F_c\epsilon RI$) on RBL cells. To examine further the conformations of IgE, both bound to this receptor and in solution, a mutant recombinant IgE ($\epsilon/C\gamma3$*) was prepared that has a cysteine replacing a serine near the C-terminal ends of the heavy chain. The introduced cysteine residues provide a means for specific modification of IgE, and the sulfhydryl groups were selectively labeled with fluorescein-5-maleimide (FM-$\epsilon/C\gamma3$*). This IgE also binds a 5-(dimethylamino)napthalene-1-sulfonyl (DNS) group in the antigen-binding sites. Resonance energy transfer experiments carried out on receptor-bound FM-$\epsilon/C\gamma3$* yielded a distance of 53 Å between fluorescein near the C-terminal end of the F_c segment and amphipathic acceptor probes at the membrane surface. The average distance between this C-terminal fluorescein and acceptor eosin-DNS in the antigen-binding sites at the N-terminal ends of the F_{ab} segments was found to be 69 Å. These results combine with those from previous structural studies to provide an unprecedented detailed description of the bent geometry of IgE bound to its receptor on the membrane. Energy transfer measured for FM-$\epsilon/C\gamma3$* in solution between fluorescein near the C-terminal end of the F_c segment and eosin-DNS at the N-terminal ends of the F_{ab} segments indicates that the average distance between these probes is about 71 Å. This contrasts with the estimated value of 175 Å for a planar Y-shaped IgE molecule. This result points to the possibility that IgE in solution is also bent such that, on average, the termini of the F_{ab} and F_c segments are closer together than they would be in the planar form.

Immunoglobulin E (IgE),[1] like other classes of Ig, acts as an adaptor molecule linking antigen to effector systems. In particular, IgE binds to its high-affinity receptor, $F_c\epsilon RI$, in a 1:1 complex on the surface of mast cells and basophils. Cross-linking of these IgE–receptor complexes by multivalent antigens leads to cellular degranulation and release of mediators of the allergic response (Metzger et al., 1986). In previous resonance energy transfer studies of membrane receptor-bound IgE, fluorescent donor probes were placed at specific sites on IgE (Holowka & Baird, 1983b; Baird & Holowka 1985) and anti-IgE antibodies (Holowka et al., 1985). The distance of closest approach between these probes on receptor-bound IgE and amphipathic acceptor probes randomly distributed at the plasma membrane surface was measured. These previous results allowed us to put some limits on the possible orientations and positions of IgE when it is bound to its receptor. In particular, the antigen-binding sites must be quite far from the membrane surface (≥100 Å), while the interchain disulfide bonds in the Cε2 domain are as close as 45 Å to this surface.

Previous studies on the region of interaction between IgE and receptor revealed that a site of trypsin cleavage between the Cε2 and Cε3 domains of rat IgE is markedly protected from proteolysis when IgE is receptor-bound (Perez-Montfort & Metzger, 1982). More recently, genetically derived mutants and segments of human IgE were used to show that a 76 amino acid polypeptide containing the carboxy-terminal portion of Cε2 and the amino-terminal portion of Cε3 inhibits binding to IgE to the receptors (Helm et al., 1987). Recently, we reported on chimeric murine IgE molecules that have one or two F_c domains exchanged with the homologous domains of human immunoglobulin G (IgG) subclass I. Human IgG1 does not interact with IgE receptors. We found that one IgE

† This work was supported by National Institutes of Health Grants AI18306 and AI22449, the Becton Dickinson Monoclonal Center, and the Leukemia Society of American (B.S.), and by a Fellowship (Y.Z.) from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the US Army Research Office.

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[1] Abbreviations: Ig, immunoglobulin; DNS, 5-(dimethylamino)-naphthalene-1-sulfonyl; DTT, dithiothreitol; ED, 1-*N*-eosinthiocarbamyl-5-*N*-DNS-cadaverine; FM, fluorescein-5-maleimide; HAE, 5-(*N*-hexadecanoylamino)eosin; HAF, 5-(*N*-hexadecanoylamino)-fluorescein; ORB, octadecyl Rhodamine B chloride; RBL, rat basophilic leukemia.

0006-2960/91/0430-9125$02.50/0