Distance between Skeletal Protein 4.1 and the Erythrocyte Membrane Bilayer Measured by Resonance Energy Transfer*

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To assess the molecular architecture of the human erythrocyte skeletal protein 4.1-bilayer interface, the distance between a donor sulphydryl-specific fluorescent probe attached to a region near the glycophorin-binding domain of protein 4.1 and an acceptor lipophilic probe in the exposed leaflet of inside-out vesicles (IOVs) was measured by fluorescence resonance energy transfer. To prevent aggregation and loss of function, protein 4.1 was labeled in situ on the surface of IOVs, purified, and rebound onto fresh IOVs. The labeled protein 4.1 was similar to the native protein in its gel electrophoretic pattern and its binding affinity to stripped-IOVs (Kd 35 ± 4 nM). Energy transfer was assessed using two donor-acceptor pairs, 5-2-[[iodoacetyl]amino]ethyl]aminonaphthalene-1-sulfonic acid and 3,3'-dioctadecyloxycarbocyanine perchlorate, or 5-iodoacetamidofluorescein and tetramethylrhodamine phosphatidylethanolamine. Using both donor fluorescence intensity and lifetime quenching measurements, an average distance of 75 ± 5 Å between the probe on the protein and the surface of IOVs was found. In parallel fluorescence resonance energy transfer studies with protein 4.1 and liposomes with a phospholipid composition similar to that of the red cell membrane, information was obtained about both the structure of protein 4.1 and the membrane bilayer. The data are also consistent with the view that the cytoplasmic tail of glycophorin is interposed between protein 4.1 and the lipids. These experiments represent the first measurement of a distance between a skeletal protein and the lipid bilayer.

The mammalian erythrocyte relies on a specialized network of skeletal proteins to maintain its structural integrity and remarkable deformability (1,2). This skeleton consists of a two-dimensional spectrin-actin network which appears to be anchored to the membrane by at least two protein linkages: the ankyrin-band 3 linkage and the protein 4.1-glycophorin linkage (3-5). Direct protein-lipid associations have also been proposed to be involved in the coupling of this skeleton to the membrane (6-9). In certain hemolytic disorders, loss of membrane lipids and production of nondeformable spherocytic cells may result from uncoupling of the skeleton from the membrane (2). Although biochemical and electron microscopic studies have provided an initial working concept of the structure of the skeleton, its precise geometry at the bilayer interface is not known. Knowledge of this geometry and its response to biochemical and physiologic stress may be important to understand the molecular mechanism of uncoupling of the skeleton from the bilayer in certain hemolytic anemias.

We have used the technique of fluorescence resonance energy transfer (RET)1 as a new approach to examine the geometric relationships between erythrocyte skeletal proteins and the lipid bilayer. In this report, the distance between the putative glycophorin-binding region of protein 4.1 and the membrane bilayer was measured in a reconstituted system using inside-out membrane vesicles (IOVs). By comparison of the distance in IOVs with the distance obtained in a reconstituted system using protein-free liposomes composed of phospholipids similar to that of the red cell membrane, information was obtained about both the structure of protein 4.1 and its interaction with the bilayer.

MATERIALS AND METHODS

Chemicals—Phospholipids (PS, PE, PC, from egg yolk) were obtained from Avanti Polar Lipids (Birmingham, AL). A fluorescent PE (TMR-PE), carbocyanines (diO-31-C18(3) and di1-C18(3)), and IAF was purchased from Molecular Probes (Eugene, OR). PE-D52 and Q-Sepharose were obtained from Pierce Chemical Co. and Pharmacia, respectively. A Protein Assay Kit was obtained from Bio-Rad. Other chemicals were obtained from Sigma.

Membrane Preparations—Large unilamellar vesicles (5.5 mM) containing FS, PE, and PC at 3:2:1 molar ratio were prepared by the reverse-phase evaporation method (10) in a buffer containing 5 mM Na-phosphate, 1 mM EDTA, 0.2 mM DFP, 130 mM KCI, 10 mM NaCl, 1 mM MgCl2, and 1 mM CaCl2.

1 The abbreviations used are: RET, fluorescence resonance energy transfer; DFP, diisopropyl fluorophosphate; di-C14(3), 1,1'-diododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; di-C18(3), 3,3'-dioctadecyloxycarbocyanine perchlorate; IAF, 5-iodoacetamidofluorescein; IOVs, inside-out vesicles; PE, phosphatidylethanolamine; POPOP, 1,4-bis(2,5-phenyloxazolyl)benzene; Na, sodium dodeyl sulfate-polyacrylamide gel electrophoresis; TMR-PE, PE labeled at its head group with tetramethylrhodamine; SDS-PAGE, 12089.
Erythrocyte Skeletal Protein 4.1 Distance to the Membrane Bilayer

0.2 mM diethiothreitol, pH 7.9 (buffer A). Vesicles of uniform size were prepared by two sequential extrusions through 0.4-μm polycarbonate filters under high pressure. The liposome phospholipid concentration was determined by the Bartlett method (11).

IOVs were prepared by spectrin depletion of red cell ghosts (12). Briefly, fresh erythrocytes were hypotonically lysed and washed in 5 mM Tris-HCl (pH 7.4) and 0.15 M NaCl, and then resuspended in 0.1 M sodium phosphate, 100 mM NaCl, 0.5 mM EDTA, 0.1% NaN3, and 10% glycerol. Following band 6 depletion with 155 mM KCl in buffer B at pH 7.6, the white ghosts were incubated for 30 min at 37 °C in a 10-fold volume of 0.1 mM EDTA, 0.2 mM DFP, pH 8.0, and washed first with 0.3 mM EDTA, 0.2 mM DFP, and then with 10 mM phosphate, 1 mM EDTA, 0.4 mM DFP (buffer C) to remove endogenous proteases (13). The membranes became spectrin-depleted and greater than 70% inside-out, as assessed by an acetylcholinesterase assay (14). Quasi-elastic light scattering and photon correlation spectroscopy analysis (15) of these IOVs showed that more than 90% were ~1 μm in diameter.

Stripped IOVs were prepared by extraction of IOVs at 37 °C for 30 min with buffer B containing 1 M KCl and 1 mM diethiothreitol (12). Subsequent washes first with buffer C containing 0.2 mM diethiothreitol and then with buffer A extracted ~50% of proteins 4.1 and 2.1.

Membrane Labeling with the Lipophilic Acceptor Probe—IOVs and stripped IOVs were labeled with the lipophilic probe, 7-diiodo-3-(3-sulfopropyl)-1,3,8-tetramethylxanthen-6-sulfonate (TMR-PE) or carboxyfluorescein by the ethanol injection method. A small amount of an ethanolic solution of the fluorophore (<1% ethanol, v/v) was added at 25 °C to membrane suspensions at 0.1-3 mol% while vortexing rapidly. Unbound probe was removed by centrifugation of the sample over an equal volume of buffer A containing 12% sucrose. The liposome-labeled IOVs were measured fluorimetrically with membranes dissolved in 1% (w/v) Triton X-100 using a standard curve of fluorescence versus known concentrations of the probe in buffer A containing 1% Triton. About 90% of the added probe was found to be associated with the membranes.

The distribution of the carboxyamine acceptor probes (dio-C18(3) and dil-C18(3)) between the two bilayer leaflets was deduced from the time course of carboxyamine fluorescence quenching with 1 mM TNBS (16). Because TNBS was found to slowly permeate the IOV membranes, the initial slope of this quenching time course was used. The distribution of the lipophilic IOVs labeled with dio-C18(3) in the outer leaflet only (labeled just prior to TNBS addition) was 0 ± 0.4 × 10^{-4} fluorescence units/s, which was 2.5 ± 0.6 times greater than that measured in IOVs labeled in both leaflets (labeled during preparation of unsealed ghosts; 4.0 ± 0.5 × 10^{-4} fluorescence units/s). The slope determined in IOVs prepared for RET studies (labeled 3 h prior to TNBS addition) was 10 ± 0.4 × 10^{-4} fluorescence units/s. A comparison of those results showed that dio-C18(3) was exclusively in the outer leaflet at the time of RET measurements. The dil-C18(3) probe was also found to be localized in the outer leaflet.

Liposomes were labeled with dio-C18(3) by inclusion of the lipophilic probe in the initial chloroform mixture of the lipids. This procedure resulted in the equal distribution of the probe into both leaflets.

Membrane Labeling with the Donor-labeled Protein 4.1—The sulphydryl groups of protein 4.1 were labeled in situ on the surface of IOVs with one of the two donor fluorophores, IAEDANS or IAF. IOVs at 1 mg of protein/ml in buffer C were mixed with an equal volume of a 2 mM stock solution of the SH-reactive fluorophore and incubated for 12 h at 4 °C in the dark. The unlabeled sample was added at 23 °C to membrane suspensions at 0.1-3 mol%.

The amount of the incorporated probe was measured fluorimetrically in IOVs prepared for RET studies (labeled 3 h prior to the time of RET measurement). The diO-C18-(3) probe was also found to be associated with the membranes.

The intramembrane distance of protein 4.1 from the bilayer center was determined by the Bartlett method (11).

Gel Electrophoresis—SDS-PAGE was performed on 7% polyacrylamide gels (19). For visualization of the fluorescent protein bands, heavy loads of 20-40 μg of protein were applied to gels. The unfixed gels were examined on a UV transilluminator, photographed using Polaroid 677 film, and stained for protein with Coomasie Blue R-250.

Spectroscopy—Fluorescence spectra were obtained at room temperature in an SLM 8000C fluorimeter. To minimize inner filter effect in the IOV experiments, measurements were performed in a short excitation path length quartz cuvette (2 × 10 mm). Absorption spectra were obtained on a Hewlett-Packard spectrophotometer. Fluorescence lifetimes were measured in an SLM 48000 multichannel fluorometer by the phase modulation method (20). A helium-cadmium laser source was used for donor excitation at 322 nm for IAEDANS or 443 nm for IAF. Emission interference filters of 420 ± 10 nm for IAEDANS and 520 ± 10 nm for IAF were used. 1,4-Bis(2-(5-phenyloxazolyl)-benzen (τ = 1.35 ns) and carboxyfluorescein (τ = 3 ns) were used as reference compounds for lifetime measurements.

The quantum yields of IAEDANS and IAF labeled 4.1 were determined using as standards either quinine sulfate in 0.1 N H2SO4 (0.7, (21)) or fluorescein in 0.1 N NaOH (0.92, (22)) All fluorescence and absorbance measurements were performed at room temperature.

Resonance Energy Transfer Calculations—The efficiency of energy transfer, E, was determined by the decrease in the quantum yield of the donor by the acceptor (23). E was measured experimentally from the ratio of the fluorescence intensities of the donor in the presence (I0) and the absence (Iq) of the acceptor at the donor emission wavelength after corrections for membrane light scattering, inner filter effect, and the contribution from the acceptor emission:

\[
E = \frac{1 - I_d/I_0}{1 - I_d/I_0}
\]

(1)

The correction for light scattering was made by subtracting the signal produced by the unlabeled sample. The correction for the contribution from the acceptor was made by subtracting the signal produced by the acceptor-labeled sample. The correction for inner filter effect was made using Beer-Lambert's Law, as follows:

\[
I_{obs} = I \left(1 - \frac{OD_{Abs} + OD_{Em}}{OD_{Abs} + OD_{Em} + 4} \right)
\]

(2)

where ODAbs and ODEm are sample absorbances at the donor excitation and emission wavelengths, I0, and Iq are one-half of the cuvette path lengths (in centimeters) in the directions of excitation and emission, and x is light relative to cuvette path length (1 cm) used for absorbance measurements.

E was also determined from fluorescence lifetimes, where correction for inner filter effect was not required:

\[
E = 1 - \tau_d/\tau_0
\]

(3)

\(\tau_d\) and \(\tau_0\) are the lifetimes of the donor in the presence and absence of the acceptor. When multicomponent lifetimes were observed, the weighted average lifetime, (τ), was used to calculate E (24, 25):
\( \tau = \frac{F_1}{F_0} \) (4)

where \( F_1 \) is the fractional contribution of \( \tau \).

The distance of closest approach, \( L \), between a point donor and an infinite plane of randomly distributed acceptors was obtained by fitting the experimental donor quenching profiles \( (L_d/L_0 \text{ versus } \rho) \) to the established theoretical curves (26-28). For liposomes in which \( L \) is expected to be less than or comparable to \( R_o \), the data were fitted to the bi-exponential approximation of the exact solutions derived by Wobler and Hudson (26); for IOVs in which \( L \) is much larger than \( R_o \), a linear approximation of the quenching profile was used (27, 28).

\[
\frac{L_d}{L_0} = (\pi / (2 \rho) L^2) + 1 \quad \text{for } L / R_o > 1.68
\] (5)

\( L \) was calculated from \( E \) determined either by donor quenching using Equation 1 \((E_{eq} \text{ and } L_{eq}) \) or lifetime using Equation 3 \((E, \text{ and } L_o) \). \( \rho \) is the surface density of the acceptor in units of number/Å², and \( R_o \) (in units of Å) is:

\[
R_o = (9.79 \times 10^8) (J \lambda^2 Q_{dn^{-4}})^{1/6}
\] (6)

where \( n \) is the refractive index of the medium between the fluorophores, \( \lambda^2 \) is the orientation factor, and \( J \) is the overlap integral between the emission spectrum of donor and the absorption spectrum of acceptor (in units of \( \text{cm}^{-1} \text{ cm}^{-1} \)). \( J \) is defined by:

\[
J = \frac{\int F_d(\lambda) \epsilon_d(\lambda) \lambda^4 d\lambda}{\int F_d(\lambda) d\lambda}
\]

where \( F_d(\lambda) \) and \( \epsilon_d(\lambda) \) are the donor emission and the acceptor molar extinction at \( \lambda \), respectively.

\( \rho \) for IOVs was calculated using the equation:

\[
\rho = 0.9(\lambda^4 / (60 [PL]))
\] (8)

where \([\lambda]\) is the initial molar concentration of the added acceptor, \([PL]\) is the molar concentration of membrane phospholipids, and 60 (in units of Å) is the average area of the headgroups of phospholipids (29). The factor 0.9 was included in the numerator to account for the 90% incorporation into the bilayer of the initial added acceptor. In the liposome samples, only one-half of the bound acceptor concentration was used to calculate \( \rho \) because acceptor molecules in the internal leaflet were too far from donors in the external leaflet to result in energy transfer.

RESULTS

To determine the distance between skeletal protein 4.1 and the lipid bilayer by RET, protein 4.1 labeled in situ with an SH-reactive donor fluorophore was purified from the labeled IOVs. The labeled protein was then reincorporated into fresh IOVs which were labeled with the lipophilic acceptor probe. The SH groups of protein 4.1 were chosen as a site for labeling because of the fortuitous clustering of all seven of them in the 30 kDa N-terminal segment which also contains the glycophorin-binding site (30).

Properties of the Labeled Protein 4.1—The sulphydryl groups of purified protein 4.1 were labeled in situ on the surface of IOVs to prevent aggregation and to preserve glycophorin-binding properties.\(^2\) To enhance the specificity of the SH-group labeling, labeling was done at low temperature (4°C), low ionic strength (buffer B), nonacidic pH (7.9), and moderate concentrations of labeling reagent (10 μmol/mg of IOV protein). This procedure resulted in the labeling of several IOV proteins (Fig. 1, lane 1). The IAEDANS-labeled IOVS showed approximately 50% of the label in ankyrin, 15% in protein 4.1, 10% in band 3, and the rest in protein 4.2 and other minor small molecular weight proteins. The labeled protein 4.1 purified from these IOVs had a low stoichiometry of labeling (0.1-0.3 mol of fluorophore/mol of protein).

Several lines of evidence showed that protein 4.1 labeled with IAEDANS by this in situ method was similar to the unlabeled protein. First, during purification by ion-exchange chromatography, the labeled protein eluted at the same ionic strength as the unlabeled protein. Second, the labeled and unlabeled protein migrated at the same positions on SDS-PAGE gels (Fig. 1). Third and most importantly, the labeled protein reassociated with stripped IOVs with a binding affinity (Kd 35 ± 4 nM) similar to the unlabeled protein (Kd 40–52 nM; 6).

Greater than 90% of the IAEDANS-labeled 4.1 appeared as the 78/80-kDa species, although some smaller species and a 100-kDa peptide were sometimes present in heavily loaded gels (Fig. 1, lane 2). These were probably native protein 4.1 isoforms (31), since they were always found in the unlabeled preparations and reassociated with affinity purified antibodies to protein 4.1 (not shown). Although these peptides also bound to the IOV membranes during reconstitution, it is unlikely that they substantially affected the distance measurements because they constituted less than 5% of the total fluorescence.

Steady-state RET Studies with Liposomes—To determine the distance between protein 4.1 and the bilayer in a model membrane system, RET studies were performed on liposomes with a phospholipid composition similar to that of red cell inner leaflet. The liposomes contained IAEDANS-labeled 4.1 as the protein donor, and dO-C14-3) as the lipophilic acceptor. This carbocyanine probe was chosen because of its high extinction coefficient (142,000 M⁻¹ cm⁻¹), the localization of its fluorophore moiety at the plane of the phospholipid head groups (32, 33), and the excellent overlap of its absorption spectrum with the emission spectrum of IAEDANS (Fig. 2).

Fig. 3 shows the emission spectrum of the singly labeled (donor alone) and the doubly labeled (donor plus acceptor) liposomes. The spectra of two controls (acceptor-labeled and unlabeled liposomes) are also shown. Comparison of the emission spectra of the donor labeled with the doubly labeled samples indicated a decrease in the emission intensity of the donor (donor quenching), and an increase in the emission intensity of the acceptor greater than that in the sample with acceptor alone (sensitized emission). To quantitate the extent of donor quenching due to energy transfer, the contribution of the signal from the sample labeled with acceptor alone was subtracted from the doubly labeled sample. In addition, corrections were made for two optical effects: membrane light scattering, given by the spectrum of the unlabeled sample, and inner filter effect, determined by sample absorbances (relative to unlabeled sample) at the excitation and emission wavelengths (0.03 A units at 360 nm and 0.13 A units at 478 nm). A transfer efficiency of 0.58 was calculated from Equation 1 (see "Materials and Methods"). Using an \( R_o \) of 57 Å

\(^2\) T. Leto, personal communication.

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**Fig. 1.** Fluorescence SDS-PAGE pattern of IAEDANS-labeled IOVs (lane 1) and protein 4.1 purified from these IOVs (lane 2). 20-40 μg of protein was applied. The unfixed gel was photographed using a UV transilluminator.
for the IAEDANS/dio-C14-(3) pair and the bi-exponential approximation described under "Materials and Methods," an L/Ro value of 0.85 ± 0.03 was determined. Thus, by donor quenching a distance of 48 ± 2 Å was found between protein 4.1 and the liposome bilayer.

Steady-state RET Studies with IOVs—To determine the distance between protein 4.1 and the bilayer in IOVs, in  

Fig. 4A shows a typical RET experiment with the IAEDANS-protein 4.1/dio-C14-(3) labeled IOVs. In analogy to the observations made for the liposomes, a decrease in the donor emission occurred together with an increase in the acceptor emission. The spectra for unlabeled and acceptor-labeled samples, which were used to correct for scattering and contribution of acceptor emission, are also shown.

Two control experiments confirmed energy transfer in the IOVs. First, the spectrum of doubly labeled IOVs was found to be different from the spectrum of a mixture of IOVs labeled separately with equivalent concentrations of donor and acceptor. In the doubly labeled sample, donor emission was decreased and acceptor emission was increased (Fig. 4B). Since the inner filter and light scattering effects were identical in both samples, these differences in the spectra were due to RET. Second, following disruption of the geometric relationship between acceptor and donor by 1% Triton X-100, the spectra from the doubly labeled IOVs and the mixture of singly labeled IOVs were nearly identical, confirming that the donor quenching and the sensitized emission in the samples without detergent was due to energy transfer between the membrane lipids and the bound protein.

Equations 1 and 5 predict a linear relationship between the ratio I_d/I_a and the acceptor surface density with a slope of (πR_o^2)/2J ~ 1. For the IAP/TMR-FP pair, R_o was 35 Å using J = 0.129 × 10^{-18} cm^{-3} m^{-1} nm^{-1}; [PL] of IAP; 3 μM dio-C14-(3).

Fig. 2. Overlap between the emission spectrum (λ_ex 360 nm) of the IOV-bound IAEDANS-labeled protein 4.1 (---) and the absorption spectrum of dio-C14-(3)-labeled IOVs (----) after subtraction of the spectra of the unlabeled IOVs. Concentrations: 70 μM [PL] of IOVs; 1.7 μM dio-C14-(3).
protein 4.1/TMR-PE and the IAF-protein 4.1/diI-C1(3)

between the fluorescein probe on protein 4.1 and the exposed

A transfer efficiency of 0.12 was calculated from the decrease

sion. By using a calculated

A. was found (data not shown) with a fitted distance of 79

pair (77 A).

Equation 5,

incorporate into empty glycophorin-binding sites. Multiple
donor-acceptor pairs were used (Table 1). For the IAEDANS/
carbocyanine pair, RET measurements were performed using
an unrelated donor-acceptor pair with a different R0. IAF-
labeled protein 4.1 was used as donor and the lipophilic probe,
TMR-PE, as acceptor. The TMR-PE incorporated into the
exposed inner leaflet of the IOVs orients with the fluorophore moity localized at the surface of the bilayer and does not flip to the other leaflet during the time course of RET measure-
ments. Comparison of the emission spectra (λem 480 nm) of the
donor-labeled, the doubly labeled, and the two control
acceptor-labeled and unlabeled samples, showed both donor
quenching (at 515 nm) and sensitized emission (at 575 nm).
A transfer efficiency of 0.12 was calculated from the decrease
in donor emission after corrections were made for light scatter-
ing, inner filter effect, and contribution of acceptor emis-

sion. By using a calculated R0 of 50 Å for this pair and
Equation 5, a 70 ± 3 Å (S.E.; n = 4) distance was determined
between the fluorescein probe on protein 4.1 and the exposed
surface of the IOVs (Table I). Given an overall (both system-
atic and statistical) error of approximately 5 Å determined for the L values (see “Discussion”), this was in agreement
with the distance calculated for the IAEDANS/carbocyanine
pair (77 ± 1 Å).

The distance between protein 4.1 and the bilayer was also
evaluated in stripped IOVs where protein 4.1 is predicted to
incorporate into empty glycophorin-binding sites. Multiple
donor-acceptor pairs were used (Table I). For the IAEDANS/
dioC1(3)-pair, donor quenching was assessed as a function of
canceptor concentration. Similar to the observations made
in IOVs, a linear relationship between the ratio I0/I and ρ
was found (data not shown) with a fitted distance of 75 ± 6
Å. Donor emission quenching was also observed with the IA-
protein 4.1/TMR-PE and the IA-protein 4.1/dioC1(3) pairs from
which distances of 75 and 66 Å, respectively, were
calculated. The distances calculated for the stripped IOVs
using multiple donor-acceptor pairs agreed well with each other and with the distances calculated for the intact IOVs.

**Lifetime RET Studies with Liposomes and IOVs—**

Resonance energy transfer was assessed in IOVs independently by measurement of the donor lifetime. At least two lifetime
components were observed for both the IAEDANS-labeled
and the IAF-labeled protein 4.1 (Table I). IAEDANS has a
complex ground state electronic structure and has previously
been shown to have multiple lifetime components ranging
from 8 to 25 ns (34). Although fluorescein in solution has a
single lifetime value of 4–5 ns, we observed two lifetime
components for the protein-bound fluorescein, similar to pre-
nious observations (35).

IAEDANS-labeled protein 4.1 bound to the liposomes had
two lifetime components of 1.27 and 9.06 ns. In the presence
of acceptor, the components decreased substantially to 0.86
and 3.97 ns, respectively. Light scattering, as determined by
the signal from unlabeled IOVs, was <5% of the signal from
labeled IOVs. By using the averaged lifetimes (calculated from
Equation 4), a transfer efficiency of 0.56 was determined. The
distance calculated from lifetime analysis (L) and the bi-
exponential approximation (see “Materials and Methods”) was
51 Å which agreed well with L0 determined by the
steady-state measurements (48 Å).

IAF-labeled protein 4.1 bound to IOVs showed two lifetime
components of 1.14 and 2.71 ns. In the presence of the acceptor, the lifetimes decreased to 0.75 and 2.25 ns. The
lifetimes from single component analysis of the data were
1.58 ns for the donor-labeled and 1.46 ns for the doubly labeled
sample. From the single lifetime analysis, a transfer efficiency
of 0.08, and an L, of 78 Å were determined. Thus, in the IOVs, distances calculated by the lifetime technique were similar to
those calculated by donor quenching using both the IAF/
TMR-PE pair (70 Å) and the IAEDANS/dioC1(3) pair (77
Å). Finally, energy transfer was observed by lifetime analysis
in the stripped IOVs using two donor/acceptor pairs (Table I)
and an average distance comparable to that in the IOVs
was found.

**DISCUSSION**

Resonance energy transfer has been used extensively to study the structure and interactions of soluble proteins, and
recently, to examine more complex membrane systems (25,
36–39). Studies in intact erythrocytes have indicated the close
apposition of intracellular hemoglobin molecules to the inner
leaflet of the bilayer (25). Other RET studies in erythrocytes
have suggested the proximity of the bilayer to both the
cysteine cluster and the tryptophan cluster of the cytoplasmic
domain of band 3 (40–42). The present studies have applied
these methods for the first time to the examination of skeletal
protein/lipid bilayer distances in erythrocyte membranes.

In both the IOV and the liposome systems, energy transfer
was observed between a donor probe bound to the sulphydryl
group(s) of protein 4.1 and acceptor probes in the exposed
leaflet of the bilayer using both steady-state and lifetime
analyses with two unrelated donor-acceptor pairs (Table I).
Control experiments provided evidence that the decrease in
donor fluorescence intensity and lifetime in the presence
of the acceptor was the result of the physical proximity of the
labeled site in protein 4.1 and the lipid bilayer. An average
donor-acceptor distance of 75 Å in the IOVs and 49 Å in the
liposomes was calculated from both the steady-state and
lifetime methods using the two donor-acceptor pairs. Based
on repeatability in multiple determinations, the statistical
errors in these values were small (±2 Å). The systematic

![Fig. 5. Relationship between the acceptor surface density (ρ) and the ratio I0/I at λem 346 nm and λem 482 nm. A linear least squares fit of the data gives a slope of 1.48 ± 0.11 x 10^5 Å² from which L0 is calculated to be 77 Å. Theoretical lines for distances from 60 to 90 Å are shown for visual comparison.](image-url)
**Erythrocyte Skeletal Protein 4.1 Distance to the Membrane Bilayer**

**Summary of the RET data used for calculation of protein 4.1:lipid distance in IOVs and in liposomes**

<table>
<thead>
<tr>
<th>System</th>
<th>Donor/acceptor pair</th>
<th>$R_0$</th>
<th>$\tau_1$</th>
<th>$F_\tau$</th>
<th>$\tau_2$</th>
<th>$\rho$</th>
<th>$E_r$</th>
<th>$E_{OQ}$</th>
<th>$L^*$</th>
<th>$L_{OQ}$</th>
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<tbody>
<tr>
<td>Liposomes</td>
<td>IAEANS-4.1/none</td>
<td>57</td>
<td>1.27</td>
<td>0.40</td>
<td>9.06</td>
<td>8.34</td>
<td>0</td>
<td>0.56</td>
<td>0.58</td>
<td>51</td>
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<td>IAEANS-4.1/dI-C14(3)</td>
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<td>0.86</td>
<td>0.28</td>
<td>3.94</td>
<td>3.73</td>
<td>21</td>
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<td>0.12</td>
<td>70</td>
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<td>1.37</td>
<td>0.80</td>
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<td>0</td>
<td>0.08</td>
<td>0.12</td>
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<td>1.34</td>
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<td>55</td>
<td>1.65</td>
<td>0.57</td>
<td>2.64</td>
<td>1.60</td>
<td>17</td>
<td>0.16</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAF-4.1/TMR-PE</td>
<td>50</td>
<td>1.15</td>
<td>0.69</td>
<td>2.88</td>
<td>1.48</td>
<td>13</td>
<td>0.12</td>
<td>0.10</td>
<td>69</td>
</tr>
</tbody>
</table>

* $F_\tau$ is the fraction of signal with $\tau_1$.
* $r$ was determined for IAEANS using Equation 4 and for IAF using single lifetime analysis.
* Distances calculated as described under "Materials and Methods" by Equation 5 for the IOVs and by the biexponential approximation (28) for the liposomes.

Errors arise from uncertainty in $Q_a$, $J$, $\rho$, and $x^2$. $Q_a$, $J$, and $\rho$ were measured accurately with a maximum composite error of ~5%. $x^2$ has a maximum theoretical error of 20%, but the error is likely to be <5%. Thus, from propagation of errors, the estimate of the maximum systematic error in $L$ was about 7% or ~5 Å. This indicates that the distances obtained by the two different methods for the IOVs were not significantly different from one another, whereas the difference in distance between liposomes and IOVs was significant.

The number and localization of labeled sites on the protein are important for the determination of $L$. Although we do not know the number of labeled sites, the low stoichiometry of labeling makes multiple labeled sites on a single molecule unlikely. Even if multiple sites were labeled, the highly compact protease-resistant 30-kDa N-terminal segment of the protein (43), which contains all of the seven sulfhydryl groups, would confine the labels to a small region. Therefore, the calculated distance probably represents an average of a very narrow range of donor-acceptor distances.

The disposition of the labeled protein 4.1 on the lipid surface can have major effects on the calculated distance in this system. Since only the 30-kDa N-terminal segment of protein 4.1 with its several amphipathic helices (31) has been shown to interact directly and specifically with PS liposomes (9), it is likely that in our experiments all of the labeled protein 4.1 molecules bind uniformly to PS liposomes with their 30-kDa domains facing the lipids. Thus, the 49-Å distance calculated in the liposomes should represent that from a point donor to the plane of the bilayer.

The specificity of the site of incorporation of the labeled protein 4.1 in the IOVs is important for interpretation of the protein-lipid distances. Three sites of interaction between 4.1 and IOV membranes are possible as shown in Fig. 6A (asterisks): the high affinity and physiologic glycoporphin-binding site ($K_d$ 40-52 x 10$^{-9}$ M, (18)), and the low affinity sites on band 3 ($K_d$ 5 x 10$^{-6}$ M; (44)) and the inner leaflet phospholipids ($K_d$ 10$^{-7}$ M; (9)). We believe that the labeled protein 4.1 was predominantly incorporated into its high affinity glycoporphin-binding site for the following reasons. First, our $K_d$ measurements indicated that the high affinity glycoporphin-binding capacity of the fluorescently labeled protein 4.1 was not altered. Second, exchange of externally added protein 4.1 with the bound protein 4.1 at the physiologically active glycoporphin site has been previously shown in studies with 4.1-defective cells (45). Third, equivalent RET was observed in stripped IOVs where the likelihood of incorporation of protein 4.1 into the empty glycoporphin sites should be even greater than exchange with the occupied sites in the IOVs. Fourth, our reconstitution studies were done at low protein 4.1 concentrations which should not favor incorporation into the relatively low affinity sites on band 3 or the bilayer. Finally, the finding of a significant difference between the distances calculated in the IOVs versus the liposomes suggests little energy transfer resulting from direct lipid binding.

The current data showing a distance of 49 Å between the labeled SH group(s) of protein 4.1 and the external plane of the bilayer in the reconstituted lipid samples implies that protein 4.1 is not globular. A globular protein of 80 kDa would have an approximate diameter of 57 Å which would localize the donor site outside of the 30-kDa SH-containing N-terminal domain (Fig. 6B, a). Instead, an elongated protein that...
extends perpendicularly away from the bilayer (as shown in Fig. 6B, b) accommodates the labeled site at the observed distance of 49 Å from the liposome bilayer. In this case, the 30-kDa domain would be at least 49 Å in length and probably not much longer because of its multihelical highly protease-resistant compact structure. Other orientations of the elongated protein (Fig. 6B, c) are inconsistent with our RET observations since they result in a decrease in L.

Our model of an elongated structure for protein 4.1 is consistent with the hydrodynamic data which suggest that the protein is highly asymmetric (46). However, electron microscopic observations of fixed or dehydrated samples suggest that protein 4.1 is globular (9, 12). We propose that protein 4.1 is an elongated molecule with multiple flexible lobes rather than a straight rod (Fig. 6A). This view of the structure of the protein is consistent with the observation of its four major proteolytic segments (43). It may also reconcile the apparent contradiction between hydrodynamic and electron microscopic observations, since the lobes which cause hydrodynamic asymmetry could fold over each other or collapse to appear globular during electron microscopy.

The observation that the average distance calculated in the IOVs is ~25 Å longer than that in the liposomes implies that, in the IOVs, protein 4.1 is 25 Å away from the bilayer. This observation is consistent with current models of membrane skeleton which interpose the cytoplasmic tail of glycophorin between protein 4.1 and the inner surface of the bilayer (Fig. 6A). The cytoplasmic tail of glycophorin C is 39 amino acids long. Although the tertiary structure is not known, this segment could have a range of dimensions from ~20 Å in diameter (if globular) to ~10 Å wide × 60 Å long (if extended and α-helical). Thus, whatever the structure of the cytoplasmic tail of glycophorin, it could account for displacing protein 4.1 by 25 Å from the bilayer. This biophysical view is consistent with current models of the architecture of the protein 4.1-bilayer junction of red cell membranes which have been deduced from biochemical and morphological evidence. Finally, this 25-Å displacement of the surface of protein 4.1 from the bilayer together with the localization of the labeled site at the extremity of the elongated 30-kDa segment (Fig. 6B) implies that protein 4.1 bound to glycophorin in IOVs does not interact directly with the lipids.

Although the present RET studies were made in IOV membranes, similar approaches should be applicable to resealed ghost membranes where changes in the skeleton-bilayer geometry could be examined during biochemical manipulations or physiological stresses. Furthermore, this type of measurement may have direct use in understanding the pathophysiology of hereditary spherocytosis where uncoupling of the membrane skeleton and the bilayer is likely to occur.

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REFERENCES

Erythrocyte Skeletal Protein 4.1 Distance to the Membrane Bilayer