Effect of Lipid Packing on the Conformational States of Purified GLUT-1 Hexose Transporter

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ABSTRACT: The purpose of this study was to determine the effect of increased lipid packing on the conformational states of the GLUT-1 hexose transporter purified in endogenous lipids. The binding of glucose results in a conformational change that can be followed by a decrease in fluorescence intensity. Lipid packing was increased by subjecting the samples to hydrostatic pressure. We have found that in the absence of ligand, the fluorescence intensity decreased approximately 20% in the 600 bar range studied. In the presence of either saturating or half-saturating amounts of D-glucose, a substantial loss in intensity (approximately 80%) was observed. Similar decreases were also seen the presence of a glucose analog, maltose, or a noncompetitive inhibitor, cytochalasin B. Changes in the accessibility of aqueous soluble quenchers (I^- and acrylamide) to GLUT-1 Trp and Tyr residues suggested that ligand binding causes interfacial fluorophores to move closer to ionic groups in the lipid head group region of the membrane. This idea was substantiated by (1) increased static quenching of the GLUT-1 fluorophores in the presence of ligand, (2) increased energy transfer efficiency between GLUT-1 fluorophores and a fluorescent membrane probe located close to the head group region, and (3) reduced change in rotational motion with temperature in the presence of ligand. Since the application of pressure results in an increase in bilayer thickness, and ligand binding causes a population of fluorophores to move closer to the membrane surface, then these interfacial interactions can be more stabilized under pressure. Studies monitoring the change in quenching of membrane probes by GLUT-1 tryptophans and energy transfer of GLUT-1 tryptophans to membrane probes support this idea.

In this study, we have investigated the role of increased lipid packing on the conformational states of the integral membrane protein GLUT-1 in its liganded and unliganded states using fluorescence spectroscopy. GLUT-1 is a facilitative transporter for glucose and other hexoses [for a review, see Baldwin (1993) and Curruthers (1990)]. GLUT-1 belongs to a family of at least six closely related proteins responsible for the facilitative transport of glucose as well as other hexose and pentoses. It has an extracellular and an intracellular binding site for a single glucose, and each site has slightly different glucose affinities. GLUT-1 is a member of a larger family of proteins that transport sugars as well as antibiotics [see Henderson (1991)]. GLUT-1 has been sequenced, and its secondary structure, based on hydrophobicity analysis, predicts 12 membrane-spanning helices. Many aspects of this model have been confirmed using bio- and immunochemical approaches [see Baldwin (1993) and Curruthers (1990)].

The binding affinities of glucose and other ligands to GLUT-1 have been measured by an array of biophysical techniques [see the review of Baldwin (1993) and Curruthers (1990)]. The most pertinent for this work is fluorescence spectroscopy. Gorga and Leinhard (1982) observed that the binding of D-glucose and D-glucose analogs to GLUT-1 is accompanied by a significant decrease in fluorescence intensity, and that the binding affinity can be determined by this decrease. Binding of cytochalasin B, a noncompetitive inhibitor that is thought to bind at or close to the inner glucose site, also produces a similar decrease in fluorescence intensity. These changes in fluorescence have been confirmed by several laboratories (e.g., Pawagi & Deber, 1990; Herbert & Curruthers, 1992; Chin et al., 1992). Implicit in the interpretation of these studies is that the quenched (liganded) and unquenched (unliganded) states of the glucose transporter reflect two distinct conformational states. Proteolysis studies indicate that ligand binding to the inner site, as opposed to the outer, produces most of the observed conformational changes (King et al., 1991). Transitions between conformational states are generally assumed to be slower than the on and off rates of glucose from the inner and outer sites (e.g., Appleman & Leinhard, 1989). Studies by Chin et al. (1992) have implicated Trp388 and -412 as key players in the observed emission changes upon ligand binding. Mutagenesis studies suggest that Trp388 plays a role in the interconversion of GLUT-1 conformational states rather than direct binding (Garcia et al., 1992; Schurmann et al., 1993).

The influence of the lipid environment on the stability of the conformational states of GLUT-1 is not known. Several years ago Reed and McElhaney (1975) and then Curruthers et al. (1989) investigated the changes in GLUT-1 activity when the membrane fluidity is altered by changes in composition. Although changes in D-glucose transport with membrane composition were observed, no clear trend with lipid packing could be found. Here, we examine the effect

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of lipid packing on the liganded and unliganded form of GLUT-1 in endogenous lipids using high pressure.

High pressure allows for an increase in the packing of lipid chains without a concomitant change in the kinetic energy and without a change in composition [for reviews, see Heremans (1982) and Weber and Drickamer (1983)]. The effect of pressure on the properties of model membranes has been characterized by several techniques. Increasing the pressure will decrease the free volume of a membrane by reducing the number of cis-gauche isomers. This straightening of the lipid chains results in an increase in bilayer thickness (Braganza & Worcester, 1986). Proteins are far less compressible than lipids, and increasing the lipid packing will increase the thickness of the bilayer without a concomitant increase in protein length. These different pressure responses could disrupt interactions between the protein and the lipid.

We have previously studied the effect of pressure on the physical properties of integral membrane proteins. For the small, integral peptide gramicidin, we found evidence that pressure promoted the disruption of protein−lipid contacts (Scarlata, 1991a). Specifically, it was found that the rotational motion of the interfacial tryptophan side chains of gramicidin channels greatly increases under pressure. This behavior is in contrast to that of fluorescent membrane probes, which show a systematic decrease in rotational motion as the lipid packing increases. On a suggestion from functional studies (O'Connell et al., 1990), the increase in rotational motion was determined to be due to the rupture of hydrogen bonds between the indole moieties and the carbonyls of the surrounding phospholipids that limited the motions of the tryptophans before pressure was applied. The presence of these hydrogen bonds has been subsequently confirmed by NMR studies (Ketchem et al., 1993). Since membrane proteins have a preponderance of Trp residues in the head group region of the lipid bilayer (Landolt-Marticorena et al., 1992), it is likely that the conformational states of other membrane proteins can be either stabilized or destabilized by lipid packing.

The purpose of this study is to use GLUT-1 to test the idea that increased lipid packing induced by high pressure may alter or stabilize the liganded or unliganded state of a protein through changes in interfacial contacts. A previous pressure study that monitored glucose transport in intact red cells suggested a significant volume change of GLUT-1 during transport (Thorne et al., 1992). Rather than reconstituting GLUT-1 in a series of lipids, we concentrate here on GLUT-1 purified from human erythrocytes in endogenous lipids since these conditions better represent the physiological environment of the protein.

MATERIALS AND METHODS

Dihydroxycytochalasin B and sugars were purchased from Aldrich and Sigma. GLUT-1 was prepared from clarified octyl glucoside extracts of alkali-stripped human erythrocyte membranes by anion-exchange chromatography as described by Baldwin and Leinhard (1985). After purification, the protein was dialyzed against 75 mM Hepes, 0.1 M NaCl, and 1 mM EDTA, pH 7.5, and subsequent studies were carried out using protein diluted in this buffer. The protein: lipid molar ratio, determined by BCA and phosphate analysis, was approximately 1:150. 6-Dodecanoyl-2-(dimethylamino)-naphthalene (Laurodan) and 2- and 12-(9-anthroyloxy)stearic acid (2-AS and 12-AS) were purchased from Molecular Probes and used without further purification.

Fluorescence measurements were taken on an ISS-K2 spectrofluorometer (ISS, Inc., Champaign, IL) equipped with Glan−Thompson polarizers. Lifetimes were measured at the National Synchrotron Light Source at Brookhaven National Laboratories. A GLUT-1 concentration of 20 μg/mL was used for each experiment. Proteoliposomes were sonicated for 30−60 s to reduce the size of the particles and eliminate settling of material during the time course of the experiments. At several points during the ligand titrations and during the pressure runs, the intensity at 340 nm was measured as a function of time to ensure that the signal was stable and events such as photobleaching and vesicle aggregation were not occurring. Unless otherwise noted, for the intrinsic fluorescence measurements an excitation wavelength of 280 nm was used. Emission intensities were isolated by a monochromator as well as a band-pass filter (WG310) in order to eliminate scattered light. Emission spectra were taken without polarizers. The reported intensities refer to the area under the emission peak as scanned from 300 to 420 nm. Since the absorption of cytochalasin B overlaps with the absorption and emission energy of GLUT-1, we estimated the correction of GLUT-1 fluorescence intensity by

$$I = I(\text{obs})10^{(A_{280}+A_{340})/2} \quad (1)$$

Ligand titration data are reported in terms of fractional quenching, which is equal to $1 - I/I_0$.

The samples containing Laurodan and 2-AS and 12-AS were prepared by adding a small volume from concentrated stocks in ethanol to the GLUT-1 proteoliposomes and sonicating briefly in a bath sonicator. Laurodan samples were excited at 340 nm, and the emission was scanned from 380 to 580 nm. Anisotropy measurements of the 2-AS and 12-AS probes were performed at λ_{ex} = 381 nm and λ_{em} = 460 nm unless otherwise noted. The intensity reported for these samples is the total intensity at those wavelengths.

Samples were pressurized in a home-built cell based on the design of Paladini and Weber (1981). The pressure was increased in 50−100 bar intervals, and the system was allowed to thermally equilibrate for at least 5 min before data were taken. Pressure studies were done at ambient temperature. Each experiment lasted approximately 60 min. Reversibility was checked by comparing the pressurized samples after release to unpressurized controls. Since the volume of the solution decreases very slightly in the 1−600 bar pressure range used (Bridgeman, 1958), corrections for increased protein concentration at elevated pressure were not necessary.

Fluorescence anisotropy measurements of GLUT-1 were taken at 290 nm. Values for an average of at least 20 measurements that were corrected for background scattering were used. Data were analyzed by the Perrin equation:

$$A_0/A - 1 = RT\tau/\eta V = \tau R \quad (2)$$

where A is the anisotropy and A_0 is the anisotropy in the absence of rotational motion and was estimated, from the temperature dependence of the anisotropy and lifetimes, to be 0.12 at 290 nm (we note that this value can be varied substantially without affecting the conclusions). R is the gas constant, T is the absolute temperature, τ is the average

fluorescence lifetime weighted by the fractional intensities, η is the viscosity, $\boldsymbol{R}$ is the rotational rate, and V is the rotational volume assuming a sphere. In the calculations, a constant V value of 150 mL/mol was used.

The change in GLUT-1 tryptophan rotational motion with temperature was analyzed in terms of the thermal coefficient of the viscosity (**b**) which relates the viscosity at a particular temperature (T) to the viscosity at a reference temperature (T_0):

$$\eta = \eta_0 e^{-\mathbf{b}(T_0 - T)} \quad (3)$$

Substituting this expression into eq 1, taking the natural logarithm, and rearranging give

$$Y = \ln(A_0/A - 1) - \ln(RT\tau/V) = \ln \eta_0 + \mathbf{b}(T_0 - T) \quad (4)$$

For simplicity, we allow T_0 to be 273 K. Therefore, if Y is plotted against the temperature in degrees centigrade, we obtain a straight line with a slope **b**.

The thermal coefficient of the viscosity can be macroscopically determined by flow viscometry or microscopically determined by fluorescence anisotropy. For free fluorophores, the **b** values determined by fluorescence anisotropy match those determined by flow (Weber et al., 1984). If one considers that the anisotropy is related to the average angular displacement of the probe during its excited state, then **b** describes the increase in this displacement as a function of temperature, or the thermal expansion of the environment surrounding the probe. For fluorophores in proteins, the **b** values obtained differ from free fluorophores due to the difference in the thermal expansion of the local environment. If a probe interacts strongly with its environment, then more energy in terms of temperature is needed for its rotational angle to expand (Scarlata et al., 1984; Rholam et al., 1984).

The local isothermal compressibility (β) was determined by first relating the anisotropy to a precession angle, γ, by $[\langle\cos^2 \gamma\rangle = (1 + 2A/A_0)/3]$ and noting that this angle is proportional to the free volume (V_f). The local compressibility can be calculated from a pressure change of p_1 to p_2 by

$$V_f(p_2) = V_f(p_1)(1 - \beta\Delta p) \quad (5)$$

A full discussion of this analysis can be found elsewhere (Scarlata, 1991b).

RESULTS AND DISCUSSION

Isothermal, Isobaric Characterization of GLUT-1−Ligand Binding. The decrease in the fluorescence intensity of GLUT-1 upon ligand binding was recorded by titrating the protein with D-glucose and cytochalasin B. Simultaneous control experiments were done using the nonbinding ligand analogs L-glucose and dihydroxycytochalasin B. In agreement with previous reports, binding was stereospecific and saturable with affinities in accord with previous studies [see Baldwin (1993) and Curruthers (1990)]; D-glucose and cytochalasin B quenched GLUT-1 fluorescence whereas L-glucose and dihydroxycytochalasin B did not alter the intensity. (For the remainder of the paper, D-glucose will be referred to simply as glucose.)

The studies were done at an excitation wavelength of 295 nm in order to preferentially view Trp emission. We repeated the titrations exciting both tryptophan and tyrosine residues (280 nm). Excitation at this wavelength yields much higher emission intensities and allows us to better discriminate fluorescence emission intensity from scattered light. Excitation at 280 nm will also allow us to view a larger fluorophore population and give more information about the conformational state of the whole protein. We note, however, that because Trp has a higher extinction coefficient than Tyr, and because Try residues can donate their excitation energy to Trp, a large fraction of the emission is expected to result from Trp residues.

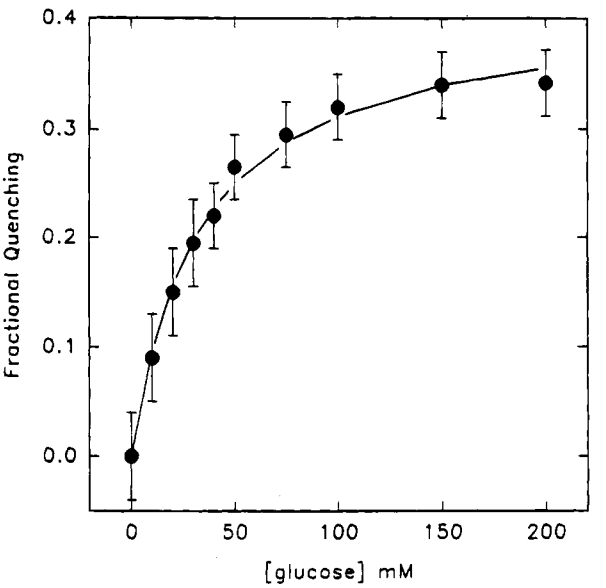

FIGURE 1: Quenching of the intrinsic fluorescence of GLUT-1 by D-glucose. The fractional area was calculated from the intensities at each concentration ($1 - I/I_0$) where the intensities refer to the areas under the emission curve scanned from 300 to 420 nm. Excitation was at 280 nm. A control study with nonbinding L-glucose showed no change in emission.

The titration curve for the binding of glucose to GLUT-1 (λ_{ex} = 280 nm; Figure 1) shows that at this excitation wavelength the decrease in intensity is close to 40%, which is much greater than the value observed when only tryptophan emission is monitored. Shown is a fitted curve for seven experiments. The affinity determined from this plot (K_d = 32.9 ± 2.6 mM) is in good agreement with previous reports (Gorga & Lienhard, 1982; Curruthers, 1986a,b; Thorne et al., 1992). A similar, saturable decrease was observed by cytochalasin B (data not shown) with a binding affinity within the range of literature values (see citations above). On the basis of the good agreement of our K_d values and others, we conducted the remainder of the intrinsic fluorescence studies using an excitation wavelength of 280 nm.

The binding of ligand is also accompanied by a change in the skewness of the spectrum such that the center of spectral mass shifts approximately 450 cm^{-1} (4 nm) toward lower energies without a significant change in the position of the emission peak. This shift indicates that upon ligand binding a population of fluorophores move toward a more polar environment. Because a large population of fluorescent species becomes quenched upon ligand binding, it is impossible to estimate the fractional shift in fluorophore environment.

Pressure Characterization of Liganded and Unliganded GLUT-1. The effect of pressure on the emission intensity

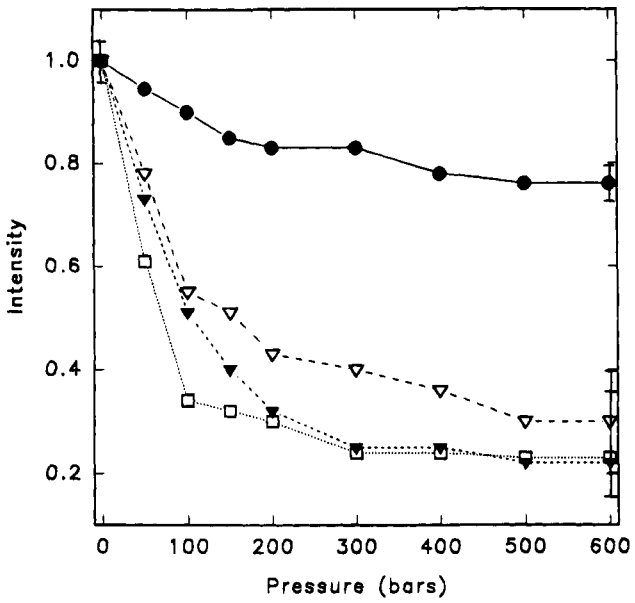

FIGURE 2: Effect of pressure on the normalized fluorescence intensity of GLUT-1 proteoliposomes in the absence of glucose (●), at 20 mM glucose (▽), at 200 mM glucose (▼), and at saturating concentrations (2 μM) of cytochalasin B (□). Data are an average of 3−5 pressure runs with the sample-to-sample error shown for the initial (lowest) and final (highest) values. Because cytochalasin B absorbs at both the exciting wavelength and the emission band, the actual values are approximately 13% higher than those shown.

of GLUT-1 under various conditions was studied. In the absence of ligand, the emission intensity decreases by approximately 20% in the first 600 bars (Figure 2) with a smaller change thereafter (we note that samples taken to 600 bars were fully reversible and those taken to 1200 bars were only frequently reversible while samples subjected to higher pressure were never reversible). In the presence of saturating glucose (0.2 M), a much more extensive decrease (approximately 80%) in intensity was observed in the first 600 bars after which the extent of decrease was comparable to the unliganded form. Half-saturating amounts of glucose (20 mM) produced a decrease in intensity closer to that seen at saturating ligand conditions, indicating a stabilization of this form under pressure.

We also studied the pressure response of the fluorescence of GLUT-1 in the presence of two ligand analogs. The first of these was cytochalasin B, which binds at or close to the inner binding site.[1] Fluorescence studies show that conformational changes of GLUT-1 brought about by cytochalasin B are similar to those by glucose although circular dichroism and proteolysis studies show that the binding of cytochalasin B does not produce the same conformational state (Chin et al., 1987; King et al., 1991). In the presence of saturating cytochalasin B (2 μM), a decrease in intensity comparable to glucose occurred in the 600 bar range studied (Figure 2). The similarity of the pressure behavior of GLUT-1 with glucose and cytochalasin B, and the extent of these changes, indicates that it is unlikely that the large pressure-induced decrease in intensity is due to a direct interaction of the ligand with a single GLUT-1 fluorophore but rather with a number of fluorophores, supporting the idea that unliganded and liganded fluorescent forms are distinct conformers.

The second ligand analog studied was maltose. Maltose, the 1−4 α dimer of glucose, binds to GLUT-1 but is not transported [see Baldwin (1993) and Curruthers (1990)]. Previous fluorescence quenching studies indicate that maltose binding changes the accessibility of GLUT-1 tryptophans to quenchers differently than glucose or cytochalasin B, thus implying a different conformational state (Pawagi & Deber, 1990), although proteolysis data argue against this. The change in intensity under pressure for samples containing saturating amounts of maltose (40 mM) closely resembles those seen for glucose and cytochalasin B (data not shown). These data show that binding of glucose or its two ligand analogs brings about the same pressure-sensitive state.

The decrease of emission intensity of GLUT-1 in the presence of ligands is very large. This decrease could be caused by the settling of proteoliposomes during the pressure experiments that occurs preferentially in the presence of ligand, movement of a large population of the fluorophores close to quenching groups caused by ligand binding, or self-quenching of the fluorophores caused by aggregation of the liganded protein. We investigated these possibilities.

To determine whether settling of the proteoliposomes in the presence of ligand occurs under pressure, we repeated the pressure studies monitoring light scattering at 90° using exciting and emitting wavelengths of 350 nm. Settling of the sample would result in a decrease in the amount of scattered light. We found that the scattering intensity remained constant throughout the 1−1200 bar pressure range. This result allows us to conclude that the decrease in fluorescence intensity with pressure is not due to a decrease in the number of particles in the light path.

Quenching Studies. Quenching studies of intrinsic GLUT-1 fluorescence were conducted to determine whether the decrease in intensity upon ligand binding is due to ligand-induced movement of a large population of fluorophores in close proximity to quenching groups. We first carried out these studies at atmospheric pressure.

The predicted secondary structure of GLUT-1 places 4 of the 6 tryptophans and 9 of the 13 tyrosines at interfacial positions [see Baldwin (1993) and Curruthers (1990)]. We note that charged species, which are found in lipid head groups, are frequently quenchers of phenol and indole fluorescence [see Eftink and Ghiron (1981)]. Thus, if ligand binding induces movement of these interfacial residues to positions closer to charged moieties in the lipid head groups, then these residues would be properly positioned to be quenched under pressure. To explore this possibility, we measured the accessibility of the Trp and Tyr residues to the quenchers, KI and acrylamide. Because of its charge, I^- will only quench residues in the aqueous phase, whereas acrylamide will quench residues exposed to the aqueous and interfacial phases as well as some internal sites. If the fluorophore population is homogeneous in terms of acces-

[1] While glucose and maltose have a very low solubility in acetone and alcohol (see Merck Index, 10th ed.) and are not expected to significantly penetrate the membrane, cytochalasin B is poorly soluble in water and is expected to completely partition into the membrane. The cytochalasin B concentration used in our pressure studies (2 μM or 10 times the K_D) is roughly 4% of the lipid concentration, and thus 4% of the membrane is occupied by the dye. We expect the dye may affect membrane properties (such as transition temperatures, microviscosity, etc.) but not the properties of the protein. Nevertheless, cytochalasin B was only used as a well-characterized, non-sugar ligand analog for this pressure experiment. We note that it is possible that initially cytochalasin B could quench nearby GLUT-1 fluorophores at atmospheric pressure and that increased pressure may enhance this effect. Therefore, the cytochalasin B pressure study should only be viewed as an indication of the behavior of GLUT-1 in the ligand state, and the quantitative aspects should not be considered.

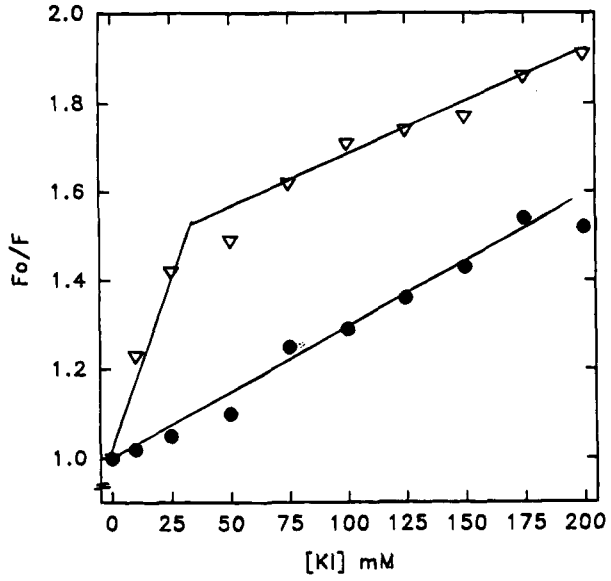

FIGURE 3: Stern–Volmer plot showing the quenching of the intrinsic fluorescence of GLUT-1 by KI. Plotted is the ratio of the intensity in the presence of quencher (F) relative to its value in the absence (F_0) of quencher: (●) no ligand and (▽) 200 mM glucose. Maximum error ± 0.08 at 200 mM KI.

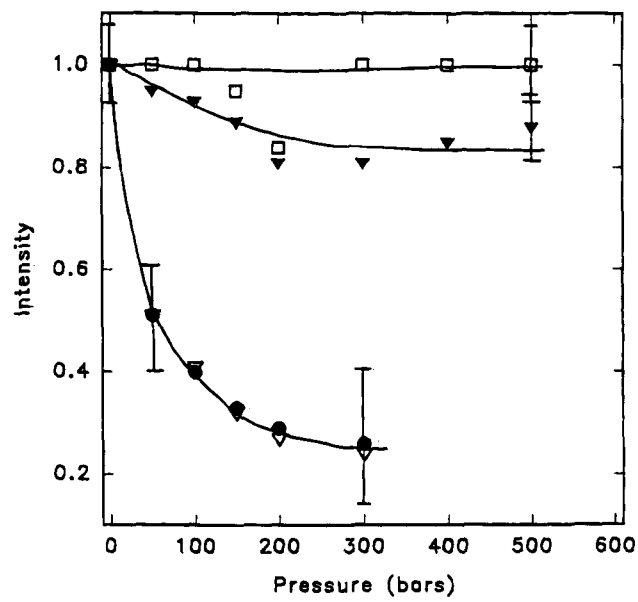

FIGURE 4: Effect of pressure on GLUT-1 fluorescence in the presence of 160 mM KI (●) and 160 mM acrylamide (▼) without ligand; and 160 mM KI (▽) and 160 mM acrylamide (□) in the presence of 200 mM glucose.

sibility, then plotting the ratio of the fluorescence intensity in the absence of quencher over the intensity in the presence of quencher (F_0/F) as a function of quencher concentration (i.e., Stern–Volmer plot) will yield a single straight line. If the fluorophore population is heterogeneous with respect to quencher accessibility, then the Stern–Volmer plot will be composed of more than one slope and appear curved. In Figure 3, we present the quenching curves of GLUT-1 in the presence and absence of saturating glucose. In the absence of glucose, the accessibility to quencher is homogeneous. In the presence of glucose, the accessibility to quencher is heterogeneous with a population of fluorophores that has a higher accessibility to KI. To ensure that the observed changes were not due to changes in electrostatic interactions, we conducted a similar experiment where the ionic strength was held constant at 0.36 M and KI was substituted for KCl. Identical data were obtained.

Acrylamide, on the other hand, quenches both liganded and unliganded GLUT-1 to the same extent (data not shown). This indicates that the difference in emission between the two forms is primarily due to an interfacial population of fluorophores that become more exposed to the aqueous solvent upon ligand binding.

To determine whether the differences in fluorescence emission we observe in the liganded and unliganded forms under pressure could be due to changes in the positions of these residues relative to the membrane surface, we studied the pressure behavior of unliganded and liganded GLUT-1 in the presence of 0.16 M I^- and acrylamide. If the decrease in emission of the liganded protein under pressure is due to a significant movement of emitting species from the hydrocarbon region of the membrane to the surface, then we should observe a more extensive loss in intensity in the presence of I^- as opposed to acrylamide. We measured the quenching by KI of the liganded and unliganded GLUT-1 under pressure and found that the intensity of both forms decreased to the same extent during the entire pressure range (Figure 4). This result allows us to conclude that the exposure of the fluorophores to aqueous solvent remains unchanged under pressure in both conformations.

Acrylamide quenches both the unliganded and liganded forms to the same extent at atmospheric pressure. Under pressure, the unliganded sample showed a small decrease in the first 500 bars while the intensity from the liganded sample was unchanged (Figure 4). Taken together, these quenching data suggest that the quencher sensitivity of the two forms of GLUT-1 under pressure is due to subtle changes in the exposure and/or dynamics of interfacial fluorophores.

The quenching data we present here differ with a previous quenching study (Pawagi & Deber, 1990), who noted a small decrease in quenching by KI and acrylamide in the presence of glucose. One or a combination of several factors may be responsible for this discrepancy. First, those investigators noted an unusual shoulder at 415 nm in the GLUT-1 emission spectrum, while the emission of our preparation appeared to be typical of a tryptophan-containing protein. Second, those authors focused on the quenching of Trp residues by exciting at 295 nm, whereas we include the quenching of both Tyr and Trp residues by exciting at 280 nm. Both the contribution of Tyr residues to the emission and the total elimination of scattered light by exciting at lower wavelengths and using band-pass filters may cause this discrepancy. Third, we determined quenching on the basis of the change in the area under the entire emission curve rather than only at the emission peak. We find that even though the emission peak barely shifts with glucose binding, the skewness of the peak increases toward larger wavelengths. Taking into account the emission of all of the fluorescent species may contribute to the differing results. Also, we offer here other corroborating studies to support the idea that ligand binding results in a change in the position of the interfacial fluorescent residues of GLUT-1.

Changes in GLUT-1 Proteoliposomes upon Ligand Binding As Detected by Fluorescent Probes. To better determine whether glucose binding produces movement of a population of membrane-buried fluorophore toward the surface, we also monitored energy transfer between the GLUT-1 tryptophan donors and fluorescent acceptor groups placed in the membrane. The first of the probes tested is 2-(9-anthroyloxy)stearic acid (2-AS). Energy transfer from tryptophan

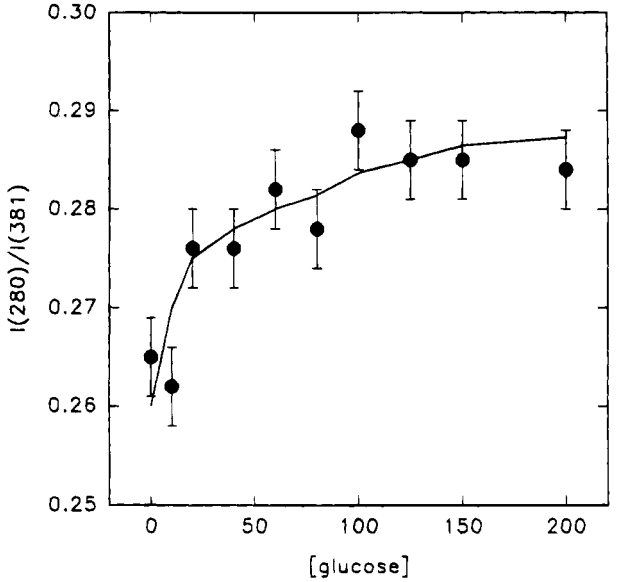

FIGURE 5: Changes in the extent of GLUT-1−2-AS energy transfer as monitored by the ratio of the emission intensity of 2-AS (1 mol %) excited at the excitation wavelength of Trp (280 nm) over the emission intensity of 2-AS excited as its excitation wavelength (381 nm) as a function of glucose concentration. The error is ±0.023 for the ratio and within the size of the points for the glucose concentrations.

residues on proteins to anthroyloxy fatty acid probes has been previously used to map out the membrane location of tryptophans of integral membrane proteins and to verify certain protein conformations (Kleinfeld, 1985; Kleinfeld & Lukacovic, 1985). The distance where 50% transfer occurs, or the critical distance, is approximately 20 Å. Experimentally, there are several methods to view energy transfer. For multiple donors, if the enhancement of acceptor fluorescence is monitored relative to the fluorescence in the absence of transfer, the efficiency can be written as an expression that is independent of the quantum yields of the donor and acceptor [see Kleinfeld (1988)]. In this case, the extent of energy transfer can be assessed by the ratio of the acceptor fluorescence when it is excited at the donor excitation wavelength (i.e., 280 nm for Trp) to when it is excited at its maximum excitation wavelength (i.e., 381 nm for 2 AS). In Figure 5, we show the change in this ratio when glucose is titrated into GLUT-1 proteoliposomes doped with 2-AS. As glucose is added, the amount of 2-AS fluorescence at 280 nm excitation relative to 381 nm excitation increases, indicating that Trp to 2-AS energy transfer increases when ligand binds to GLUT-1. Moreover, this curve closely resembles the glucose titration curve that is obtained when the intrinsic fluorescence is viewed (Figure 1). Since the fluorescent group of 2-AS is located close to the head group region, the data in Figure 5 are consistent with movement of a population of fluorophores toward the membrane surface upon ligand binding. Analogous experiments using a probe whose fluorescent group is more deeply buried in the membrane (12-AS) and using a probe whose fluorescent group is located on the membrane surface [6-dodecanoyl-2-(dimethylamino)naphthalene or Laurodan] did not show any glucose-dependent changes in the extent of energy transfer. Since these probes have a similar critical distance to 2-AS, these results suggest that although movement of fluorophores toward the surface may be occurring, it is not extensive enough to change the extent of transfer to the membrane surface or to very buried probes.

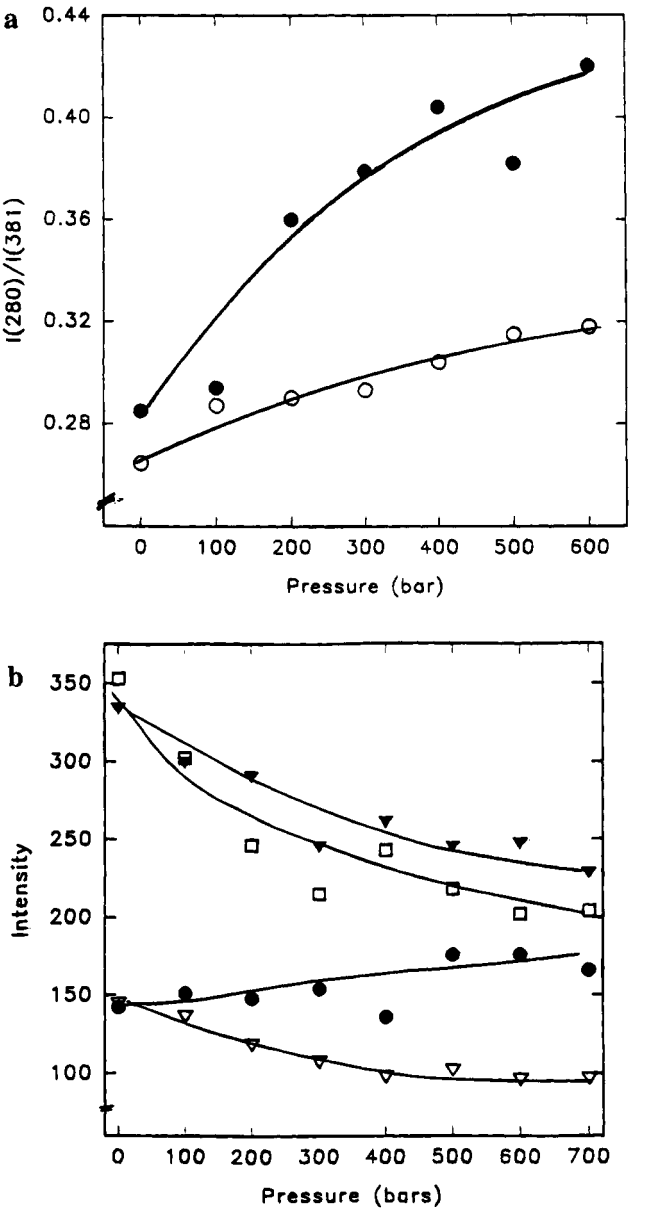

FIGURE 6: (a) Effect of pressure as seen by changes in the extent of Trp−2-AS energy transfer (see Figure 6) in the absence of (●) and presence (○) of 200 mM glucose with an error of ±0.0033. (b) Analogous study showing the pressure behavior of the 2-AS intensity in the absence (▽) and presence (●) of glucose; and for 12-AS in the absence (□) and presence (▼) of glucose. Maximum error ±20 counts.

We also monitored Trp−2-AS energy transfer as a function of pressure in the absence and presence of saturating ligand. Without glucose, the amount of energy transfer increases with pressure, most likely due to the decrease in distance between the species caused by the decrease in volume (Figure 6a). In the presence of glucose, energy transfer between GLUT-1 Trps and 2-AS increases more extensively, indicating a greater reduction in the distance between these species in the presence of ligand. Both samples showed a similar increase when GLUT-1 Trp−12-AS energy transfer was monitored.

To obtain more corroborating evidence that movement of a fluorophore population is occurring upon ligand binding, we also conducted quenching studies using 2-AS. 2-AS will be quenched by interfacial tryptophan residues (Haigh et al., 1979), and thus changes in 2-AS fluorescence intensity will be indicative of changes in the average Trp−AS distance in

this region of the membrane. In model membranes composed of both saturated and unsaturated lipids in the absence of proteins, the intensity of both 2-AS and 12-AS remains constant in the 0−2000 bar pressure range (Scarlata, 1990), and we have no reason to expect that the probes in endogenous lipids in red blood cell membranes behave differently. Thus, if the average distance between the Trp residues and the probe remained constant under pressure, we would not expect to observe any significant changes in AS intensity. However, we do expect that under pressure the average distance decreases as the free volume of the membrane decreases.

The intensity of 12-AS incorporated into GLUT-1 proteoliposomes decreases under pressure corresponding to a decrease in the average distance between the GLUT-1 Trp residues and 12-AS (Figure 6b). The extent of this decrease is similar in the absence and presence of glucose. The intensity of 2-AS in the absence of glucose decreases to a similar extent. In contrast, the intensity of 2-AS in the presence of glucose increases, corresponding to an increase in distance. These data show that upon binding of glucose there is a change in the average position of the tryptophan residues that are close to the membrane surface. The AS behavior of GLUT-1 in the presence of glucose can be compared to that observed for bleached bacteriorhodopsin (Scarlata, 1993), where pressure increased the distance between the interfacial Trp residues and the AS probes due to the increase in bilayer thickness as the lipid chains straighten under pressure.

Assuming that ligand binding causes interfacial residues to move closer to the membrane surface, the reason for the extensive decrease in intrinsic fluorescence of the liganded form under pressure is unclear. It is possible that in the liganded form, pressure may induce a mismatch between the hydrophobic regions of the protein and the hydrocarbon chains of the lipid, leading to protein aggregation in the membrane and self-quenching of the fluorophores. Self-quenching due to the close proximity of fluorophores of this magnitude has been previously observed for the membrane peptide gramicidin due to stacking of two Trp residues (Scarlata, 1988) and for oligomerization of fluorescein-labeled melittin monomers to tetramers (Runnels and Scarlata, unpublished observations), and self-quenching has even been used to monitor protein polymerization (Sims & Weidmer, 1984). Alternately, repositioning of the GLUT-1 fluorophores closer to the membrane surface may make these residues more susceptible to quenching by charged species in the lipid head group region as the bilayer thickness increases under pressure (Braganza & Worcester, 1986). To discriminate between these two mechanisms, we conducted a series of studies again using fluorescent probes incorporated into the proteoliposomes. If a fluorescent probe is dispersed in a proteoliposome that has a homogeneous distribution of proteins, and pressure causes the proteins to segregate into domains, then the environment around the probe is expected to shift to one that is rich in lipids, unless, of course, the solubilities of the probe in the lipid-rich and protein-rich domains are identical, which is unlikely. In this way, monitoring changes in the local physical properties measured by fluorescent probes may point to protein aggregation. We note that although the protein:lipid mole ratio is only 1:150 in the GLUT-1 proteoliposomes, the ratio of the membrane volume occupied by GLUT-1 versus lipid is on the order of 1:9 based on a rough calculation. On the basis of this large protein volume, and considering that annular lipids may be associated with the protein, the formation of protein domains should be observable by changes in the environment of the fluorescent probes used.

We studied the pressure sensitivity of two sets of probes incorporated into the GLUT-1 membranes. First, we monitored the changes in the emission energy under pressure of a polarity-sensitive probe, Laurodan [see Weber and Farris (1979)], incorporated into GLUT-1 proteoliposomes. We find that from 1 to 600 bars the emission shifts to higher energies and that the magnitude of this shift is identical in the presence and absence of saturating glucose and is identical to the shift of the probe seen in DOPC bilayers. This result shows that the dielectric properties of the GLUT-1 proteoliposome head group region undergo minor changes that are typical of lipid membranes under pressure, and are not altered by the presence of ligand. These data argue against the formation of protein-rich domains of liganded GLUT-1 under pressure.[2]

In a second series of studies, we monitored the fluorescence anisotropy and intensity of 12-AS incorporated into GLUT-1 membranes in the presence or absence of saturating glucose and related these parameters to the local compressibility around the 12-AS probe (eq 5). Both sets of samples showed identical behavior: an increase in anisotropy and a small decrease in intensity in the first few hundred bars. Similarly, the local compressibilities calculated from the pressure region where the intensities were constant give comparable values (6.9 ± 0.3 and 7.3 ± 0.4 K^{-1}, in the presence and absence of glucose, respectively). If ligand binding promoted protein aggregation under pressure, then we would expect to observe differences in compressibility between the two samples. Moreover, we would expect the formation of protein-rich domains would cause the probe to be in a more lipid-rich environment. Since lipids are more compressible than proteins,[3] then the local compressibility observed by the probe would be higher. The similar compressibilities in the liganded and unliganded forms indicate that the liganded form of GLUT-1 remains dispersed in the membranes. The observation that energy transfer between GLUT-1 Trps and 12-AS increases by the same amount also argues against the formation of domains. Taken together, the Laurodan and 12-AS studies suggest that the large decrease in fluorescence intensity of the liganded GLUT-1 under pressure is due to increased quenching by charged species in the lipid head group region caused by the greater thickness of the bilayer as pressure is applied.

Effect of Ligand Binding on the Rotational Behavior of GLUT-1 Fluorophores. To gain more insight into the

[2] We also observed that the fluorescence intensity of Laurodan increased under pressure, and so it is unlikely that the decrease in intrinsic fluorescence observed for GLUT-1 is due to settling of the proteoliposomes during the pressure experiments. This result corroborates the findings of the light-scattering measurements.

[3] The compressibilities of several aqueous soluble proteins have been measured, and these values are much lower than lipids [see Heremans (1982) and Weber and Drickamer (1983)]. However, the compressibility of integral membrane proteins is unknown. In general, compressibilities reflect the strength of interactions between the species comprising the sample. For the hydrocarbon region of lipids, these interactions are weak. In contrast, the known and proposed structures of integral membrane proteins show hydrogen-bonding patterns in the helical secondary structures as well as other dipole−dipole and dipole-induced dipole interactions throughout the protein. On this basis, the compressibility of integral membrane proteins is expected to be lower than lipids.

underlying structural changes that occur when glucose binds, we studied the rotational motion of GLUT-1 fluorophores at atmospheric pressure. Unfortunately, the limited emission signal of the protein precluded us from monitoring the change in rotational motion under pressure since a significant fraction of light is lost through the windows of the pressure cell. On the basis of studies of both water-soluble and integral membrane proteins, we reasoned that if many of the GLUT-1 fluorophores are interfacial and interact strongly with the lipid head groups through hydrogen bonding, their rotational motion would be limited. Alternately, if these residues were more buried in the membrane, or if they were immersed in the aqueous solvent, they would rotate much more freely.

We measured the change in anisotropy and lifetime of GLUT-1 upon binding to either glucose or cytochalasin B. The anisotropy of tryptophan is very low when exciting a 280 nm, and to obtain higher values, we conducted these studies exciting at 290 nm. At this wavelength, we will primarily be viewing emission from tryptophan residues. At 22 °C, the rotational rate (***R***, see eq 2) decreased significantly from 0.465 to 0.287 ns^{-1} with ligand binding. The decrease in rotational rate with ligand binding corresponds to a restriction of rotational motion of the GLUT-1 tryptophans. This decrease was almost completely due to an increase in fluorescence anisotropy since the lifetime showed only a small increase with binding [(1.58−1.66) ± 0.1 ns]. The lifetime data show that the intensity changes in Figure 1 are caused either by fluorescence from tyrosine residues, which is unlikely due to the magnitude of the change, or by static quenching of interfacial tryptophans. Monitoring glucose binding by anisotropy closely followed the curves in Figure 1, giving the same K_d as the fluorescence intensity and other techniques.

Considering the decrease in rotational rate with ligand binding, the decrease in emission intensity, the red shift of the center of spectral mass, the increased amount of KI quenching, and the increased energy transfer between 2-AS and GLUT-1 Trp residues, we are led to the idea that a large population of the fluorophores in the liganded state are at the interface and that the binding of glucose shifts the average positions of the interfacial fluorophores closer to the lipid head groups where they become quenched under pressure. If these residues interact strongly with the lipid head groups, then their motions should be locked tightly and should not be perturbed by changing the temperature. Alternately, if the unliganded form of the transporter is more buried (as implied by the anisotropy, KI quenching, and emission energy), then its rotational motion should increase with increasing temperature due to the increase in kinetic energy of the system and to the increase in the fluidity of the system. If there are no strong interactions between the fluorophore residues and the lipid head groups, then the change in fluorophore motion with temperature should follow the change of lipid probes embedded in the hydrocarbon interior of the membrane.

We measured the change in anisotropy and average lifetime as a function of temperature and analyzed the data using eq 4 (see Materials and Methods), which yields the parameter **b**, the thermal coefficient of the viscosity which can be regarded as a thermal expansion coefficient (Scarlata, 1989). We find that from 12 to 37 °C GLUT-1 displays two **b** values with a transition at ~23 °C whether ligand is present or not (data not shown). This transition is due to either a large-scale change in the interaction of the protein with the lipid matrix or a transition of the lipid matrix itself. A transition at 23 °C has also been observed monitoring the turnover of GLUT-1 in red blood cells as well as motions of an EPR probe embedded in red cell membranes (Whitesell et al., 1989). We also find that in the absence of glucose, the magnitudes of the **b** values of GLUT-1 are similar to those seen by 12-AS in the same proteoliposomes and are much greater than those seen in the absence of glucose. Although these **b** values are a composite of rotational motions from a very heterogeneous population of Trp residues with varying contributions to the average anisotropy and lifetime, the data indicate that in the absence of glucose the change in rotational motion with temperature of GLUT-1 fluorophores is similar to a probe embedded in the hydrocarbon interior but in the liganded state the change in motion with temperature of these residues is more limited. These data support the idea that in the unliganded protein the interfacial residues are more buried and subject to an environment more similar to the membrane whereas upon ligand binding the residues move closer to the surface.

Suggested Model for the Stabilization of the GLUT-1 Conformational State by Increased Lipid Packing. In this study, we have found that the fluorescent residues of GLUT-1 undergo a substantial decrease in intensity upon binding of ligand (Figure 1). This decrease is accompanied by a shift in the spectral mass toward lower energy and by a large increase in anisotropy without a significant change in lifetime. The binding of ligand also increases the accessibility of the fluorescence quencher KI to tryptophan and/or tyrosine residues (Figure 3). Energy transfer and quenching studies of a probe located close to the lipid head group region show more extensive interaction with GLUT-1 tryptophans in the presence of glucose than in the absence (Figures 5 and 6). Pressure studies show a substantial quenching of GLUT-1 emission in the presence of ligand as opposed to in the absence (Figure 2). Studies employing other fluorescent probes located close to the surface and in the interior of the membrane indicate that the large decrease of GLUT-1 fluorescence in the presence of ligand is not due to protein aggregation. Further, temperature studies focusing on the rotational motion of the probes support the notion that in the unliganded form, the interfacial fluorophores are more buried within the membrane.

On the basis of these observations, we propose the simplest model that can explain the experimental results. We first assume that the liganded and unliganded forms of GLUT-1 can be thought of as at least two distinct conformational states characterized by different fluorescence emission intensity, energy, access to ionic quenchers, and rotational freedom. While it is remotely possible that these changes result from a direct effect of the ligand without a conformational change, the fact that identical behavior is seen with cytochalasin B, which binds close to but not at the inner glucose binding site, and the fact that the fluorescence undergoes such an extensive decrease with ligand binding make this possibility highly unlikely. While many studies point to the validity of the two-state model, other work suggests that more than two states may exist. For example, proteolysis studies show two major conformational states of GLUT-1 brought about by glucose binding to the intracellular site, and a distinct proteolysis pattern is observed with cytochalasin B (King et al., 1991). Circular dichroism studies show that binding of glucose but not cytochalasin B alters the secondary structure of GLUT-1 (Chin et al., 1987). However, all fluorescence

studies show that binding of glucose and cytochalasin B produces a similar decrease in emission intensity (Gorga & Leinhard, 1982). Thus, the conformational change associated with alterations in the interfacial residues appears to occur independently from the other changes that characterize the liganded state.

Even though the fluorescence properties of the liganded and unliganded states are very different, they need not be due to large scale conformational changes of the protein. The proposed secondary structure of GLUT-1 places most of the fluorophores at interfacial positions [see Baldwin (1993) and Curruthers (1990)], and thus a small change in the positions of these residues relative to the lipid surface could produce large fluorescence changes. Therefore, the fluorescence of GLUT-1 can be used as a sensitive monitor of changes in interfacial contacts with lipid packing. Several fluorescence parameters suggest that upon ligand binding the fluorophores of GLUT-1 move closer to the membrane surface, resulting in much stronger interactions with the lipid head groups. In contrast, the fluorophores of unliganded GLUT-1 appear more buried and less rotationally restricted. How does lipid packing affect these two forms? Since increased lipid packing increases the thickness of the bilayer (Braganza & Worcester, 1986), then the liganded form of GLUT-1, which has a larger population of Trp and Tyr close to the interface, is stabilized by pressure or other conditions that stabilize interfacial contacts.

Previously, in studies of gramicidin (Scarlata, 1991a) and bacterioopsin (Scarlata, 1993), we have found that increased lipid packing will destabilize hydrogen bonding between indole protons and hydrogen-bond acceptors on the lipid head groups (presumably carbonyl groups). The above discussion further points to surface interactions being responsible for changes in conformational stability. A small change in conformation that primarily alters surface interactions is not necessarily high in energy if the major expenditure is the loss of indole hydrogen bonds. Assuming that the hydrogen bonds on the lipid head group re-form with either water or a neighboring lipid, then the energy lost for the complete rupture of six indole hydrogen bonds is ~6 kcal (using a value of 2 kcal/mol per indole hydrogen bond as estimated for gramicidin; Scarlata, 1991a). Rather than energetic, the advantage of using interfacial residues to switch conformations may be kinetic since it would not involve slower movements of the protein backbone.

Our data suggest that transport of ligand through the membrane involves movements of the protein against the aqueous phase since the fluorophores move closer to the surface. If upon ligand binding the protein expanded against the lipid chains, then we would not expect a stabilization of the unliganded form with increased lipid packing unless the liganded form had a central pore that could fill with solvent which could counteract the lateral compression by the lipid chains. Our results here instead show that ligand binding causes an expansion against the aqueous phase rather than the lipid phase. Expansion against the aqueous phase offers a lower energy route for conformational changes since water is much more easily displaced than lipid chains. Thus, conformational changes of this type may be a general strategy for membrane transport proteins.

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