A Method for Determining Transmembrane Helix Association and Orientation in Detergent Micelles Using Small Angle X-Ray Scattering

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ABSTRACT Solution small angle x-ray scattering can be used to study the association of transmembrane proteins solubilized in detergent micelles. We have used the α-helical transmembrane domain of the human erythrocyte glycophorin A (GpA) fused to the carboxyl terminus of monomeric staphylococcal nuclease (SN/GpA) as a model system for study. By matching the average electron density of the detergent micelles to that of the buffer solution, the micelle contribution to the small angle scattering vanishes, and the molecular weight and the radius of gyration of the proteins can be determined. SN/GpA has been found to dimerize in a zwitterionic detergent micelle, *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB), whose average electron density naturally matches the electron density of an aqueous buffer. The dimerization occurs through the transmembrane domains of GpA. With the aid of the nuclease domain scattering, the orientation of the helices within a dimer can be determined to be parallel by radius of gyration analysis. The association constant of a mutant (G83I) that weakens the GpA dimerization has been determined to be 24 μM in the DDMAB environment. The experimental methods established here could be used to apply solution small angle x-ray scattering to studying the association and interactions of other membrane proteins.

INTRODUCTION

Many membrane proteins contain transmembrane α-helices that interact with each other in a side-to-side association that determines their structure. It is also observed that similar helix association governs the formation of higher order quaternary structures as well. The study of the interactions between transmembrane helices in folding and oligomerization has been difficult, since the application of classical solution methods is compromised by the need to solubilize these regions of structure using detergent environments. Consequently, the emergence of new technical approaches to the study of oligomerization broadens the scope of physical biochemistry as it pertains to membrane proteins.

In this study we explore the possibility of applying small angle x-ray scattering (SAXS) to measure the association and conformation of transmembrane proteins in detergent micelle solutions. SAXS measures the absolute molecular weight, the radius of gyration, and the global conformation of macromolecules in solution. It has been used effectively in studying protein–protein interactions, protein quaternary structures, and protein folding problems (for reviews see Moore, 1982; Lattman, 1994; Trewhella, 1997). Although the parameters obtained from solution SAXS are comparable to those from static laser light scattering, solution SAXS is more suitable for studying protein size and conformation changes because the distance scale probed by SAXS is between 500 and 300 Å, within the size range of most proteins. Small angle neutron scattering (SANS) has also been used to study the structure and aggregation state of proteins in detergent micelles (Yeager, 1976; Perkins and Weiss, 1983; Pachence et al., 1987; Jeanteur and Pattus, 1994). Neutron scattering contrast variation methods are readily implemented by varying the deuteration of water to change the contrast between the buffer and the protein or the detergent, facilitating measurement of the molecular weight of the protein, the location of the protein in the detergent micelles or in lipid, and the number of bound detergent molecules. SAXS does not have a wide contrast variation range as compared to SANS. Nevertheless, we show here that, by contrast matching solvent with the average electron density of the detergent micelles, SAXS provides a feasible method to study the association states and conformation of membrane proteins in detergent micelles, which should enable expanded use of the approach given that x-ray facilities are more generally available.

We use the fusion protein, staphylococcal nuclease/glycophorin A (SN/GpA), with the transmembrane domain of glycophorin A (GpA tm) of human erythrocytes fused to the carboxyl terminus of staphylococcal nuclease (SN) to explore the application of SAXS to transmembrane protein association in detergent micelles. GpA has a single transmembrane helix that lacks strongly polar side-chain groups and forms stable dimers in detergent micelles (Lemmon et al., 1992a,b; MacKenzie et al., 1996, 1997). Mutagenesis studies and a recent NMR structure show that van der Waals interactions mediate stable and specific associations between the two transmembrane helices (Lemmon et al., 1992a,b; MacKenzie et al., 1997). The GpA transmembrane domain has also been found to dimerize in phospholipid bilayers (Bormann et al., 1989; Adair & Engelman, 1994) and in a natural biological membrane environment (Langosch et al., 1996; Leeds and Beckwith, 1998; Russ and

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Engelman, 1999). The hierarchy of mutational sensitivity of GpA tm dimerization is found to be similar in detergent micelles and in membranes, indicating that key features of the GpA tm oligomerization are conserved in the environments provided by detergent micelles and natural membranes (Russ and Engelman, 1999). Nevertheless, some differences do exist: mutations to polar residues, which generally disrupt GpA tm dimers in detergent micelles, are found to be less sensitive in the membrane environment.

RATIONALE FOR USING SAXS TO STUDY TRANSMEMBRANE PROTEIN ASSOCIATION IN MICELLE SOLUTIONS

For a protein dissolved in a dilute aqueous solution (about 1–10 mg/mL), the intermolecular interference can be ignored, and the scattered intensity from the protein $I_2(Q)$ in a sample volume V, after buffer subtraction, can be expressed as

$$I_2(Q) = \left[\int_{V_2} (\rho_2(r) - \rho_1) \frac{\sin Qr}{Qr} \, dV\right]^2 = I_2(0)P_2(Q), \quad (1)$$

where $Q = 4\pi \sin\theta/\lambda$ is the magnitude of the scattering vector and 2θ is the scattering angle; $\rho_2(r)$ is the local electron density of the macromolecule, ρ_1 is the electron density of the solvent, and V_2 is the volume of the protein; $P_2(Q)$ is the form factor that is related to the size, shape, and the internal structure of the macromolecule. $I_2(0)$ is the forward scattering intensity, and is related to the protein molecular weight M_{w2} by (Pessen et al., 1973; Timasheff, 1973; Glatter and Kratky, 1982; Moore, 1982),

$$I_2(0) = \kappa(\rho_2 - \rho_1)^2 V_2^2 N_2 \quad (2a)$$

or

$$I_2(0) = \kappa(\rho_2 - \rho_1)^2 \bar{\nu}_2^2 c_2 M_{w2}/N_a, \quad (2b)$$

where κ is a constant that includes the x-ray beam intensity, the instrument configuration, transmission of the x-rays by the sample, the sample thickness, and the scattering from a single electron; ρ_2 is the average electron density of the protein; N_2 is the number of protein molecules in the scattering volume; V_2, $\bar{\nu}_2$, and c_2 are the volume, the partial specific volume, and the concentration (in mg/mL) of the protein, respectively; N_a is the Avogadro number. In the small angle region ($QR_{g2} \leq 1$), $P_2(Q)$ can be expressed by the Guinier approximation as (Guinier and Fournet, 1955)

$$P_2(Q) = \exp(-Q^2 R_{g2}^2/3), \quad (3)$$

where

$$R_{g2} = \left(\frac{\int_{V_2}(\rho_2(r) - \rho_1) r^2 \, dV}{\int_{V_2}(\rho_2(r) - \rho_1) \, dV}\right)^{0.5}$$

is the radius of gyration of the protein. The radius of gyration and the forward scattering intensity can be obtained from the initial slope and the $Q^2 = 0$ intercept of a plot of Ln $I_2(Q)$ versus Q^2.

Practically, for molecular weight determination, the $I_2(0)$ of the protein and several protein molecular weight standards dissolved in the same buffer are measured without changing the instrument configuration. The partial specific volume and the electron density of the protein can be computed from the amino acid composition (Eisenberg and Crothers, 1979). The $I_2(0)/\bar{\nu}_2^2 c_2$ of the protein standards are plotted versus their molecular weights to obtain a line with a slope of κ according to Eq. 2b. The molecular weight of a protein can then be determined using the experimental value of κ.

Equations 1–3 are for the scattering from a two-component system composed of the large protein molecules and the small aqueous solvent molecules. In such a system the density fluctuations of the solvent can be ignored in the Q range measured by SAXS (0.01–0.3 $Å^{-1}$), and the solvent can be subtracted as a homogeneous, incompressible background (Cotton, 1991). However, a membrane protein dissolved in a detergent micelle solution is a three-component system. This three-component system is composed of the protein–detergent complex, the free detergent micelles, and the aqueous buffer. The micelles used to dissolve the membrane proteins are typically ~40 Å in diameter, comparable to the length of the transmembrane helices. On this distance scale, the concentration fluctuations of the micelles and the composition fluctuations between the micelles and the proteins cannot be ignored. Thus, the measured scattered intensity contains information on the concentration and composition fluctuations of the protein–micelle complex as well as information on the size and shape of the individual protein molecules and micelles. Moreover, the protein bound detergent molecules make the protein–detergent complex heterogeneous: above a certain Q value, the scattering intensity profile should also reflect such an internal structural heterogeneity. Therefore, the experimental difficulty in determining the molecular weight and the size of the membrane protein is in the identification and separation of the contribution of different species to the scattered intensity.

The scattering from a multicomponent system has been discussed by several authors (Stockmayer, 1950; Pessen et al., 1973; Timasheff, 1973; des Cloizeaux and Jannink, 1980; Cotton, 1991; Higgins and Benoit, 1994) and was put into the simple partial structure factor form by Higgins and Benoit (1994). In a solution composed of more than one species of large particles, the solvent can still be treated as an incompressible medium and subtracted as background. In the case of a solution of membrane protein–detergent complex and free detergent micelles, the scattered intensity after buffer subtraction can be expressed as

$$I(Q) = \kappa[\Delta\rho_2'^2 V_2'^2 S_{22}(Q)' + \Delta\rho_2' \Delta\rho_3 V_2' V_3 S_{23}(Q) + \Delta\rho_3^2 V_3^2 S_{33}(Q)], \quad (4)$$

where $S_{22}(Q)' = N_2P_2(Q)' + N_2^2Q_2(Q)'$ is the partial structure factor of the protein–detergent complex, which corresponds to the intraparticle interference $P_2(Q)'$ and interparticle interference $Q_2(Q)'$ of the protein–detergent complex, $S_{33}(Q) = N_3P_3(Q) + N_3^2Q_3(Q)$ is the partial structure factor of the micelles, which corresponds to the intramicelle interference term $P_3(Q)$ and the intermicelle interference term $Q_3(Q)$, and $S_{23}(Q)$ is the cross interference term between the micelle and the protein–detergent complex; $\Delta\rho_2' = \rho_2' - \rho_1$ where ρ_2' is the electron density of the protein–detergent complex, and V_2' is the volume of the complex; $\Delta\rho_3 = \rho_3 - \rho_1$ where ρ_3 is the average electron density of a micelle, N_3 is the number of micelles, and V_3 is the volume of the micelle.

Equation 4 shows that both the second and the third terms contribute to the measured forward scattering intensity. Simple subtraction of the scattering from the detergent micelle as a solvent, in the best case, will eliminate the third term but leave the large second term unattended, preventing a correct determination of the protein molecular weight or radius of gyration. However, if we let the electron density of the buffer match the average electron density of the detergent micelle $\Delta\rho_3 = \rho_3 - \rho_1 = 0$, the second and third terms in Eq. 4 both become zero. When the protein concentration is low enough so that the intermolecular interaction can be ignored, Eq. 4 becomes

$$I_2(Q)' = \kappa(\rho_2' - \rho_1)^2V_2'^2\,N_2P_2(Q)'. \qquad (5)$$

Equation 5 is an expression for the protein–detergent complex, but the $(\rho_2' - \rho_1)V_2'$ term can be expanded as

$$\begin{aligned}\sum e_2' - \rho_1V_2' &= (\sum e_2 + \sum e_d) - \rho_1(V_2 + V_d)\\ &= (\sum e_2 - \rho_1V_2) + (\sum e_d - \rho_1V_d)\\ &\approx (\rho_2 - \rho_1)V_2 + (\rho_3 - \rho_1)V_3,\end{aligned}$$

where $\Sigma\, e_2'$ is the number of electrons in the protein–detergent complex, $\Sigma\, e_2$ is the number of electrons in the protein, $\Sigma\, e_d$ is the number of electrons of the detergent molecules bound to the protein, V_2 is the volume of the protein, V_d is the total volume of the detergent molecules bound to the protein. At the match point, $\rho_3 - \rho_1 = 0$, the measured forward scattering intensity should reflect the scattering from the protein in the protein–detergent complex,

$$I_2(Q)' = \kappa(\rho_2 - \rho_1)^2V_2^2\,N_2P_2(Q)'. \qquad (6)$$

when $Q \to 0$, $P_2(Q)' \to 1$, and the molecular weight of the protein alone can thus be determined by Eq. 2.

Equations 4–6 show that contrast matching the average electron density of the micelles also has the advantage of masking the strong intermicelle and micelle–protein interference effect in an ionic detergent solution in which the scattering from the highly charged micelles may be strongly correlated. Charged detergent and nondilute detergent micelle solutions can be used without interfering with the single molecular property determination as long as the average micelle electron density matches that of the buffer.

In the Guinier region, the detergent micelles can be approximated as homogeneous particles and $P_2(Q)' = \exp(-Q^2R_{g2}'^2/3)$. The measured apparent radius of gyration is (Yeager, 1976; Moore, 1982)

$$R_{g2}'^2 = XR_{g2}^2 + (1 - X)R_{g3}^2 + X(1 - X)L_{23}^2, \qquad (8)$$

where $X = (\rho_2 - \rho_1)V_2/[(\rho_2 - \rho_1)V_2 + (\rho_3 - \rho_1)V_d]$ is the volume fraction of the protein in the protein–detergent complex weighted by the contrast of the protein, $(1 - X)$ is the volume fraction of the detergent bound to the protein in the complex weighted by the contrast of the detergent. L_{23} is the distance between the centers of mass of the protein and the bound detergent. When the electron density of the solvent matches the averaged electron density of the detergent micelles $1 - X = 0$ and $R_{g2'} = R_{g2}$, the radius of gyration of the protein alone can be measured without considering the bound detergent molecules.

The above calculations of the protein molecular weight and the radius of gyration apply in the small angle region, where the Guinier approximation and the homogeneity assumption for the protein–detergent complex are valid. At larger scattering angles, the intraparticle heterogeneous properties of the protein–detergent complex and the micelle become apparent (Cabane, 1986; Philipse et al., 1989). The scattering intensity profile from such a heterogeneous complex will show a local intensity maximum around the Q values where the scattering intensity from a homogeneous particle of comparable size become close to zero (see Fig. 1 *A*).

MATERIALS AND METHODS

Sample preparation

The fusion proteins WtSN/GpA and SNG83I/GpA mutant were overexpressed and extracted from *Escherichia coli* strain HMS174(DE3) (from Novagen, Madison, WI) containing the plasmid pT7SN/GpA and the mutant substitute as described by Lemmon et al. (1992a,b). The extracted proteins were extracted in 2% Thesit (Boehringer Mannheim Inc., Indianapolis, IN), 25 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.025% NaN_3, pH 7.9 at a concentration of about 3 mg/mL. The protein was purified as described by Flanagan et al. (1993) except that a detergent, 1% Thesit was present in each step. The protein extract was dialyzed against 0.5% Thesit, 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.025% NaN_3 at 4°C for 4 h and loaded over DEAE cellulose column ~40 mL resin per liter of culture. The DEAE column was pre-equilibrated with 0.5% Thesit, 25 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.025% NaN_3, pH = 7.9. The DEAE column was washed with 2 column volumes pre-equilibration buffer. The DEAE flow-through and the wash were loaded onto an SP cation-exchange column (EM Science, Cincinnati, OH) ~20 mL resin per liter of culture. The SP column was pre-equilibrated with 0.5% Thesit, 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.025% NaN_3, pH = 7.9. The SP column was washed with at least 10 column volumes of pre-equilibration buffer, and then eluted with 0.5% Thesit, 50 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 1 mM PMSF, 0.025% NaN_3, pH = 7.9.

The purified fusion proteins were dialyzed against 0.1% Thesit, 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.025% NaN_3, pH = 7.9 for about 4 h, and loaded again over the SP column ~1 mL resin per

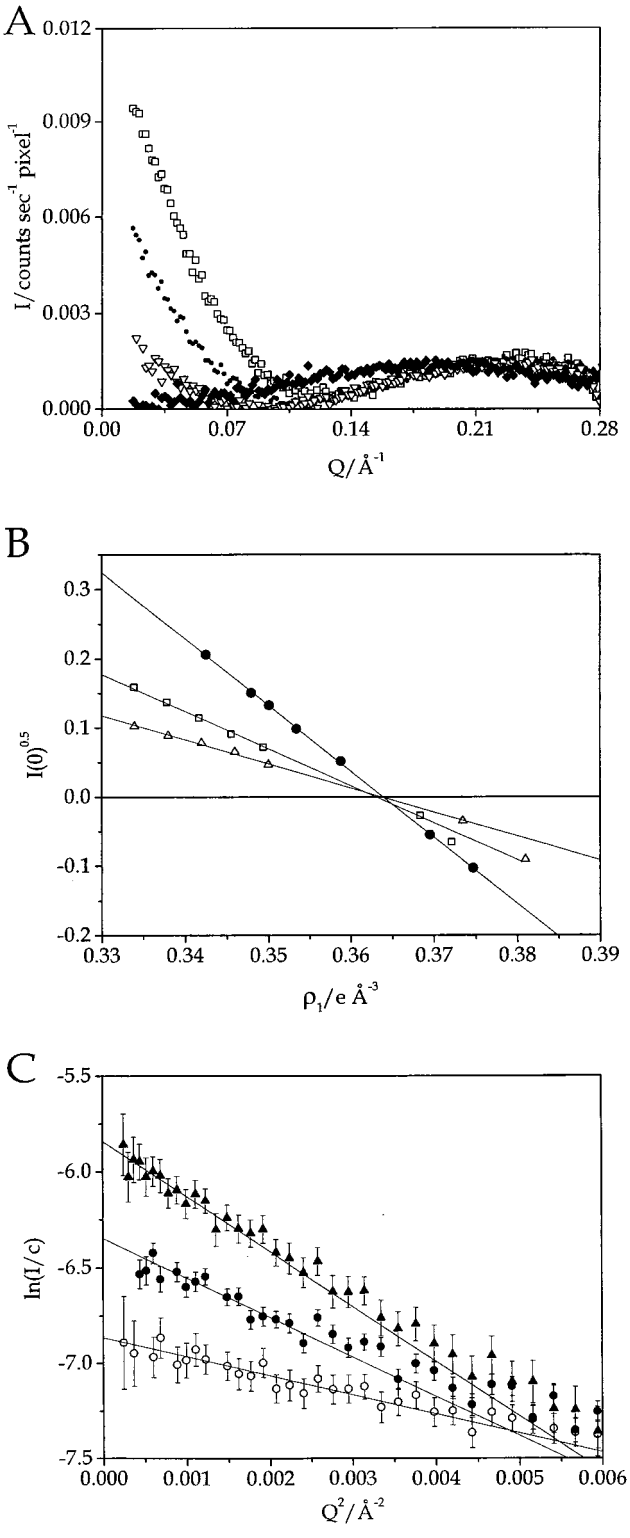

FIGURE 1 (*A*) $I(Q)$ versus Q 19.5 mg/mL β-OG at different sucrose concentrations. (□) No sucrose added; (●) in 70 mg/mL sucrose; (▽) in 140 mg/ml sucrose; (◆) in 281 mg/mL sucrose. (*B*) The $I(0)$ of β-OG detergent micelles at different buffer electron density ρ_1 as adjusted by sucrose. (●) 80 mg/mL β-OG in 250 mM Na_2HPO_4/NaH_2PO_4, pH = 7.0 buffer; (□) 40.1 mg/mL β-OG in 200 mM NaCl, pH = 6.5 buffer; (△) 19.5 mg/mL β-OG in 200 mM NaCl, pH = 6.5 buffer. At $\rho_1 = 0.365e^-/Å^3$, the scattering from the micelles disappears, suggesting that the buffer electron density matches the averaged electron density of the micelles. (*C*) Guinier plots of protein molecular weight standards in 19.5 mg/mL β-OG at the match point. (○) 7.4 mg/mL chymotrysinogen A; (●) 5.4 mg/mL overalbumin; (▲) 5.6 mg/mL albumin. The scattering intensities were normalized by the protein concentration.

10 mg protein. The SP column was pre-equilibrated with 0.5% Thesit, 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 0.025% NaN_3, pH = 7.9. The SP column was washed with at least 10 volumes of 50 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, 1 mM EDTA, pH = 7.9 containing 2% *n*-Octyl-β-D-glucopyranoside (β-OG, from Sigma, St. Louis, MO) or 1% *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB, from Calbiochem, La Jolla, CA) for detergent exchange. The protein was eluted with 50 mM Na_2HPO_4/NaH_2PO_4, 2 M NaCl, 1 mM EDTA, pH = 7.9 and 1% DDMAB (or the desired detergent concentration), and then dialyzed against 50 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, 1 mM EDTA, pH = 7.9, 2% β-OG or 1% DDMAB (or the desired detergent concentration) at 4°C for at least 4 days before SAXS experiment. The dialysate detergent/buffer solution was used for SAXS background subtraction.

Pure soluble staphylococcus nuclease (SN) and gel filtration molecular weight markers (from Phamacia, Bridgewater, NJ, see Table 1) were used to examine whether SAXS can correctly determine the molecular weight and the radius of gyration of a protein in a nondilute detergent solution. These soluble proteins were also used as standards in the determination of the molecular weight of SN/GpA99. The proteins were dissolved in 50 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, 1 mM EDTA, pH = 7.9, 2% β-OG or 1% DDMAB and dialyzed against the same detergent/buffer solution as the SN/GpA99 before the SAXS experiment. Protein concentration was determined before and after the SAXS measurements by ultraviolet absorbance. The extinction coefficients of the SN and the protein standards were taken from CRC Handbook of Biochemistry and Molecular Biology (Fasman, 1977).

SAXS experiments and data analysis

The SAXS instrument was as described previously (Bu et al., 1998). The sample-to-detector distance was 2.3 m. This enabled a scattering vector magnitude range of $0.01 < Q < 0.30$ Å^{-1} to be covered. The sample solutions were pipetted into a 2.0 mm quartz cell mounted on a temperature controlled sample holder. Data collection time was 2–8 h each for the protein/detergent solutions and the detergent solution background, depending on the protein concentrations. SAXS measurements were at 25°C.

The scattering images were circularly averaged and reduced to linear $I(Q)$ versus Q plots. The diffraction pattern of a polycrystalline pellet of ammonium sulfate mounted ~2.5 cm in front of the beam stop and 40 cm in front of the detector was used to monitor changes in the incident beam intensity as well as the differences in absorption of x-rays by the sample solutions and the buffer as previously described (Bu et al., 1998). The scattering intensity from a protein/detergent solution was first multiplied by a factor of $A_{peak,3}/A_{peak,2}$ before detergent solution background subtraction, where $A_{peak,3}$ and $A_{peak,2}$ are the peak areas of the beam monitor diffraction patterns when the detergent solution and the protein/detergent solution were measured, respectively. The subtracted scattered intensity was then multiplied by a factor $A_{peak,1}/A_{peak,3}$ where $A_{peak,1}$ is the peak area of the beam monitor diffraction pattern from water. The subtracted scattering intensity can be expressed as

$$I(Q) = \frac{A_{peak,1}}{A_{peak,3}}\left[\frac{A_{peak,3}}{A_{peak,2}} I_2(Q) - I_3(Q)\right]. \qquad (9)$$

The instrument geometric configuration was kept consistent during the measurements. Equation 9 ensured that the scattering intensity from different sample solutions is normalized on the same intensity scale for molecular weight measurements. Guinier analysis was performed in the $QR_g \leq 1$–1.5 on the $\ln I(Q)$ versus Q^2 plot.

TABLE 1 The radius of gyration of protein standards, SN and SN/GpA99

	M_w	$\bar{\nu}_2$ (mL g^{-1})	R_g (Å)		
			In Buffer	In β-OG	In DDMAB
Ribonuclease A	14.0	0.693			16.0 ± 0.3
Chymotrypsinogen A	25.7	0.718	17.5 ± 0.2	17.1 ± 0.5	18.3 ± 0.6
Ovalbumin	42.9	0.726	24.4 ± 0.6	25.3 ± 0.7	25.4 ± 0.5
Bovine serum Albumin	66.1	0.716	29.6 ± 0.2	30.1 ± 0.6	64 ± 4
SN	16.9	0.725	18.3 ± 0.3		32 ± 2
SN/GpA99	21.1*	0.731			65 ± 4

*Monomeric molecular weight.

RESULTS AND DISCUSSION

The molecular weight and the radius of gyration of proteins are correctly measured in nonionic detergent micelle solutions at the match point

Figure 1 *A* is the scattering profile $I(Q)$ versus Q of β-OG at different sucrose concentrations. When sucrose was added, the electron density of the buffer was increased and the contrast between the β-OG micelles and the buffer was changed. The scattering intensities varied when the contrast was changed. The forward scattering intensity at different contrast can be determined from the Guinier analysis. Figure 1 *B* shows $I(0)^{0.5}$ of the β-OG micelle as a function of the electron density of the buffer ρ_1 at three detergent concentrations. The linear relationship of $I(0)^{0.5}$ versus ρ_1 indicates that the aggregation number, the micelle volume, and the structure are not changed by the addition of sucrose, since, according to Eq. 2, the forward scattering is related to the molecular weight or the volume of the micelles. At $\rho_1 = 0.365e^-/Å^3$, the $I(0)^{0.5}$ of β-OG micelle became zero, indicating that the averaged electron density of the β-OG micelle is $\rho_3 = 0.365e^-/Å^3$.

Figure 1 *C* shows the Guinier plots of three protein molecular weight standards added to β-OG micelle/sucrose solutions in which the buffer electron density matches the average electron density of the β-OG. The normalized forward scattering intensity $I_2(0)/(\bar{\nu}_2^2 c_2)$ correctly reflects the molecular weight relationships of these protein standards (see Fig. 2). The radii of gyration of these proteins determined in β-OG micelle solutions are essentially the same as those measured in a buffer solution (Table 1).

The molecular weight and the radius of gyration of protein standards and SN/GpA99 can be correctly measured in a naturally matched zwitterionic detergent micelle solution

The above experiments on soluble protein molecular weight standards demonstrate that, by contrast matching the averaged electron density of the detergent micelles in the Guinier region, it is possible to measure the molecular weight and the radius of gyration of a protein dissolved in nondilute detergent micelle solutions. However, when the fusion protein SN/GpA99 protein was added in β-OG micelle solutions at the match point, the protein formed highly nonspecific aggregates. An alternative, zwitterionic detergent DDMAB, was selected to solve this problem. DDMAB has been found to be a nondenaturing detergent for extraction and separation of mycoplasm membrane protein antigens as judged by the functions of the extracted proteins (Brenner et al., 1995; Jan et al., 1996). Physical characteristics of DDMAB have been reported in a series of papers by Chevalier (Kamenka et al., 1995a,b; Chevalier et al., 1996). Dynamic light scattering studies have shown that DDMAB forms monodisperse, spherical micelles with an average hydrodynamic radius of 2.33 nm.

By contrast variation experiments, SAXS can also determine the radius of a micelle. If the micelle is spherical and monodisperse, the radius of the micelle can be determined by the occurrence of a common intersecting point $QR = 4.4935$ at different contrasts, where R is the radius of the micelle (Philipse et al., 1989; Hickl and Ballauff, 1996). From contrast variation solution SAXS experiments, we found that the radius of the DDMAB micelle was 2.0 ± 0.1

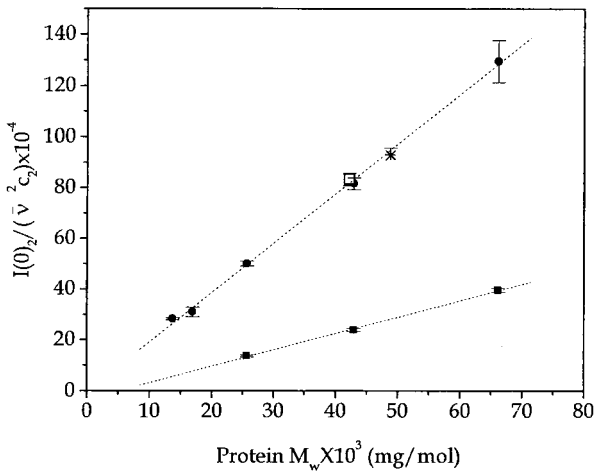

FIGURE 2 $I(0)_2/(\bar{\nu}^2 c_2)$ of (■) protein molecular weight standards in 2% β-OG, sucrose solution in which the electron density of the buffer matches the detergent micelles; (●) protein molecular weight standards in 33.3 mM DDMAB solutions; (□) SN/GpA99 in 33.3 mM DDMAB solution; (*) 3.61 mg/mL SN/GpA131 G83I mutant in 66.7 mM DDMAB solution. The buffer for all the proteins is 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH = 7.9.

nm. We believe that this is a better estimation of the radius of the micelle than the hydrodynamic radius determined by dynamic light scattering measurements. The aggregation number calculated from this radius was 66. The average electron density of a micelle calculated from the number of electrons in a micelle and the micelle volume is $\rho_3 = 0.331e^- \text{ Å}^{-3}$, which is very close to the electron density of water (of molecular volume 30 Å^{-3}) of $0.333e^-$ Å, so the average electron density of DDMAB nearly matches that of water (with a 0.6% mismatch). The physical parameters of DDMAB detergent micelles are listed in Table 2.

A 0.6% mismatch in electron density will generate about 3.5% systematic error in molecular weight. This error from electron density mismatch is lower than the usual 10–15% error in the SAXS determined molecular weight that typically arises from protein concentration determination and the Guinier plot fittings.

Figure 3 *A* is the scattering profile of 33.3 mM DDMAB in 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH = 7.9 after buffer subtraction: the intensity is approximately zero in the entire *Q* region measured. This confirms that the average electron density of the DDMAB detergent matches the buffer electron density.

The radii of gyration of the protein molecular weight standards except BSA in detergent solution were close to those measured in buffer (see Table 1), showing that the radius of gyration can be measured correctly in detergent solution. The radii of gyration also suggested that chymotrypsinogen A and ovalbumin are folded in DDMAB detergent solutions. In contrast, bovine serum albumin is evidently denatured by the detergent, as seen from the large radius of gyration.

Figure 3 *A* also shows the scattering profiles of an 8.8 mg/mL SN/GpA99 dissolved in 33.3 mM DDMAB, 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4 solution after detergent solution and buffer subtractions, respectively. The net scattering from the protein after buffer subtraction is essentially the same as that seen using detergent solution as background for subtraction, indicating that the detergent micelles do not contribute.

Figure 3 *B* shows the Guinier plots for SN in buffer and in 33.3 mM DDMAB, 200 mM NaCl, 50 mM $Na_2HPO_4/$

TABLE 2 Properties of DDMAB detergent molecule and micelles

Chemical formula	$C_{12}H_{25}(CH_3)_2N^+(CH_2)_3CO_2^-$
Critical micelle concentration (mM)	4.3
No. of electrons per detergent molecule (e^-)	168
Molecular volume, V_{mol} (nm^3) (ref)	0.506
Electron density of detergent molecules (e^-/nm^3)	332.0
Micelle radius (nm)	2.0
Micelle volume, $V_{micelle}$ (nm^3)	33.51
Aggregation number	66
No. of electrons per micelle (e^-)	11088
Average electron density of micelle ρ_3 (e^-/nm^3)	331

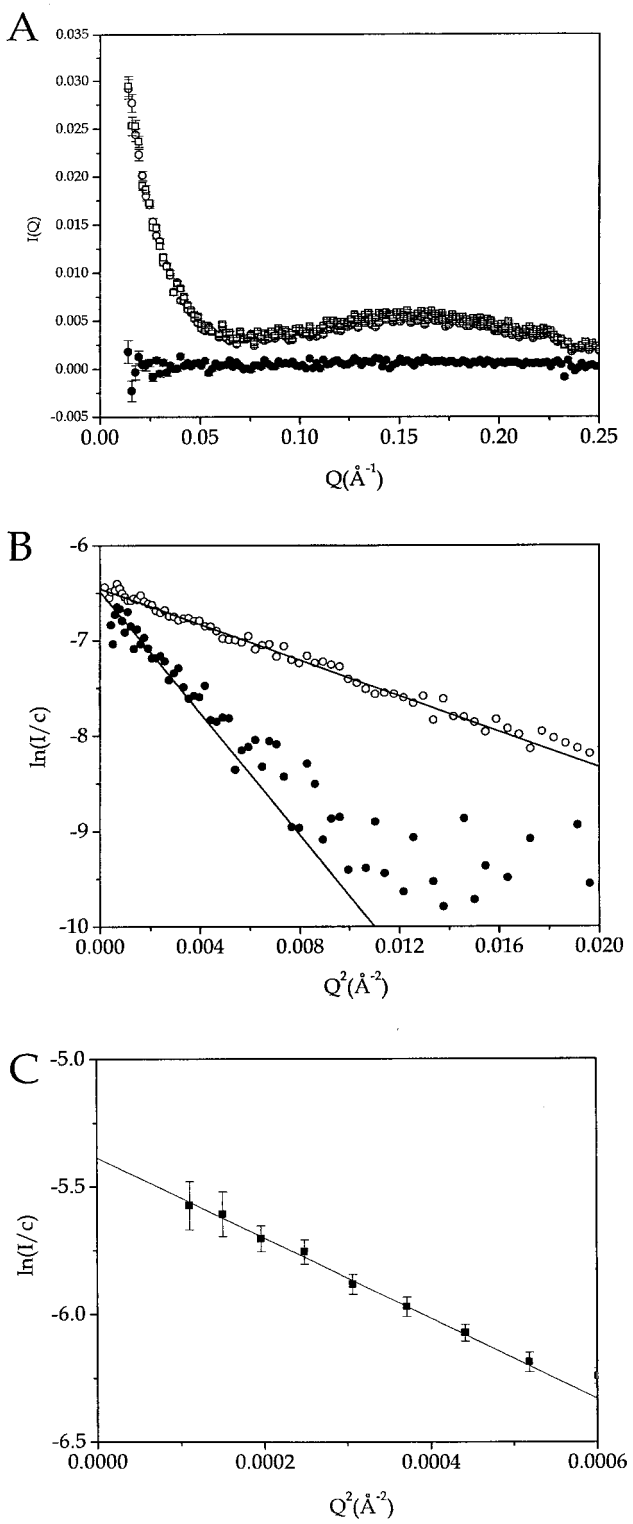

FIGURE 3 (*A*) Scattering profile of (●) 33.3 mM DDMAB in 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH = 7.9 solution after a buffer subtraction; (○) 8.77 mg/mL SN/GpA99 in 33.3 mM DDMAB after subtraction of detergent solution as background; (□) 8.77 mg/mL SN/GpA99 in 33.3 mM DDMAB after subtraction of buffer as background. (*B*) Guinier plot of (○) 5.4 mg/mL SN in buffer solution; (●) 4.7 mg/mL SN in 33.3 mM DDMAB solution. (*C*) Guinier plot of 8.77 mg/mL SN/GpA99 in 33.3 mM DDMAB, 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH = 7.9 solution. $R_g = 65 \pm 4$ Å.

NaH_2PO_4, pH = 7.9 solution scaled on the same intensity scale and normalized by the protein concentration, respectively. In buffer, the radius of gyration of SN was 16.7 ± 0.2 Å. In DDMAB detergent solution, the radius of gyration of SN was 32 ± 2 Å. This value was close to the R_g = 33 ± 1 Å of SN denatured in 8 M urea solution (Flanagan et al., 1993) suggesting that SN, like BSA, is denatured in DDMAB solution. However, the $I_2(0)/(c_2\ \bar{\nu}_2^2)$ of SN in buffer and in detergent solution had the same values, so SN is unfolded in DDMAB solution, but remains monomeric. In a DDMAB detergent solution, the unfolded SN domain should therefore not change the oligomeric state of the SN/GpA fusion protein, i.e., the dimerization of the fusion SN/GpA99 is due only to the dimerization of the GpATM domain. From a linear fit to the Guinier plot of SN/GpA in 33.3 mM DDMAB, 200 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH = 7.9 solution (Fig. 3 *C*), the $I_2(0)/(c_2\bar{\nu}_2^2)$ and the R_g of SN/GpA can be determined (Table 1, Fig. 2). By comparing the $I_2(0)/(\bar{\nu}_2^2 c_2)$ of SN/GpA99 in detergent solution with the $I_2(0)/(\bar{\nu}_2^2 c_2)$ of other proteins in detergent solution (see Fig. 2), the SAXS data showed that SN/GpA99 is a dimer in DDMAB solutions.

Figure 4 *A* shows the $I_2(0)/c_2$ of SN/GpA99 versus the detergent concentration. The $I_2(0)/c_2$ does not depend on the amount of detergent added, showing that the electron density of the buffer is a good match to the average electron density of the micelles. Otherwise, the interparticle interaction effects ($S_{23}(Q)$ and $S_{33}(Q)$ terms as in Eq. 4) would become increasingly strong, changing the $I_2(0)/c_2$ as the solution becomes increasingly crowded with detergent micelles. It also shows that the DDMAB micelles can be approximated as having homogeneous electron densities in the Guinier region.

Figure 4 *B* demonstrates that the $I_2(0)/c_2$ of SN/GpA99 is independent of the protein concentration. The protein concentrations used in this study were low enough so that the intermolecular interaction effects (the $Q_2(Q)$ term in Eq. 4) can be ignored.

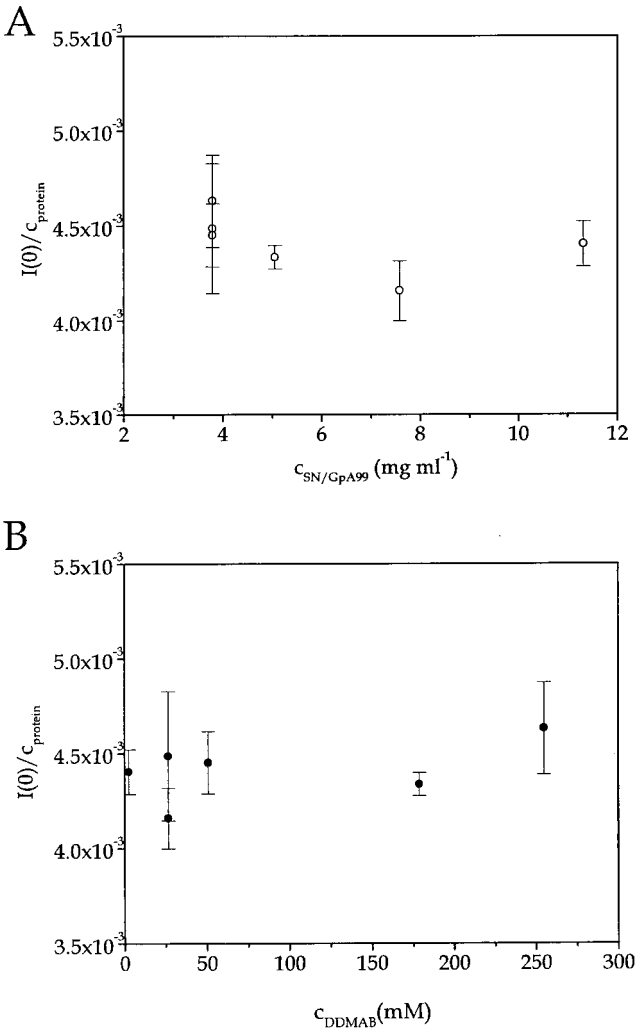

FIGURE 4 (*A*) $I(0)_2/c_{protein}$ of SN/GpA99 at different protein concentrations. $I(0)_2/c_{protein}$ is independent of protein concentrations in the range of protein concentrations studied, indicating the absence of intermolecular interference effect. (*B*) $I(0)_2/c_{protein}$ of SN/GpA99 at different detergent concentrations. In the protein concentration range studied, the $I(0)_2/c_{protein}$ is independent of detergent concentration.

The transmembrane helices of the GpA dimer are parallel

In DDMAB detergent solution, the maximum dimension $D_{max,SN}$ of the denatured SN domain was estimated from the $P(r)$ function to be 80 ± 10 Å. Like the radius of gyration, this D_{max} value in DDMAB detergent solution was also close to SN denatured in 8 M urea (D_{max} = 75 Å, Flanagan et al., 1993). (Note: for a folded protein, the SAXS measured D_{max} is usually in agreement with that computed from the Protein Data Bank coordinates.) The radius of gyration and the maximum dimension of a GpATM domain monomer plus the linker can be calculated from the NMR structure to be $R_{g,GpATM,m}$ = 18.7 Å and $D_{max,GpATM,m}$ = 65 Å, respectively (MacKenzie et al., 1997; PDB file). The distance between the centers of mass of the SN domain and the GpATM domain can be calculated from their maximum dimensions to be L_1 = 72 ± 5 Å (see Fig. 5 *A*). Errors in L_1 and the subsequently calculated distances were propagated from errors in $R_{g,SN}$ and $D_{max,SN}$ according to the standard error propagation procedure (Bevington and Robinson, 1992). The radius of gyration of a monomeric SN/GpA99, $R_{g,SN/GpA,m}$, can be calculated by the parallel axis theorem (Moore, 1982),

$$R^2_{g,SN/GpA,m} = W_{SN}R^2_{g,SN} + W_{GpATM,m}R^2_{g,GpATM,m} + W_{SN}W_{GpATM,m}L_1^2, \quad (9)$$

to be $R_{g,SN/GpA,m}$ = 41 ± 2 Å, where W_{SN} and $W_{GpATM,m}$ are the weight fractions of the SN domain and the GpATM domain in the assumed monomeric fusion protein, respectively.

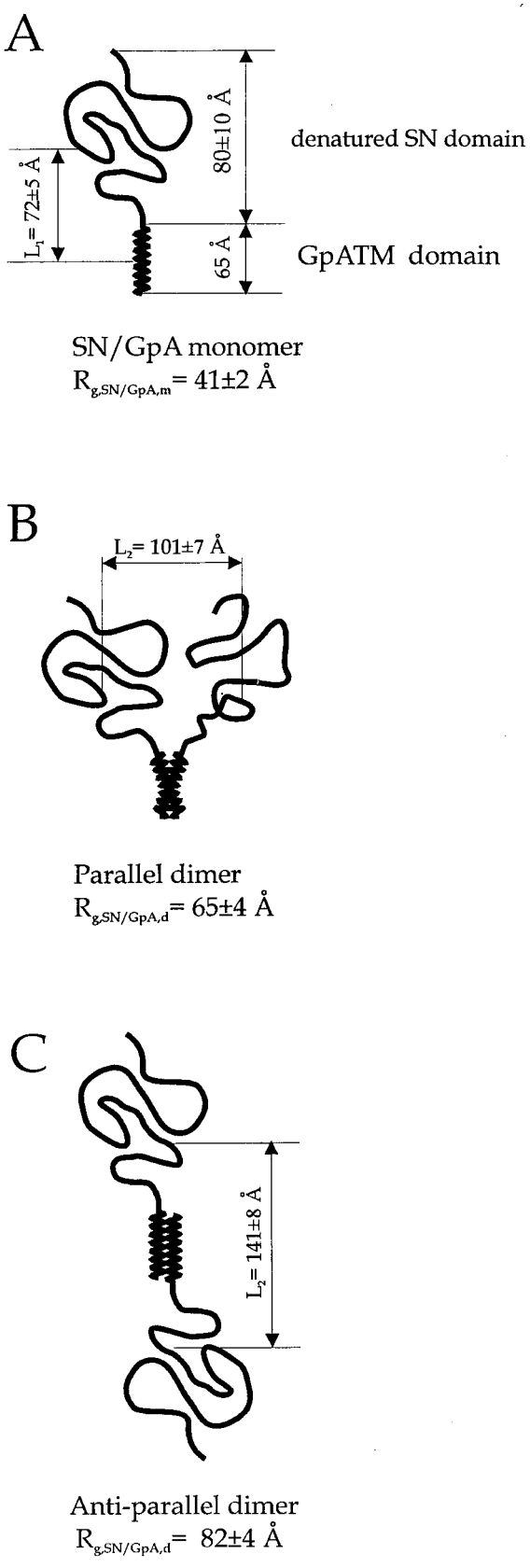

FIGURE 5 (*A*) Diagram of a SN/GpA99 monomer. (*B*) Diagram of a SN/GpA99 parallel dimer. From the parallel axis theorem calculation, this parallel orientation is the preferred configuration of the SN/GpA99 dimer. (*C*) Diagram of a SN/GpA99 antiparallel dimer.

The radius of gyration of the SN/GpA99 dimer can be calculated by using the parallel axis theorem again,

$$R^2_{g,SN/GpA,d} = 0.5R^2_{g,SN/GpA,m} + 0.5R^2_{g,SN/GpA,m} + 0.25L_2^2, \quad (10)$$

where L_2 is the distance between the centers of mass of the two SN/GpA99 monomers in the dimer complex. For an antiparallel dimer, L_2 can be calculated to be 141 ± 8 Å (Fig. 5 *C*), predicting a $R_{g,SN/GpA,d}$ of 82 ± 4 Å, much larger than the measured R_g of 65 ± 4 Å. Analysis of L_2 and the R_g of the SN/GpA99 therefore indicates that the SN/GpA99 is a parallel dimer in DDMAB. This is consistent with the NMR structure (MacKenzie et al., 1997) in dodecylphosphocholine and the combined mutagenesis and computation modeling structure (Lemmon et al., 1992b; Treutlein et al., 1992). By using Eq. 10, the L_2 of a parallel dimer was calculated to be 101 ± 7 Å (see Fig. 5 *B*).

The dissociation constant of the transmembrane helix dimer of a GpA mutant can be measured

The dissociation constant of wt SN/GpA99 in DDMAB has been estimated by fluorescence resonance transfer to be 40 nM in 25 mM DDMAB solutions (Fisher, personal communication). This dissociation concentration is much below the concentration limit that could be measured using our SAXS apparatus. Nevertheless, a G83I mutant that weakens the GpA dimer could be measured. Figure 6 is the $I_2(0)/c_2$ of a Glycine 83 to Isoleucine mutant (G83I) as a function of protein concentration. This mutant has an additional C-terminal 32 residues of monomeric molecular weight of 25.1. When scaled on the same intensity scale as the other proteins, the $I_2(0)/c_2$ of SN/GpA131 G83I demonstrated that it is a dimer of molecular weight 50 at protein concentrations of above 0.08 mM. Below 0.08 mM, the dimer starts to dissociate. Under the dissociation equilibrium condition,

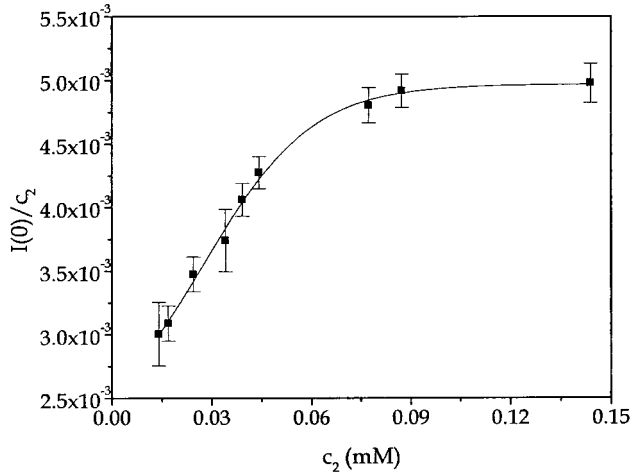

FIGURE 6 $I(0)_{protein}/c_{protein}$ of SN/GpA131 G83I at different protein concentrations. The association of this mutant breaks apart at protein concentration c_2 = 0.08 mM.

the forward scattering intensity $I(0)_2$ can be expressed as the weight averaged forward scattering intensity of the concentration normalized, monomer $I(0)_m$ and the forward scattering intensity of the concentration normalized, dimer $I(0)_d$,

$$\frac{I(0)_2}{c_2} = \frac{\alpha I(0)_m^2 + (1-\alpha)I(0)_d^2}{\alpha I(0)_m + (1-\alpha)I(0)_d},$$

where α is the mole fraction of the monomer and $I(0)_d = 2I(0)_m$. The dissociation constant determined using $K_d = \alpha^2 m_2/4(1-\alpha)$, where m_2 is the total molar concentration of the protein, 24 μM using the data from Fig. 6.

CONCLUSION

By matching the average electron density of a detergent micelle to that of the solvent, we are able to use small angle x-ray scattering to measure the molecular weight and the radius of gyration of transmembrane proteins in detergent micelle solutions. The detergent DDMAB is a particularly convenient choice, because its micelle electron density is nearly equal to that of water. We have used a fusion protein with the α-helical transmembrane domain of the glycophorin A fused to the carboxyl terminus of monomeric staphylococcal nuclease as a model system for study. With the help of the nuclease domain, it is possible to determine the orientation of the transmembrane helices to be parallel in glycophorin dimers. Contrast matching can be achieved by either adding sucrose to the micelle solution, or selecting a detergent micelle whose average electron density naturally matches that of the buffer. If nondenaturing detergents are found for a given complex, the radius of gyration, measured in micelles, can be a useful structural parameter. The method established here can be used to study protein interactions or to measure molecular weights for membrane protein complexes in detergent environments.

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