Adenine nucleotide translocase greatly increases the partition of trinitrophenyl-ATP into reduced Triton X-100 micelles

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ABSTRACT The presence of adenine nucleotide translocase (ANT) was found to greatly enhance the partitioning of the ATP analog 2′,3′-O-(2,4,6-trinitrophenyl)-adenosine 5′-triphosphate (TNP-ATP) into reduced Triton X-100 micelles. The protein's effect was studied through the quenching of fluorescence of purified ANT, irreversibly inhibited by carboxyatractyloside (CAT), solubilized in reduced Triton X-100 micelles. The dependence of quenching of the protein's time-resolved tryptophan fluorescence on TNP-ATP concentration was measured and found to follow a Stern-Volmer mechanism. However, the calculated quenching constant was too large to be accounted for by the aqueous TNP-ATP concentration. Experiments were therefore conducted to determine the partitioning of the quencher between the three phases present: aqueous, protein-free micelle, and protein micelle; a system also described by the equation of Omann, G. M., and M. Glaser (1985. *Biophys. J.* 47:623–627.). By measuring the dependence of the apparent quenching rate constant on the protein concentration and protein/micelle ratios, this equation was used to calculate both the quencher partition coefficient into protein-free micelles (P_m) and into protein-micelles (P_{pm}), as well as the bimolecular quenching rate constant (k_{pm}) in protein micelles. From the quenching experiments, $k_{pm} = 5.0 \times 10^8\ M^{-1}\ s^{-1}$, $P_m = 290$ and $P_{pm} = 7.0 \times 10^3$. P_m was also determined independently by a pyrene quenching experiment to be 325, and by a rapid filtration experiment to be 450. Clearly, the presence of the integral membrane protein ANT-CAT in reduced Triton X-100 micelles greatly increases the partition of TNP-ATP into the micelle. ANT alters the properties and thus, the structure of the detergent micelle, which has direct implications for the use of detergent micelles as a model system for membrane proteins and may indicate that analogous effects occur in the mitochondrial membrane.

INTRODUCTION

Adenine nucleotide translocase (ANT) is an integral membrane protein located in the inner mitochondrial membrane that cotranslocates cytosolic ADP into the mitochondrial matrix and ATP from the matrix to the cytosol with a 1:1 stoichiometry. It is the most abundant mitochondrial membrane protein (1) and has been studied extensively because it plays an important role in the bioenergetics of the cell (2). ANT has been isolated from beef heart mitochondria in its native dimer form with either the irreversible inhibitor carboxyatractyloside (CAT) bound (3) or with palmitoyl Coenzyme A (pCoA) bound reversibly (4). Both procedures solubilize the protein into micelles of the nonionic detergent Triton X-100.

One method often used to study ANT examines the binding and translocation of adenine nucleotide analogs by the protein. Schlimme et al. (5) found that the analog 2′,3′-O-(2,4,6-trinitrophenyl)-adenosine 5′-diphosphate (TNP-ADP) and TNP-ATP reversibly inhibit adenine nucleotide translocation in rat liver mitochondria. We have recently determined that TNP-ATP specifically binds to ANT which has been solubilized in reduced Triton X-100 and purified with pCoA, with a dissociation constant of ~20 μM. The binding was found to be inhibited by the addition of the ANT-inhibitor CAT (Tummino and Gafni, unpublished data). These results were obtained by following the partial quenching of intrinsic tryptophan fluorescence of the ANT upon TNP-ATP addition; the quenching of ANT-pCoA between 0–50 μM being saturable indicating binding, while the quenching at higher TNP-ATP concentrations was not saturable reflecting dynamic quenching. With CAT bound to ANT, no tight binding of the nucleotide analog was observed and only dynamic quenching measured. Interestingly, the apparent rate constant for TNP-ATP quenching of ANT-CAT derived from these experiments using bulk solution concentration of quencher was unreasonably high (in the range of $10^{12}\ M^{-1}\ s^{-1}$), and indicated that the quencher strongly partitions into Triton X-100 micelles. An important question that arose from this was whether the integral membrane protein is affecting the micelle environment in a manner that increases quencher solubility in micelles, compared to its solubility in protein-free micelles. The goal of this study was to quantify any effect by comparing the quencher partitioning into micelles with and without ANT, as well as to determine the bimolecular quenching rate constant.

Omann and Glaser (6) presented an equation that describes the quenching of a micelle-solubilized fluorophore by a quencher that partitions between an aqueous phase, an unlabeled micelle, and a fluorophore-micelle phase. By measuring the dependence of the apparent quenching rate constant on the micellar volume, this equation can be used to determine the bimolecular quenching rate constant and one of the partition coeffi-

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Abbreviations used in this paper: ANT, adenine nucleotide translocase; CAT, carboxyatractyloside; FITC-dextran 2000, fluorescein isothiocyanate dextran (2,000 kD); Mops, 3-[*N*-morpholino] propanesulfonic acid; pCoA, palmitoyl Coenzyme A; ANS, 8-anilino-1-napthalenesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-adenosine 5′-triphosphate.

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cients if the other partition coefficient is known. This approach is applicable to solubilized ANT, since the quencher (TNP-ATP) partitions between water and both protein-free micelles and protein-containing micelles. Since the ANT-micelle size had been determined (7), the equations may be used in a different manner so that the TNP-ATP partition coefficients into both reduced Triton micelles (P_m) and into ANT-reduced Triton micelles (P_{pm}), as well as the bimolecular quenching rate constant (k_{pm}), can be determined from one set of experiments. This was done by measuring changes in the apparent quenching rate constant versus changes in protein concentration and protein/micelle ratios.

Our results indicate that the integral membrane protein greatly enhances the solubility of an amphiphilic quencher in the micelle. Specifically, the presence of ANT increases the partition of TNP-ATP into the reduced Triton X-100 micelles by greater than 24-fold. This is in contrast to previous reports (8, 9) where integral proteins were found to have no effect or even induce a decrease in amphiphilic partitioning into membranes. Possible explanations for the difference between these previous results and our results are presented, along with the direct significance of the results for the effects of integral proteins on detergent micelles and potential effects on lipid membranes.

MATERIALS AND METHODS

Materials

CAT, pCoA, reduced (hydrogenated) Triton X-100 (a polydisperse preparation of *p*-(1,1,3,3,-tetramethylbutyl)cyclohexyloxypolyoxyethylene glycols with an average of 9.5 oxyethylene units per molecule), Brij 58, pyrene, 8-anilino-1-napthalenesulfonic acid, molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), FITC-dextran 2000 and Sepharose CL-6B were purchased from Sigma Chemical Co (St. Louis, MO). Triton X-100 (protein grade) and the BCA protein assay reagent were purchased from Pierce Chemical Co. (Rockford, IL). Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, CA). TNP-ATP was purchased from Molecular Probes, Inc (Eugene, OR). Centricon-10 filters were purchased from the Amicon Division, W. R. Grace and Co. (Beverly, MA).

Methods

General methods

Concentrations of reduced Triton X-100 were determined according to the method of Garewal (10). The standard curves constructed using known amounts of reduced Triton had excellent linear fits ($R^2 > 0.99$) with slopes similar to those obtained by Garewal. Unlike Garewal's results, however, the Triton assay used was completely unaffected by the presence of protein (bovine serum albumin). Protein concentrations in the presence of detergent were determined according to the method of Smith et al. (11), using bovine serum albumin as a standard and with the identical reduced Triton X-100 concentration as present in the protein sample. The critical micelle concentration of reduced Triton was determined under conditions used in the fluorescence quenching experiments using the method of De Vendittis et al. (12). The fluorescent probe used was 8-anilino-1-napthalenesulfonic acid (ANS), and the sample was incubated at 3°C in 0.6 M NaCl, 10 mM Mops at pH 7.2. Under these conditions, the critical micelle concentration was determined for reduced Triton X-100 to be 0.185 mM.

ANT isolation

Adenine nucleotide translocase was isolated from beef heart mitochondria according to the procedure of Klingenberg et al. (3) and Aquila et al. (13) with the modifications described by Woldegiorgis et al. (4). The protein was isolated with either the inhibitor CAT or with pCoA bound to it due to the lability of the unliganded protein (3). A high salt concentration of 0.6 M NaCl in the isolation buffer (10 mM Mops, pH 7.2) was employed in order to maximize membrane protein solubilization by the detergent. The same buffer was used in all subsequent experiments. Reduced Triton X-100 was used instead of Triton X-100 because its absorbance and fluorescence, which overlap those of tryptophan, are greatly reduced. The reduced detergent was found to have very similar solubilizing properties to the nonreduced form in the purification of ANT, consistent with a previous report (14) on the extraction of other membrane proteins. SDS-PAGE was carried out to determine protein purity as described by Laemmli (15) using a 12% acrylamide gel. The protein sample was prepared for electrophoresis by precipitation from the reduced Triton solution with trichloroacetic acid (5% final) and centrifugation (10,000 *g* for 15 min). The precipitation and centrifugation were repeated three times followed by washing the pellet to neutral pH with distilled water. After electrophoresis, the protein bands were stained with Coomassie blue, relative band intensities were measured with a Hoefer densitometer and the data analyzed using Gel Scan software. The major band present in the gels made up 85–90% of the total protein in the sample, and had a molecular weight (determined using molecular weight standards and Gel Scan software) of 29.5 kD.

Gel filtration chromatography

Gel filtration on Sepharose CL-6B was performed separately for reduced and for nonreduced Triton X-100 micelles to estimate and compare their size. The elution buffer contained 0.5% wt/vol Triton X-100 (reduced or nonreduced), 0.6 M NaCl, and 10 mM Mops at pH 7.2. The elution was performed at 25°C with a column size of 1.5 × 30 cm and an elution rate of approximately 8 ml/h. 0.20 ml of a solution of 50 mg/ml FITC-dextran 2000 was first loaded onto the column; its elution volume used to determine the void volume (V_0). The column was then washed with 5 ml of elution buffer and 0.30 ml of 6% wt/vol Triton X-100 (reduced or nonreduced) was loaded onto the column and the run continued. Again 5 ml after the Triton was loaded, 0.5 ml of 100 mg/ml potassium ferricyanide was applied; its elution volume used to determine the total volume (V_t) of the column. The samples were loaded separately to avoid any possible effect on the elution of the standards by the high Triton concentration. The elution profiles of reduced and nonreduced Triton were monitored by fluorescence (excitation 290 nm, emission 312 nm). The void volume and the total volume were determined for both reduced and nonreduced Triton experiments and no difference was found. From the elution volume (V_e) measured for each detergent, K_d was calculated, $K_d = (V_e - V_0)/(V_t - V_0)$.

Determination of the partition coefficient of TNP-ATP into reduced Triton X-100 micelles using rapid filtration

The partition coefficient was determined by centrifuging a solution of TNP-ATP in aqueous reduced Triton through a centricon-10 filter, which retains all Triton micelles, and measuring the TNP-ATP concentrations in the filtrate and in the original solution. In a typical experiment, a solution of 50 μM TNP-ATP in 1.0% wt/vol reduced Triton X-100, 0.6 M NaCl, 10 mM Mops, pH 7.2 at 3°C was filtered through the centricon-10 membrane at 5,000 *g*; this procedure was performed twice in order to equilibrate the centricon membrane with TNP-ATP solution. An identical TNP-ATP + reduced Triton solution was then centrifuged through the washed centricon, with half the volume being filtered through (although the amount filtered through did not affect

the TNP-ATP concentration in the filtrate). The filtrate was diluted into the same buffer containing reduced Triton (1.0% final), and the concentration of nucleotide was determined from its steady-state fluorescence (excitation 404 nm, emission 533 nm). This fluorescence is enhanced 17.5-fold in the presence of 1.0% reduced Triton. The optical density of all samples at 404 nm was less than 0.07, and background fluorescence was subtracted from each value. As a control, an otherwise identical TNP-ATP sample containing no reduced Triton was filtered through the centricon membrane and the concentration of nucleotide in the filtrate determined as described above. The partition coefficient for ATP into reduced Triton X-100 micelles was determined in the same manner, except that the ATP concentration was determined from the absorbance at 259 nm. The partition coefficients for TNP-ATP and ATP into reduced Triton micelles was calculated using Eq. 1, where ATP absorbance is substituted for fluorescence:

$$P_m = \frac{(\text{Fluor}_{(\text{total})} - \text{Fluor}_{(\text{filtrate})})/V_{(\text{micelle})}}{\text{Fluor}_{(\text{filtrate})}/V_{(\text{aqueous})}} \quad (1)$$

and $\text{Fluor}_{(\text{total})}$ is the fluorescence of the original uncentrifuged sample. The $\text{Fluor}_{(\text{filtrate})}$ was multiplied by the $\text{Fluor}_{(\text{filtrate})}/\text{Fluor}_{(\text{total})}$ obtained for the control, which had a value of 0.945 ± 0.06. The $V_{(\text{micelle})}$ = (total Triton volume − Triton monomer volume), where the Triton volume was calculated using the partial specific volume for Triton X-100 reported by Tanford et al. (16) of 0.908 cm^3/gram. The Triton monomer volume was calculated using the critical micelle concentration of 0.185 mM determined for reduced Triton X-100 under these conditions (see General Methods), and the $V_{(\text{aqueous})}$ = total volume − Triton volume.

Determination of the partition coefficient of TNP-ATP into reduced Triton X-100 micelles using pyrene quenching

The quenching of pyrene fluorescence in Triton micelles by TNP-ATP at different micelle concentrations was used to determine the TNP-ATP partition coefficient as follows: 10 μM pyrene was solubilized in 1.0% wt/vol reduced Triton X-100, 0.6 M NaCl and 10 mM Mops, pH 7.2. The Triton micelle concentration (104 μM) was tenfold higher than that of pyrene in these experiments. It was assumed that partitioning into the micelle is unaltered by the fluorophore and this system was considered to consist of only two phases: micelle and aqueous. The 0.5 ml sample, at 3°C, was excited at 321 nm and the fluorescence intensity measured at 372 nm in order to minimize inner filter effects by TNP-ATP absorption at longer wavelengths. The fluorescence intensity was monitored as a function of TNP-ATP addition up to 50 μM final. The absorbance of 50 μM TNP-ATP at 372 nm was 0.018 with the 1.0 millimeter pathlength used. All fluorescence values were corrected for inner filter effects and for dilution. The quenching experiment was repeated four more times with the original pyrene/Triton sample diluted 2, 4, 8, and 16-fold with 0.6 M NaCl, 10 mM Mops, pH 7.2.

Stern-Volmer plots were generated from the quenching experiments performed at each micelle concentration. The apparent quenching rate constant (k_{app}) for each micelle concentration was derived from the slope, and its reciprocal plotted versus relative micelle concentration (α_m). Eq. 2, from Lakowicz and co-workers (17), describes quencher partitioning between two phases and was used to determine the partition coefficient into Triton micelles (P_m) and the quenching rate constant (k_m).

$$\frac{1}{k_{app}} = \alpha_m\left(\frac{1}{k_m} - \frac{1}{k_m P_m}\right) + \frac{1}{k_m P_m} \quad (2)$$

α_m is defined as $V_{micelle}/V_{total}$, $P_m = [Q]_{micelle}/[Q]_{aqueous}$, and the units for k_m and k_{app} are M^{-1}. The bimolecular quenching rate constant for quenching of pyrene by TNP-ATP was determined from the quenching rate constant (k_m) and the fluorescence lifetime of pyrene in reduced Triton X-100 micelles (excitation wavelength 290 nm, emission wavelength 372 nm, 3°C). The method used for fluorescence lifetime determination is described in the following section.

Protein fluorescence measurements

These experiments were conducted with protein samples incubated at 3°C, with an excitation at 290 nm and emission measured at 345 nm. This choice of excitation and emission wavelengths maximizes the ANT/reduced Triton X-100 fluorescence. Steady state fluorescence was measured with a Spex Fluorolog II fluorometer, with all samples diluted to an optical density of 0.09 or less at the excitation wavelength. The steady-state fluorescence of a reduced Triton X-100 sample with a concentration equal to that in the protein sample was used to correct for the contribution of detergent fluorescence to the total fluorescence of the sample. The detergent contribution under the conditions used in the present study varied between 4.6% and a maximum of 19%.

Time-resolved fluorescence was used in the ANT quenching experiments to avoid artifacts due to inner filter effects. The decay curves were obtained by the correlated single photon counting method using an instrument previously described (18). The fluorescence decay of the ANT-CAT sample was measured with no TNP-ATP present, and again following each TNP-ATP addition. Upon addition of an aliquot of TNP-ATP, the sample was allowed to incubate for at least 3 min before a fluorescence decay was measured in order for the system to equilibrate, although no time dependence to the degree of quenching was observed. All decay kinetics were analyzed in an identical manner by a Marquardt nonlinear least squares algorithm (19) over the same number of channels with none of the lifetimes fixed to avoid any artifacts from the analysis. For each experiment, the fluorescence decay of ANT-CAT in aqueous reduced Triton without and with TNP-ATP was analyzed, along with the fluorescence decay of aqueous reduced Triton X-100 alone. The latter decay parameters were unaffected by TNP-ATP addition, and were subtracted, after an appropriate scaling, from the parameters of each ANT-CAT + reduced Triton sample to obtain the fluorescence decay due solely to the ANT-CAT. In scaling the reduced Triton decay parameters, the preexponential terms (ai) were chosen so that the ratio of the time-integrated emission of the detergent to that of the protein was the same as the ratio of steady state fluorescence intensities of Triton and protein solutions. The degree of quenching of ANT upon addition of TNP-ATP was then determined as the quenching ratio ($\Sigma\, ai_0\tau i_0/\Sigma\, ai\tau i$).

Analysis of fluorescence quenching data

The apparent rate constant for quenching, k_{app}, of a fluorophore in a micelle by a quencher (Q) which partitions between an aqueous (W), a fluorescently-labeled micelle (A), and an unlabeled micelle phase (B) was described by Omann and Glaser (6):

$$k_{app} = \frac{k_A}{[\alpha_A + (P_B\alpha_B + 1)/P_A]} \quad (3)$$

where $\alpha_A = V_{(A)}/V_{(total)}$, $\alpha_B = V_{(B)}/V_{(total)}$, k_A is the bimolecular quenching rate constant in the fluorescently-labeled micelle, and the partition coefficients for the quencher are $P_A = [Q]_A/[Q]_W$, $P_B = [Q]_B/[Q]_W$. Eq. 3 assumes the total micelle volume/total volume to be negligible. Omann and Glaser measured the dependence of k_{app} on either α_A or α_B and used the equation to calculate the bimolecular quenching rate constant and one of the partition coefficients, with knowledge of the other partition coefficient.

The system studied in the present work also involves the partitioning of a quencher (TNP-ATP) between three phases, of which only one contains the fluorophore (ANT). Below are the equations of Omann and Glaser written specifically for our experiments. These equations are used to calculate both partition coefficients and the quenching rate constant from one experiment, using the detergent aggregation number for the protein micelle (to calculate protein micelle volume), as previously determined for ANT-Triton X-100 (7). For a quencher that partitions between the aqueous solution (w), the protein-free micelles (m), and the protein micelles (pm), one can write:

$$[Q]_{total}V_{total} = [Q]_mV_m + [Q]_{pm}V_{pm} + [Q]_wV_w \quad (4)$$

In our experiments, the protein-micelle phase represented a small portion (0.5–2.5%) of all micelles. Quenching of fluorescence is described by the Stern-Volmer Eq. 5, where only quencher molecules in the protein micelle are assumed to contribute to the quenching.

$$\frac{\tau_0}{\tau} = \frac{\sum a_i\tau_{i0}}{\sum a_i\tau_i} = 1 + k_{pm}\tau_0[Q]_{pm} = 1 + k_{app}\tau_0[Q]_{total} \quad (5)$$

τ and τ_0 are the average fluorescence lifetimes with and without quencher present, respectively, $\Sigma\, a_i$ is normalized to 1, and k_{pm} is the bimolecular quenching rate constant in protein micelles. Protein quenching by aqueous TNP-ATP is ignored since the concentrations of this species were in all cases much too small to make any significant contribution, even if quenching is assumed to be diffusion limited. α_m is defined as V_m/V_{total}, $\alpha_{pm} = V_{pm}/V_{total}$ and the partition coefficients for the quencher into free micelles and protein micelles as $P_m = [Q]_m/[Q]_w$, $P_{pm} = [Q]_{pm}/[Q]_w$. Substituting these values into Eqs. 4 and 5 yields the following expression for the reciprocal apparent quenching rate constant:

$$\frac{1}{k_{app}} = \alpha_{pm}\left(\frac{1}{k_{pm}} - \frac{1}{k_{pm}P_{pm}}\right) + \alpha_m\left(\frac{P_m}{P_{pm}k_{pm}} - \frac{1}{k_{pm}P_{pm}}\right) + \frac{1}{k_{pm}P_{pm}}. \quad (6)$$

If P_m and $P_{pm} \gg 1$ (this assumption is verified by the results), then Eq. 6 simplifies to:

$$\frac{1}{k_{app}} = \alpha_{pm}\left(\frac{1}{k_{pm}} + \frac{\alpha_m/\alpha_{pm}}{k_{pm}}\frac{P_m}{P_{pm}}\right) + \frac{1}{k_{pm}P_{pm}}. \quad (7)$$

While Eqs. 7 and 3 are interconvertible, the derivation of Eq. 7 did not assume the total micelle volume to be negligible. This equation can therefore be used regardless of micelle volume if P_m and $P_{pm} \gg 1$. A plot of $1/k_{app}$ versus α_{pm} at constant α_m/α_{pm} (experiment performed by serially diluting the ANT + detergent solution with buffer containing no detergent) has a slope, $S1 = 1/k_{pm} + [(\alpha_m/\alpha_{pm})/k_{pm}](P_m/P_{pm})$ and an intercept, $I1 = 1/k_{pm}P_{pm}$. A second plot of $1/k_{app}$ versus α_m at constant α_{pm} (derived from quenching experiments where the protein concentration, and hence α_{pm} is kept constant while the detergent concentration is varied) has a slope, $S2 = (1/k_{pm})(P_m/P_{pm})$ and an intercept, $I2 = 1/k_{pm}(\alpha_{pm} + (1/P_{pm}))$. Therefore, $S2/I1 = P_m$ and $S1/I1 = P_{pm} + P_m(\alpha_m/\alpha_{pm})$. A plot of S1/I1 versus α_m/α_{pm} thus, has an intercept of P_{pm}.

α_{pm} was calculated from the known protein concentration and the value of 150 Triton monomers/ANT-Triton micelle (Hackenberg and Klingenberg, [7]). α_m = (total micelle volume − protein micelle volume)/total volume.

Calculation of R_0 for ANT/TNP-ATP as a donor/acceptor pair for energy transfer

The Förster distance, R_0 (in Å), is the distance at which the transfer efficiency between an energy donor and an acceptor is 50% (see [20] for review) and is shown in Eq. 8.

$$R_0 = (J\kappa^2Q_0n^{-4})^{1/6} \times (9.7 \times 10^3)\text{Å}. \quad (8)$$

J is the spectral overlap integral; κ^2, the orientation factor between donor and acceptor dipoles; Q_0, the fluorescence quantum yield of the donor in the absence of acceptor; and n is the refractive index of the medium. The spectral overlap integral, which reflects the overlap of the donor fluorescence and acceptor absorption spectrum, was evaluated to be 2.034×10^{-14} cm^3 M^{-1} for the ANT/TNP-ATP pair. The value of ⅔ was used for κ^2, n is approximated as 1.42 for the reduced Triton, and Q_0 was determined to be 0.176 from the ratio of the fluorescence lifetime of ANT to the radiative decay time of tryptophan (3.51 ns/20 ns). Using these values, R_0 was approximated to be 28 Å.

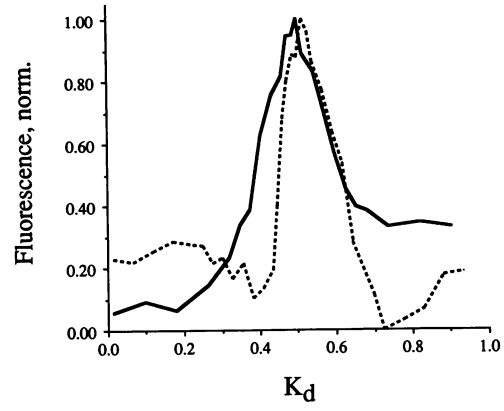

FIGURE 1 Sepharose CL-6B gel filtration of Triton X-100 (*solid line*) and reduced Triton X-100 (*dotted line*) with both peaks normalized to 1.0. V_0 and V_t were determined by gel filtration of FITC-dextran 2000 and potassium ferricyanide, respectively, and their values were both determined for elution buffer containing Triton X-100 and reduced Triton X-100 (see Methods for a complete description). 0.0 on the x-axis indicates the void volume while 1.0 indicates the total elution volume.

RESULTS

Comparison of reduced and nonreduced Triton X-100 micelle size

There appears to be no reports in the literature of micelle size for reduced Triton X-100. Therefore, this size was directly compared to the known size of nonreduced Triton X-100 micelle by gel filtration chromatography. The results in Fig. 1 show no measurable difference between the K_d value for Triton and for reduced Triton: K_d (Triton) = 0.50, K_d (reduced Triton) = 0.51; therefore, micelles of reduced and nonreduced Triton X-100 are the same size. The broad elution band of each detergent probably reflects the significant distribution of micelle size. The K_d for both detergents was used to estimate the molecular weight of the micelle to be ~90,000, consistent with the known molecular weight of the Triton micelle [21].

Determination of P_m for TNP-ATP and ATP into reduced Triton X-100 micelles using rapid filtration

There was no measurable amount (<1%) of reduced Triton in the filtrate of the centricon-10, as determined by detergent fluorescence (excitation 290 nm, emission 312 nm), which indicates that the filtration effectively separates the micelle phase from the aqueous phase. This was expected since the Triton micelle size of ~90 kD is much greater than the 10 kD molecular weight cutoff of the filter while the amount of Triton in monomer form is

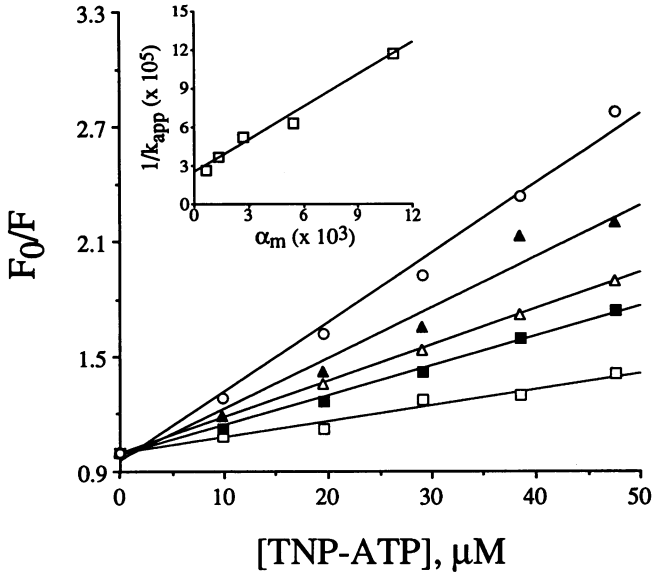

FIGURE 2 Stern-Volmer plots of TNP-ATP quenching of pyrene fluorescence in reduced Triton X-100 micelles at five different values of α_m. Excitation wavelength 321 nm, emission monitored at 372 nm, temperature = 3°C. (□) Pyrene, 10 μM, in 1.0% (wt/vol) reduced Triton X-100, 0.6 M NaCl, 10 mM Mops, pH 7.2. (■) two-fold dilution of the original concentration with buffer containing no detergent, (△) four-fold dilution of the original concentration, (▲) eight-fold dilution of the original concentration, (○) sixteen-fold dilution of the original concentration. (*Inset*) Reciprocal apparent quenching constant versus α_m, where the k_{app} values were derived from the slopes in the main figure.

~1.2% of total detergent, according to the critical micelle concentration determined (see General Methods). The TNP-ATP concentration in the filtrate was found not to change as the retentate volume decreased, with $[\text{TNP-ATP}]_{(\text{filtrate})}/[\text{TNP-ATP}]_{(\text{total})} = 0.182 \pm 0.003$ ($n = 3$). From these values, the TNP-ATP partition coefficient into reduced Triton micelles was calculated; $P_m = 450 \pm 7$. Similar filtration experiments using ATP yielded a $[\text{ATP}]_{(\text{filtrate})}/[\text{ATP}]_{(\text{total})}$ value of (0.945, $n = 1$), which is essentially identical to the value obtained for the control (which contained no detergent), indicating that ATP does not partition into the detergent micelles.

Determination of P_m for TNP-ATP into reduced Triton X-100 micelles using pyrene quenching

Fig. 2 shows a high degree of quenching of pyrene fluorescence by TNP-ATP. From the low concentrations of quencher used, it is clear that this is solely due to quencher partitioning into reduced Triton X-100 micelles. The quenching ratio (F_0/F) versus TNP-ATP concentration data shown in the main figure fit well to a linear slope ($R^2 > 0.97$). The figure inset shows the dependence of the reciprocal apparent quenching rate constant ($1/k_{app}$) on the relative micelle volume (α_m). Using Eq. 2, the slope = 8.34×10^{-3} M = $(1/k_m - (1/k_m P_m))$ and the intercept = 2.57×10^{-5} M = $1/k_m P_m$. From these values, P_m was determined to be 325 and $k_m = 120\ \text{M}^{-1}$. The fluorescence decay of pyrene in reduced Triton micelles fit well to one component ($\chi^2 = 1.12$) with a lifetime of 209 ns, and therefore the bimolecular quenching rate constant for this experiment equals $5.8 \times 10^8\ \text{M}^{-1}\ \text{s}^{-1}$.

Time-resolved fluorescence experiments

TNP-ATP specifically binds to ANT isolated with pCoA (a reversible inhibitor) in the 0–50 μM range with an approximate $K_d = 20$ μM, as determined by monitoring quenching of steady-state fluorescence of ANT tryptophans (Tummino and Gafni, unpublished results). At higher TNP-ATP concentrations (up to 300 μM), there was additional quenching with Stern-Volmer characteristics. When ANT was isolated with the TNP-ATP binding site blocked by the irreversible inhibitor CAT, Stern-Volmer quenching was still observed. Notably, Hiratsuka (22) also reported TNP-ATP quenching of protein fluorescence, specifically the intrinsic fluorescence of heavy meromyosin upon binding, but reported no dynamic quenching. As shown in Figs. 3–5, the quenching was very efficient, yielding apparent quenching rate constants too large to be accounted for even by diffusion-limited quenching. Since the rapid filtration and pyrene quenching experiments demonstrated that TNP-ATP partitions into free reduced Triton X-100 micelles, the following quenching experiments were performed to determine if the presence of ANT in the micelles affected the TNP-ATP partition coefficient so as to increase its local concentration and thus account for the efficient Stern-Volmer quenching.

The decay kinetics of the intrinsic fluorescence of CAT-inhibited adenine nucleotide translocase can be fit well to three components, as shown in Fig. 3 (*top panel*), with an average lifetime ($\Sigma\ a i_0 \tau i_0$) = 3.51 ns in the absence of quencher. The bottom panel of Fig. 3 depicts the decay in the presence of 96.8 μM TNP-ATP, yielding $\Sigma\ a i \tau i$ = 1.82 ns. Therefore, under these conditions, the quenching ratio ($\Sigma\ a i_0 \tau i_0 / \Sigma\ a i \tau i$) equals 1.93. Fig. 4 shows the Stern-Volmer plots from similar quenching experiments performed at four different protein-micelle concentrations (α_{pm}). In all cases, the addition of TNP-ATP dynamically quenched the ANT fluorescence. As α_{pm} (along with α_m) was decreased by dilution, the quenching rate increased as indicated by the greater slopes. In all the experiments shown in Fig. 4, the sample free-micelle volume/ANT-micelle volume ratio (α_m/α_{pm}) was fixed at 29.1. In four subsequent sets of experiments, increasing amounts of reduced Triton were added to the sample to increase α_m/α_{pm} and quenching experiments identical to those shown in Fig. 4 were conducted (results not shown). As in Fig. 4, all experiments fit well to linear slopes as expected for Stern-Volmer

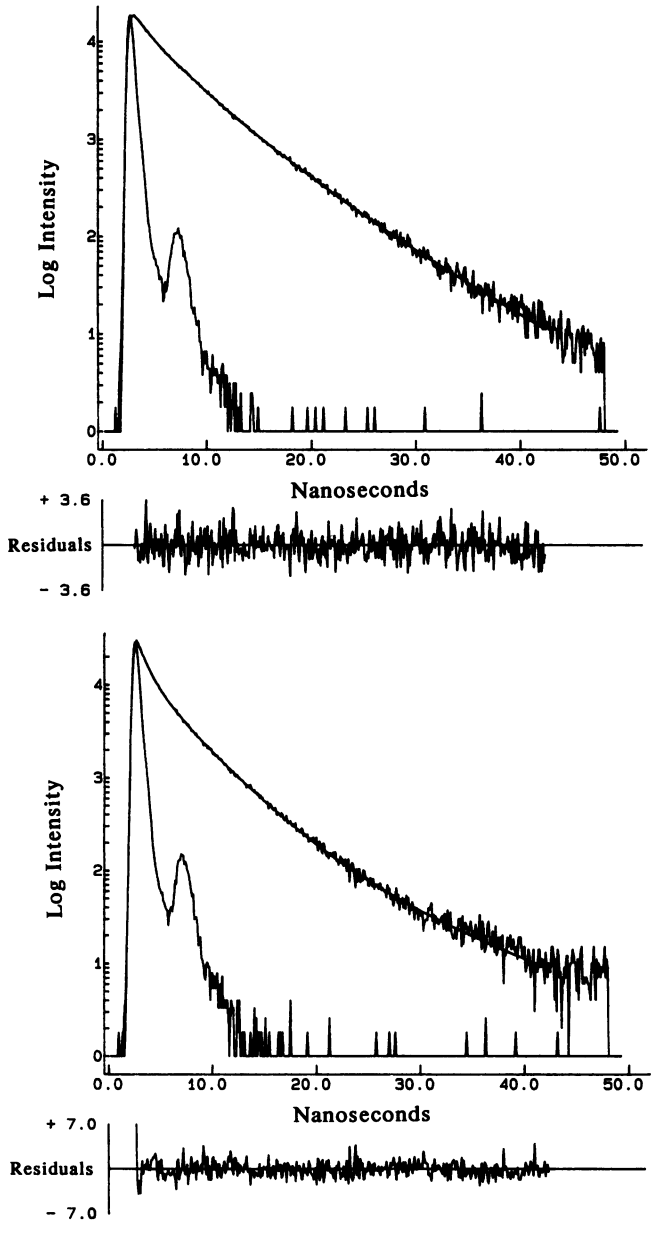

FIGURE 3 The fluorescence decays of ANT-CAT without (*top*) and with (*bottom*) TNP-ATP. Excitation wavelength 290 nm, emission wavelength 345 nm, temperature = 3°C. The fluorescence decay due to the reduced Triton X-100 in the sample has been subtracted as described in Methods. (*Top panel*) ANT-CAT, 0.171 mg/ml, in 0.934% (wt/vol) reduced Triton X-100, 0.6 M NaCl, 10 mM Mops, pH 7.2. A three-exponential decay fits the data; $a_1 = 0.37$, $\tau_1 = 1.48$ ns, $a_2 = 0.53$, $\tau_2 = 4.18$ ns, $a_3 = 0.10$, $\tau_3 = 7.48$ ns and $\Sigma\ a_i\tau_i = 3.51$ ns. $\chi^2 = 1.12$. (*Bottom panel*) ANT-CAT, 0.0214 mg/ml, + 96.8 μM TNP-ATP in 0.117% (wt/vol) reduced Triton X-100, 0.6 M NaCl, 10 mM Mops, pH 7.2. A three-exponential decay fits the data; $a_1 = 0.75$, $\tau_1 = 1.09$ ns, $a_2 = 0.24$, $\tau_2 = 3.76$ ns, $a_3 = 0.01$, $\tau_3 = 9.87$ ns and $\Sigma\ a_i\tau_i = 1.82$ ns. $\chi^2 = 1.56$.

quenching, with an average $R^2 = 0.980$. The reciprocal values of the quenching rate constants derived from these experiments were plotted versus α_{pm} according to Eq. 7, and are presented in Fig. 5 where the different slopes correspond to different α_m/α_{pm} values. The intercept of these slopes, I1, has an average value of $(2.844 \pm 0.062) \times 10^{-13}$ M·s ($n = 4$).

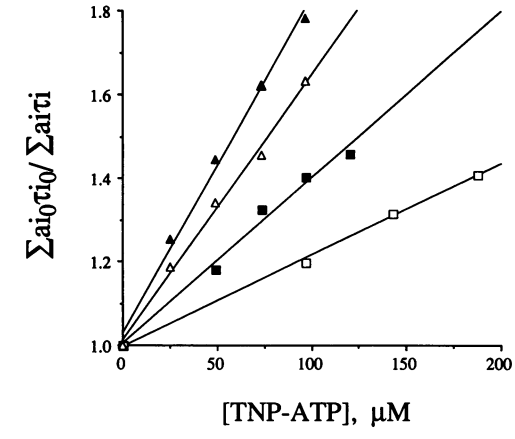

FIGURE 4 Stern-Volmer plots of TNP-ATP quenching of the fluorescence decay of ANT-CAT in reduced Triton X-100 micelles at four different values of α_{pm}. (□) ANT-CAT, 0.214 mg/ml, in 0.934% (wt/vol) reduced Triton X-100, (■) two-fold dilution of the original concentration with buffer containing no detergent, (△) four-fold dilution of the original concentration, (▲) eight-fold dilution of the original concentration. The excitation wavelength was 290 nm with emission monitored at 345 nm. TNP-ATP was added to the sample from a 3.0 mM stock and TNP-ATP concentrations shown on the *x*-axis were adjusted for dilution (see Methods for complete description). The *y*-axis shows the average decay time of ANT-CAT without TNP-ATP added ($\Sigma\ a_i\tau_i$) divided by ANT-CAT the corresponding values with TNP-ATP present, also called the quenching ratio.

The values of $1/k_{app}$, as derived from the Stern-Volmer plots in Fig. 5 at an arbitrary α_{pm} value of 1.0×10^{-4}, were plotted against α_m to generate Fig. 6. The slope of this plot, S2, has a value of 1.158×10^{-10} M·s.

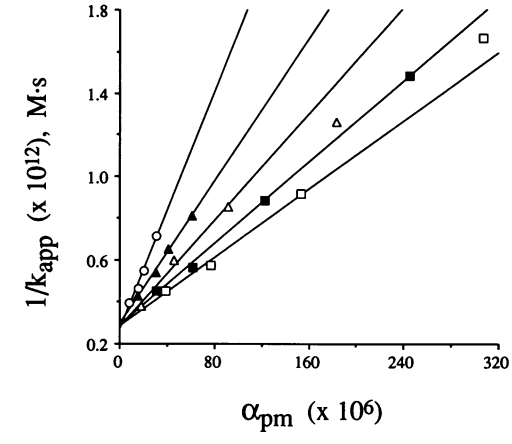

FIGURE 5 Reciprocal apparent quenching constant versus α_{pm} at constant α_m/α_{pm}. (□) $\alpha_m/\alpha_{pm} = 29.1$, (■) $\alpha_m/\alpha_{pm} = 36.7$, (△) $\alpha_m/\alpha_{pm} = 49.2$, (▲) $\alpha_m/\alpha_{pm} = 74.3$, (○) $\alpha_m/\alpha_{pm} = 149.7$. The first set of points (□) were calculated from the slopes shown in Fig. 3. The subsequent sets of points were obtained in the same manner using samples which contained less ANT-CAT and increasing amounts of reduced Triton X-100; however, each set of points has a constant α_m/α_{pm}. The slopes and intercepts from this plot are referred to as S1 and I1, respectively.

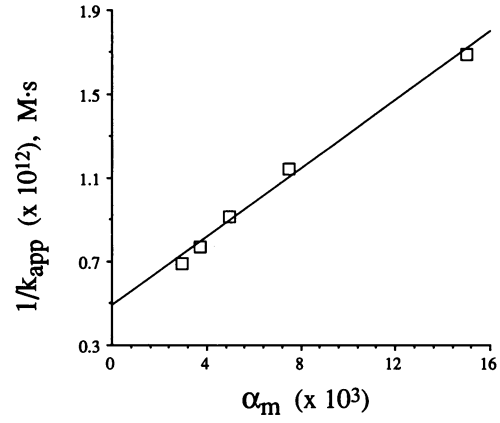

FIGURE 6 Reciprocal apparent quenching constant versus α_m at $\alpha_{pm} = 1.0 \times 10^{-4}$. The points shown were derived from the data presented in Fig. 5 by using each slope to calculate the value of $1/k_{app}$ at an arbitrary value of $\alpha_{pm} = 1.0 \times 10^{-4}$. Each $1/k_{app}$ value was then plotted against its corresponding α_m value. The slope from this plot is referred to as S2.

The value of P_m, derived from S2/I1 as described in Methods, was found to be 290, similar to the values derived from the rapid filtration and pyrene quenching experiments. The five slopes in Fig. 5 differ in their α_m/α_{pm} ratios, obtained by adding reduced Triton to the ANT sample. The slope/intercept values of these curves were plotted against the corresponding α_m/α_{pm} values and shown in Fig. 7. In this plot, the value for P_{pm} was obtained directly from the y-intercept as $P_{pm} = 7.0 \times 10^3$. Finally, the value of $k_{pm} = 5.0 \times 10^8\ M^{-1} \cdot s^{-1}$ was calculated from the equation, $k_{pm} = 1/(I1 \times P_{pm})$.

DISCUSSION

The major finding of this study is that the partitioning of TNP-ATP into reduced Triton X-100 micelles is greatly enhanced by the presence of dissolved ANT. This has direct implications for the study of membrane proteins which are frequently dissolved in detergent micelles as a model system, and may indicate important analogous effects for membrane proteins in the lipid bilayer.

Triton X-100 is a nonionic detergent frequently used to isolate membrane proteins in their native state. One disadvantage of this detergent is its significant absorption and fluorescence which overlap those of tryptophan, making the study of solubilized proteins by optical spectroscopy difficult. Complete reduction of the phenyl group of Triton X-100 decreases ultraviolet absorption and fluorescence one thousand-fold (14), making it possible to measure protein absorbance and fluorescence. In order to compare the micellar characteristics of the reduced detergent to those of the nonreduced form, gel filtration experiments were performed and showed that the reduction of Triton X-100 does not change the size and shape of the micelle. Also, the critical micelle concentration for the reduced Triton X-100 under our experimental conditions was found to be 0.185 mM, similar to the value for nonreduced Triton determined by Kushner and Hubbard (23). Tiller et al. (14) also reported a similar critical micelle concentration for the reduced form, and demonstrated that the reduced detergent has the same solubilizing characteristics as the nonreduced detergent. These studies indicate that reduced Triton X-100 micelles have the same physical properties as micelles of the nonreduced form, particularly with relation to size. For this reason, it is reasonable to assume that the aggregation number for ANT-reduced Triton micelles is the same as that for nonreduced Triton, whose value was determined by Hackenberg and Klingenberg (7). This value was, therefore, used in our fluorescence quenching calculations.

The partition coefficient for TNP-ATP into reduced Triton micelles, P_m, was determined by three independent methods. The value of $P_m = 450$ was calculated using the rapid filtration method, $P_m = 325$ from the pyrene quenching method, and $P_m = 290$ using the ANT fluorescence quenching data as described in Methods. The two independent quenching methods yielded very similar values, and thus, the pyrene experiment confirms the results from the ANT experiments while the value from the filtration method is somewhat higher. The only significant difference between the reduced Triton X-100 micelles used in the three experiments was the presence of some phospholipid (16 mole/mole protein) only in the micelles used in the ANT quenching experiments due to the protein isolation (7). However, since the P_m values derived from the pyrene experiment (without phospholipid) and from the ANT experiments (with phospholipid) are very similar, phospholipid appears not to affect partitioning. It should be noted that filtration experimental artifacts, such as TNP-ATP adsorption to the centricon filter or incomplete removal of

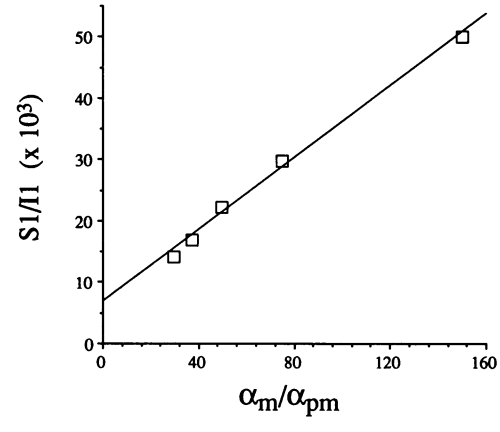

FIGURE 7 Slope 1/Intercept 1 versus α_m/α_{pm}. The slope/intercept value for each set of points in Fig. 5 was calculated and is plotted here versus α_m/α_{pm}. The intercept of this plot equals the partition coefficient of TNP-ATP into ANT-reduced Triton X-100 micelles; the value obtained is $P_{pm} = 7.0 \times 10^3$.

water from the membrane that would dilute the filtrate, were controlled for by the TNP-ATP filtration without detergent present. Note that while two of the P_m values are in very good agreement and one is somewhat higher, all three are only a small fraction of the P_{pm} value. Interestingly, no partition of ATP into the detergent micelles under the same conditions was observed indicating that the trinitrophenyl moiety of the quencher is responsible for the high P_m values.

The equation of Omann and Glaser (6) describes the partitioning of a quencher between three distinct phases, only one of which contains the fluorophore. In the present work, this equation was applied to the quenching of tryptophan fluorescence of a membrane protein dissolved in micelles of known size. In such cases, quenching data as presented here allow for the determination of the partition coefficients of quencher into both protein-free and protein micelles along with the bimolecular quenching rate constant from the same set of experiments. Comparison of the partition coefficients is therefore less affected by experimental artifacts. This is a useful technique because quenching experiments are often used to probe the structure of membrane proteins without consideration of partitioning. Determination of any partitioning by quencher will allow for accurate calculation of quenching rate constants from Stern-Volmer plots. The aggregation number for the protein micelle must be known to use the equation in this manner, and it has been determined for many solubilized membrane proteins by hydrodynamic studies. Also, if either k_{pm} or P_{pm} is known, the same quenching experiments can be used to directly determine micelle size, and therefore detergent aggregation number.

TNP-ATP dynamically quenches the intrinsic fluorescence of ANT-CAT with a bimolecular quenching rate constant of $5.0 \times 10^8\ M^{-1}\ s^{-1}$, which is very similar to the value determined here for TNP-ATP quenching of pyrene ($5.8 \times 10^8\ M^{-1}\ s^{-1}$). This value for ANT-CAT, comparable to the one reported for collisional quenching of carbazole-labeled phospholipid vesicles by 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (6), appears to be high for the collisional quenching of tryptophan residues in a detergent-bound protein. It is, however, pertinent to note that TNP-ATP quenching of ANT-CAT probably involves a long-range mechanism, due to the strong spectral overlap between the protein emission and the quencher absorption. Indeed, from our data for the ANT/TNP-ATP pair we calculate the Förster distance (R_0) to be approximately 28 Å. Thus, the TNP-ATP quenching rate constant for ANT-CAT is not a measure of the quencher diffusional rate, as has been the interpretation of other quencher rate constants measured (17). However, since Eq. 7 was developed with the sole assumption that the quenching follows Stern-Volmer kinetics, as indeed observed, it is clear that the equation can be used to calculate partition coefficients whether the quenching is due to a collisional or to a long-range mechanism(s).

The addition of ANT to reduced Triton X-100 micelles increases the partition of TNP-ATP into the micelles by greater than 24-fold. The high TNP-ATP solubility in protein micelles can not, however, be due to a high-affinity binding of the quencher to the ANT since the protein has been inhibited irreversibly with CAT and is incapable of binding the nucleotide. Moreover, from the partition coefficients determined, the TNP-ATP/ANT ratio in the protein micelle in our experiments was as high as 44, too high to be accounted for by distinct quencher binding sites on the protein. Even under these conditions, only insaturable dynamic quenching was observed. The increased partitioning is also not due to electrostatic interaction between the $TNP\text{-}ATP^{4-}$ and the basic ANT, with an overall net charge of +36 per dimer (24), since at the high TNP-ATP/ANT ratio measured, any electrostatic effect would be masked, in contrast to our observation that dynamic quenching still occurs. Finally, phospholipid can not be responsible for the great increase in partition since, as previously mentioned, both protein-free micelle and protein micelles contain phospholipid (2) from the ANT isolation. Even though phospholipids may be present in somewhat higher amounts in protein micelles due to an affinity for ANT (25), they are not responsible for the large partition increase because the P_m values determined with and without phospholipid present are very similar. Thus, the partition increase is specifically due to the ANT.

The current study does not reveal the mechanism of increased partitioning, but consideration of previous findings on the ANT-reduced Triton micelle indicates possible causes. The detergent micelle appears to be elongated in the presence of the integral protein, and this may increase hydration of the micelle and enhance TNP-ATP solubility. The results of Hackenberg and Klingenberg (7) indicate an elongation of the ellipsoid micelle to a small degree, in comparison to the Robson and Dennis (21) Triton X-100 micelle model (to be described in further detail later). Another potential cause for increased partitioning is less efficient detergent monomer packing due to disruption by the integral membrane protein surface, as suggested by Leonard et al. (26). This poorly packed micelle may be more hydrated, leading to greater quencher solubility.

Although the current evidence is insufficient to actually determine the cause(s) of the large increase in partitioning, the finding is important for two reasons. The first more direct reason involves detergent micelles as a model system for membrane proteins. This study indicates that partitioning should be considered in a protein micelle system whenever using a substrate or inhibitor with a nonpolar moiety. More generally, it is clear that the protein-micelle environment may be quite different from that of the protein-free detergent micelle. The sec-

ond reason is that since an integral membrane protein alters partitioning into detergent micelles, there may be analogous alterations to the local membrane bilayer in vivo. Although the detergent micelle is different from the lipid bilayer, ANT may have similar effects through disordering of local phospholipids. Partitioning is an important biological phenomenon, and it may be altered greatly by integral proteins. Further studies on the detail of protein-induced micelle changes, possibly in liposome systems, should yield important insight into the effects of the ANT on its local membrane environment.

An important study on the effect of integral proteins on membrane properties was conducted by Conrad and Singer (8), who reported over a 1000-fold decrease in the partition coefficient for small amphiphilic molecules in biological membranes versus the values determined in pure phospholipid vesicles. The authors stated that the presence of large amounts of integral membrane proteins, along with other differences, induce an "internal pressure" in biological membranes that causes the large drop in the partition coefficient. Subsequent work by Gains and Dawson (9) disputed this conclusion and reported no change in partition of ANS into submitochondrial particles with protein versus partition of the dye into Triton X-100 micelles without protein. Our results clearly differ from those obtained in both studies and show a large increase in the partition coefficient of an amphiphilic quencher into nonionic detergent micelles upon addition of an integral membrane protein. Direct comparison of the studies, though, is difficult because of the difference in experimental conditions. The studies of Conrad and Singer were conducted with lipid bilayers, while Gains and Dawson used both lipids and detergent and the current work was performed in detergent. Also, all changes observed in the current study can be specifically attributed to the presence of ANT since purified protein was used, while both previous groups used membranes containing many different integral proteins along with peripheral proteins, cholesterol, and neutral lipid. Any of these components may be responsible for the changes observed; an explanation Conrad and Singer did note.

More recently, three different groups (26–28) have shown a two- to threefold increase in the partitioning of amphiphilic molecules into lipid membranes due to the presence of integral membrane proteins. The study of Leonard et al. (26), in particular, concludes that integral membrane proteins nonspecifically alter the properties of the lipid bilayer resulting in increased partitioning. The similar results of these three studies along with the findings of the current work indicate that protein-induced partition increases may be common for integral membrane proteins and not specific for the ANT. Further partitioning studies should be conducted employing a number of integral membrane proteins in lipid membrane systems to determine the generality of this effect.

A model of the ANT-reduced Triton micelle can be constructed by examining our quenching experiments in conjunction with previously reported structural information. Hydropathy plot analysis (29) shows that the bovine ANT monomer consists of two hydrophilic extramembrane regions connected by a hydrophobic membrane spanning region. Two of the five tryptophans per monomer are in the membrane-spanning region. Based on the results of Robson and Dennis (21) on the Triton micelle structure, the ANT-reduced Triton X-100 micelle may be described as an oblate ellipsoid with the ANT membrane-spanning region solely in the hydrophobic detergent core, enlarging it. Since only nonpolar amino acids, therefore hydrophobic protein regions, can be in contact with the hydrophobic detergent core and still maintain detergent-micelle stability (30), the extramembrane ANT regions are probably solvated in the hydrophilic detergent shell, extending out to the aqueous solvent.

TNP-ATP probably partitions solely into the hydrophilic region of the micelle. Evidence for this is provided by our observations that TNP-ATP does not partition at all from water into cyclohexane, a solvent whose hydrophobicity is comparable to that of the octylcyclohexyl core of the micelle and that ATP^{4-} does not partition at all into Triton micelles (results not shown). These observations are not surprising since the triphosphate portion of the adenine nucleotide is densely charged and, when conjugated to TNP-, would still require significant hydration so as to reside either near or at the surface of the hydrophilic micelle shell. This is relevant because the model presented above for the ANT indicates that two tryptophans per monomer are buried in the micelle hydrophobic core, and thus inaccessible to direct interaction with quenchers in the hydrophilic region. Quenching of the fluorescence of these residues is possible only by long-range interactions. Since the Förster distance for energy transfer between ANT and TNP-ATP was estimated to be 28 Å (see Methods), and the radius of the Triton micelle ranges from 27 to 52 Å (21), TNP-ATP can effectively quench the hydrophobic core tryptophans along with those in the hydrophilic shell. Considering the ANT-micelle model described above, TNP-ATP should be able to quench ANT tryptophan fluorescence to some extent from any position within the protein micelle, thus, providing for the observed dynamic quenching. Clearly this behavior is not unique to the ANT, and it may be anticipated that the interaction of other integral membrane proteins with detergent micelles can be studied by the same approaches described here.

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