Relating Surfactant Properties to Activity and Solubilization of the Human Adenosine A_3 Receptor

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ABSTRACT The effects of various surfactants on the activity and stability of the human adenosine A_3 receptor (A_3) were investigated. The receptor was expressed using stably transfected HEK293 cells at a concentration of 44 pmol functional receptor per milligram membrane protein and purified using over 50 different nonionic surfactants. A strong correlation was observed between a surfactant's ability to remove A_3 from the membrane and the ability of the surfactant to remove A_3 selectively relative to other membrane proteins. The activity of A_3 once purified also correlates well with the selectivity of the surfactant used. The effects of varying the surfactant were much stronger than those achieved by including A_3 ligands in the purification scheme. Notably, all surfactants that gave high efficiency, selectivity and activity fall within a narrow range of hydrophile-lipophile balance values. This effect may reflect the ability of the surfactant to pack effectively at the hydrophobic transmembrane interface. These findings emphasize the importance of identifying appropriate surfactants for a particular membrane protein, and offer promise for the development of rapid, efficient, and systematic methods to facilitate membrane protein purification.

INTRODUCTION

G-protein coupled receptors (GPCRs) are thought to be targets for more than half the currently approved therapeutics worldwide, yet they and integral membrane proteins as a whole are grossly underrepresented in terms of available structural information (1). The number of known GPCRs is expected to increase as the methods to categorize receptors improve; of the nearly 2000 GPCRs identified to date, an increasing number are so-called ''orphan'' receptors of which the function and agonists remain unknown (2). This gap in knowledge impacts drug discovery efforts, and limits our understanding of the signal transduction mechanisms underlying many biological processes. Currently high-resolution structural information is available for only one GPCR, bovine rhodopsin, thanks in part to its natural abundance (3). Detailed and efficient purification procedures for bovine rhodopsin have been established that allow recovery of a large fraction of the expressed protein as well (4). Unfortunately, such methods have not translated successfully to other GPCRs. Therefore, expression and purification remain largely trial-and-error processes (5–7). These obstacles limit the overall ability to characterize GPCR structure and biochemical properties, especially because their relative abundance when overexpressed in mammalian systems is typically <1 mg/L (8).

Another major limitation in characterizing GPCRs is knowledge of solution conditions that promote activity and solubility, because NMR, protein crystallography, or other biophysical methods require highly purified samples for analysis. Despite numerous studies, no general pattern has emerged as to which surfactant properties are critical for efficient removal of active GPCRs from the membrane (9–12). More specifically, nonionic surfactants at concentrations near the critical micelle concentration have been found to work best in solubilization of membrane proteins, although notable exceptions such as the anionic CHAPS, sodium cholate, and sarkosyl exist (13). Therefore, screening a wide range of surfactants and carrying out numerous functional assays are the usual methods to assess and optimize purification. Obtaining sufficient receptor is often a limiting factor, which makes such surfactant screens inefficient and costly.

We sought to address these difficulties by characterizing the effects of a wide range of surfactants on the purification and activity of the human adenosine A_3 receptor (A_3) expressed in a stable mammalian cell line. Specifically, through relating surfactant properties to activity and solubility of A_3, we hope to understand the factors most important in identifying solution conditions favorable for purification and crystallization.

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Abbreviations used: GPCR, G-protein coupled receptor; A_3, human adenosine A_3 receptor; ^{125}I-AB-MECA, N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; NECA, 5′-(*N*-ethylcarboxamido)adenosine; R-PIA, (R)-N^6-(1-methyl-2-phenylethyl)adenosine; HLB, hydrophile-lipophile balance; CMC, critical micelle concentration; DM, *n*-decyl-β-D-maltoside; DDM, *n*-dodecyl-β-D-maltoside; OG, *n*-octyl-β-D-glucoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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A_3 belongs to the superfamily of GPCRs, and is the most recently identified of the four known adenosine receptors (14). The pharmacological profile of A_3 most closely resembles that of the adenosine A_1 receptor (A_1), and both are thought to mediate cardioprotection, although tissue distributions have identified isoforms in the brain, lungs, liver, and testes as well (14). Studies of cardiac myocyte models suggest that A_3 couples to phospholipase D whereas A_1 couples to phospholipase C, both of which in turn stimulate diacylglycerol accumulation and phosphokinase C response to mediate the protective effects of adenosine (15). Interestingly, the duration of A_3 response upon activation is much longer than that of A_1, suggesting that they may work synergistically to greater effect. Thus, we anticipate that our analysis of A_3 will be useful for further characterization of this important receptor, and will help facilitate studies of other GPCRs and integral membrane proteins.

MATERIALS AND METHODS

Materials

Vector pCEP4, Platinum Taq polymerase, T4 DNA ligase, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Antibiotics and cell culture reagents were from Gibco (Invitrogen). Media was sterile filtered using a 0.2-μm filter kit (Millipore, Billerica, MA) and tested periodically for contamination using a Mycoplasma Plus PCR primer set (Stratagene, La Jolla, CA). Ten percent NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) with MES running buffer were used for SDS-PAGE and Western blotting according to manufacturer's instructions. Rabbit anti-hexahistidine and HRP-conjugated goat anti-rabbit antibodies were obtained from Covance (Princeton, NJ). SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and ECL Plus Western blotting detection system (Amersham, Buckinghamshire, UK) were used for antibody detection. [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (^{125}I-AB-MECA) was purchased from Perkin-Elmer Life Sciences (Wellesley, MA). 2-Chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (Cl-IB-MECA), 5′-(*N*-ethylcarboxamido)adenosine (NECA), and (R)-N^6-(1-methyl-2-phenylethyl)adenosine (R-PIA) were from Sigma (St. Louis, MO). Optifluor was from Wallac Oy. Ni-NTA Superflow resin and Maxiprep DNA purification kit were from Qiagen (Valencia, CA). Protein concentration was determined using a membrane protein compatible BCA assay (Pierce). Sodium dodecyl sulfate, Brij, Tween 20, PEG 4000 distearate, and PEG 6000 distearate were obtained from Sigma, whereas digitonin (high purity) was from Calbiochem (San Diego, CA). All other surfactants used (Table 1) were purchased from Anatrace (Maumee, OH).

Cloning, transfection, and expression

Full-length cDNA encoding the 957-bp human adenosine A_3 receptor (ADORA3) was a kind gift of Marlene Jacobson (Merck, Rahway, NJ). The A_3 fragment was amplified using standard polymerase chain reaction (PCR) techniques. The forward primer 5′ ttttttttt*ggatccgctagc***gccgccaccatgg**ccaacaacagcactgctctgtca 3′ introduced *Bam*HI and *Nhe*I restriction endonuclease sites (shown in italics) as well as an in-frame Kozak sequence (shown in bold). The reverse primer 5′ ttttttttt*ctcgag*tgataa**caccatcaccatcaccat**ctactcagaattcttctcaatgct 3′ introduced an *Xho*I restriction endonuclease site (shown in italics) and a hexahistidine tag (shown in bold) to facilitate purification and detection of the receptor. The PCR product was digested using *Nhe*I and

TABLE 1 Summary of activity (Eq. 7), efficiency (Eq. 5), and selectivity (Eq. 6) for entire surfactant set

		Activity	Efficiency	Selectivity
1	Fos-Fenth	0.12	0.09	0.03
2	Nonopol-Fos	0.00	0.20	0.07
3	Fos-choline	0.16	0.14	0.04
4	Fos-choline 11	0.47	0.18	0.06
5	Fos-choline 13	0.48	0.18	0.05
6	Fos-choline 15	0.46	0.17	0.05
7	C_8-β-D-thioglucoside	0.06	0.08	0.03
8	C_7-β-D-glucoside	0.11	0.46	0.21
9	C_8-β-D-glucoside	0.22	0.31	0.15
10	C_9-β-D-glucoside	0.42	0.21	0.11
11	C_{10}-β-D-glucoside	0.51	0.17	0.10
12	C_8-β-D-thiomaltoside	0.63	0.56	0.25
13	C_8-β-D-maltoside	0.31	0.18	0.10
14	C_{10}-β-D-maltoside	0.61	0.33	0.20
15	C_{11}-β-D-maltoside	0.81	0.36	0.24
16	C_{12}-β-D-maltoside	0.70	0.46	0.35
17	HEGA8	0.24	0.12	0.05
18	HEGA9	0.42	0.40	0.18
19	HEGA10	0.60	0.48	0.22
20	HEGA11	0.66	0.55	0.27
21	CYMAL3	0.05	0.05	0.05
22	CYMAL4	0.08	0.07	0.07
23	CYMAL5	0.28	0.11	0.11
24	CYMAL6	0.46	0.16	0.16
25	C_8E_4	0.14	0.34	0.25
26	C_8E_5	0.50	0.36	0.23
27	$C_{10}E_4$	0.01	0.34	0.21
28	$C_{10}E_5$	0.21	0.09	0.06
29	$C_{12}E_4$	0.00	0.25	0.18
30	$C_{12}E_5$	0.16	0.05	0.03
31	$C_{12}E_{10}$	0.5	0.12	0.07
32	Brij 35	0.08	0.18	0.18
33	Brij 76	0.24	0.27	0.26
34	Brij 78	0.49	0.33	0.30
35	Brij 93	0.21	0.12	0.11
36	Brij 96	0.64	0.50	0.36
37	CHAPS	0.07	0.05	0.06
38	Big CHAPS	0.36	0.04	0.05
39	DDAO	0.08	0.16	0.08
40	Sodium cholate	0.46	0.12	0.00
41	Sarkosyl	0.16	0.24	0.06
42	Digitonin	0.82	0.78	0.40
43	C_7-β-D-thioglucoside	0.04	0.40	0.21
45	C_9-β-D-thioglucoside	0.00	0.29	0.17
46	C_{10}-β-D-thioglucoside	0.00	0.25	0.13
48	C_9-β-D-thiomaltoside	0.55	0.2	0.14
49	C_{10}-β-D-thiomaltoside	0.52	0.59	0.29
50	C_{12}-β-D-thiomaltoside	0.35	0.75	0.38
51	Surfonyl 485	0.06	0.02	0.02
52	Triton X-305	0.02	0.00	0.01
53	Triton X-405	0.10	0.00	0.00
54	Triton X-705	0.00	0.01	0.00
55	Tween 20	0.11	0.10	0.00
56	Brij 700	0.00	0.00	0.01
57	PEG 4000 distearate	0.00	0.00	0.02
58	PEG 6000 distearate	0.00	0.02	0.02

In all cases, the surfactant concentration was 20 mg/mL.

*Xho*I and ligated into mammalian expression vector pCEP4 according to the manufacturer's instructions. Maxiprep purified DNA suitable for transfection had a 280:260-nm absorbance ratio below 1 as determined by ultraviolet spectrometry using a Beckman DU 7400 spectrophotometer.

HEK293E cells were seeded into six-well, 100-mm cell culture dishes and grown to an approximate density of 10^6 cells per milliliter for transfection, corresponding to >90% confluency. After removal of media, the cells were rinsed briefly with pH 7, 50 mM potassium phosphate buffer and Opti-MEM reduced serum was added to a final volume of 1 mL. The ratio of DNA/lipofectamine and total DNA-lipofectamine complex/media volume were varied to identify optimal conditions for transfection; in general, successful transfections favored high DNA to lipofectamine ratios and high overall complex/media volume. Posttransfection (48 h), media was removed and rinsed briefly with PBS to remove residual lipofectamine, after which 1 mL of nonselective media was added. After 24 h incubation, confluency was ~50%. At this point, cells were rinsed gently with nonselective media to remove traces of lipofectamine and then incubated with selective media containing 150 μg/mL hygromycin B for 8–11 days. During this time, media were replaced every 3–4 days without passing cells, over which time confluency decreased to ~10–20%. Individual colonies were then expanded and expression levels verified by SDS-PAGE and Western blotting as described below. Stable cell lines producing A_3 remained viable for at least two months, with an apparent doubling time roughly half that of control cells transfected using pCEP4. For large-scale A_3 expression, T-175 flasks containing 50 mL of media were seeded 1:10 and grown to >90% confluency. Cells were harvested by repeated rinsing and resuspending in 10 mL of lysis buffer (pH 7, 50 mM potassium phosphate buffer containing 100 mM NaCl and 50 mM EDTA). This suspension was centrifuged for 5 min at 4°C, 4000× *g*, the supernatant decanted, and cell pellet stored at −80°C.

Purification and extraction

Frozen cell aliquots were thawed on ice and resuspended in 1–5 mL of lysis buffer containing 100 μM PMSF; for SDS-PAGE, each cell pellet was from 100 mL of saturated media, whereas for immunoblotting and ligand binding, each cell pellet was from 10 mL of saturated media. Throughout purification, each lane for SDS-PAGE was loaded with 20-μL samples using 10% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) with MES sample buffer and run at 80 V, 50 mA for 6 h at 4°C. Gels were stained and visualized using GelCode Plus staining reagent (Pierce). For immunoblotting, samples were transferred using Invitrolon PVDF filter paper and blotted using 1:1000 rabbit anti-A3 primary and 1:250 HRP-conjugated goat anti-rabbit antibodies (Covance) as per manufacturer's instructions. Visualization was achieved using SuperSignal West Pico chemiluminescent substrate (Pierce) and ECL Plus Western blotting detection system (Amersham).

Unless otherwise stated, all purification and extraction steps were carried out at 4°C. To homogenize the pellet and lyse cells, this suspension was sonicated on ice for 1 min at a 10% duty cycle with 10% output using a Branson 450 sonicator and then pressed 40 times through a Dounce homogenizer on ice. The lysate was centrifuged for 1 h at 90,000× *g* to collect membrane material, after which the pellet was resuspended in 1–5 mL of extraction buffer (pH 7, 25 mM potassium phosphate) and sonicated on ice for 1 min at a 10% duty cycle with 10% output. Total mass of membrane protein was determined using the micro BCA protein assay kit (Pierce) with BSA as a standard. To minimize interference from lipids when using the BCA method, the cell membrane pellet was resuspended in 1–5 mL of extraction buffer containing 2% (w/v) SDS (16). The solution was incubated with gentle mixing for 2 h and then centrifuged at 90,000× *g* for 1 h. Generally, no pellet was observed after solubilization with SDS. The amount of A_3 present per mass of total membrane protein used in calculating efficiency (*D* in Eq. 5) was determined by competitive ligand binding experiments as discussed below.

Purification of A_3 was achieved using several different protocols. An overview of each procedure used and its relationship to the activity, efficiency, and selectivity measurements is given (Fig. 1). To determine extraction efficiency, 1 mL aliquots of membrane suspension at a total membrane protein concentration of 1 mg/mL were mixed with 0.1 mL of a concentrated test surfactant solution such that the concentration of surfactant was 20 mg/mL. Generally, this concentration of test surfactant chosen fell within a range of 15–30 × CMC for the entire set of surfactants examined, with at least 500 test surfactant molecules present per membrane protein molecule. This ensures adequate test surfactant is present to solubilize the membrane. The membrane-surfactant mixture was incubated with gentle mixing for 48 h and then centrifuged at 90,000× *g* for 1 h.

The concentration of solubilized membrane protein used in calculating selectivity (E in Eq. 6) was determined by the BCA method. For the BCA measurement, the test surfactant concentration was reduced to 1% (w/v) in all cases except for the alkyl polyglucosides, CHAPS, and digitonin, where it was instead reduced to 0.1% (w/v), by diluting into SDS solution such that the final total surfactant concentration was 2% (w/v) as per the manufacturer's recommendations. The additional SDS was necessary to maintain a minimum surfactant/protein ratio of 500 and prevent potential membrane protein aggregation or precipitation, which would limit the accuracy of the concentration measurement. As an additional correction, all protein concentrations measured using the BCA method were blanked against buffer containing the same total surfactant concentration and composition. The solubilized membrane protein solution (0.5 mL) was then added to 50 μL of Ni-NTA resin containing 5% (v/v) glycerol and incubated with gentle agitation for 6 h; glycerol was found to help prevent nonspecific binding. The suspension was centrifuged for 10 min at 4000× *g* to collect resin and resuspended in 0.5 mL of wash buffer (pH 6, 50 mM MES, 10 mM imidazole) containing the same concentration of test surfactant. This wash step was repeated twice to remove nonspecifically bound material. The test surfactant was then exchanged for digitonin by washing twice with 0.5 mL of a 3.5-mM stock solution in wash buffer. Competitive ligand binding was then performed on the immobilized receptor as discussed below to determine the amount of A_3 extracted using a given test detergent to calculate both efficiency (*C* in Eq. 5) and selectivity (*C* in Eq. 6).

For experiments to determine A_3 activity in various detergents, a 3% (w/v) digitonin solution was prepared by dissolving 30 mg of digitonin in 1 mL extraction buffer, heating for 10 min at 90°C, cooling for 12–16 h at 4°C, and centrifuging at 20,000× *g* for 1 h to remove insoluble material. The supernatant was stored at 4°C until further use. The stock digitonin solution (1 mL) was added to 5 mL of membrane suspension and mixed with gentle agitation for 48 h. This solution was centrifuged for 1 h at 90,000× *g* to remove insoluble material, supernatant decanted, and added to 25 μL of Ni-NTA resin. This sample was mixed with gentle agitation for 6 h and resin collected by centrifugation for 10 min at 4000× *g*. The resin with bound receptor was washed twice with pH 6, 50 mM MES buffer containing 10 mM imidazole and 3% (w/v) digitonin to remove nonspecifically bound material. To determine the amount of bound receptor after washing used in calculating activity (*B* in Eq. 7), competitive ligand binding was performed as described below.

For detergent exchange, 100 μL of washed resin with bound receptor was diluted into 1 mL of pH 6, 50 mM MES buffer containing a test detergent at a concentration of 20 mg/mL, which ensures that at least 500 detergent molecules are present per receptor. As was the case previously when measuring efficiency (Eq. 5) and selectivity (Eq. 6), this choice of surfactant concentrations ensures that adequate surfactant is present to solubilize the receptor, thereby minimizing the possibility that loss of activity is due to lack of surfactant. The resin was washed again with 1 mL of the new detergent solution to remove residual digitonin, and mixed with gentle agitation for 24 h. The resin was then collected by centrifugation at 4000× *g* for 10 min. The concentration of active A_3 in the presence of a given test detergent used to calculate activity (*A* in Eq. 7) was determined by competitive ligand binding as described below. To ensure that activity measurements were not biased by having the protein immobilized, control experiments were also performed where the surfactant-solubilized receptor was eluted from the particles using 0.2 M imidazole, diluted 10-fold into buffer at the same surfactant concentration, allowed to equilibrate for 16 h in the absence of particles, then incubated with Ni-NTA resin and competitive ligand binding

performed as before (not shown). No significant difference in activity was measured between samples that were immobilized 2 h after extraction or immobilized after equilibrating for 16 h, supporting the idea that ligand-binding experiments performed on Ni-NTA particles accurately reflect conditions in solution.

Competitive binding assay

For competitive binding experiments, 130 μL of detergent solution containing immobilized A_3 on Ni-NTA resin was combined with 10 μL of a 13.5 nM ^{125}I-AB-MECA stock solution (final concentration 0.9 nM) and 10 μL of stock NECA solutions spanning a range of final concentrations from 10 to 1000 nM. The samples were incubated in Millipore MultiScreen 96-well filter plates for 2 h at 20°C, with gentle agitation. Nonspecific binding was measured in each assay by subjecting mock-transfected cells to the same conditions and procedures as the cells expressing A_3. Binding reactions were terminated by removing unbound ligands using a Millipore MultiScreen vacuum manifold. The retentate was washed 3 times with 250 μL of ice-cold extraction buffer containing an appropriate concentration of detergent. Optifluor (Wallac Oy) (250 μL) was then added to each well. Radioactivity was measured using a Perkin-Elmer 1450 Microbeta scintillation counter with Microbeta workstation software version 3.01.005. In most cases, detergent concentrations at an detergent/A_3 ratio of 500:1 were most amenable to ligand-binding experiments, whereas higher detergent concentrations led to significant nonspecific binding and often gave results that were not interpretable, probably due to partitioning of the hydrophobic ligands into detergent-rich domains formed on the resin surface. Therefore, a surfactant concentration of 20 mg/mL was used in all cases. Each measurement was performed in triplicate and the results averaged. Data were fit to the following competitive binding isotherm using Kaleidagraph 5.0 for Macintosh (17):

$$Y = B + \frac{T - B}{1 + \left(\frac{X}{IC_{50}}\right)^{n}}. \quad (1)$$

T refers to maximum specific activity, B is the minimum specific activity, X is the competitor ligand concentration, and n is the Hill coefficient. K_d values for hot and cold ligands were obtained using the Cheng-Prushoff equation (18).

Saturation binding assay

For saturation binding experiments, 150 μL of cell membrane suspension was added to increasing concentrations of ^{125}I-AB-MECA in a range of 0.1–20 nM. Solutions were incubated and treated, and the data collected as described above for the competitive ligand-binding assay. Three sets of independent measurements were taken over the entire range of concentrations. The results were averaged and fit to a single-site, noncooperative binding isotherm using Kaleidagraph 5.0 for Macintosh (17):

$$Y = \frac{B_{max}X}{K_d + X}. \quad (2)$$

B_{max} refers to the maximum binding, X is the ligand concentration, and K_d is the equilibrium ligand dissociation constant.

Surfactant characterization

HLB values for *n*-alkyl-β-D-glucosides (n = 7–16) and *n*-alkyl-β-D-thioglucosides (n = 8–18) were determined from correlations based on experimentally measured HLB values, the form of which is similar to Eq. 8 (19). The results of these correlations agree well with other experimentally determined HLB values for *n*-octyl-β-D-glucoside as well as with the proposed range of HLB values for alkyl polyglucosides (20). HLB values for *n*-alkyl-β-D-maltosides were estimated from experimentally determined values in a similar manner to the *n*-alkyl-β-D-glucosides (21). HLB values for polyoxyethylene and additional surfactants were calculated from Eq. 8 using tabulated hydrophilic and lipophilic group numbers (22). In the case of the PEG distearates and Sulfonyl 485, data provided by the manufacturers were used in estimating HLB values according to Eq. 8. Surfactants 1–6, 17–20, 21–24, and 37–41 (Table 1) were not included in the HLB calculations, as adequate hydrophilic and lipophilic group numbers (Eq. 8) were not available.

To calculate packing parameters (Eq. 9), cross-sectional headgroup areas for alkyl polyglucosides were taken from literature values (20,23,24). For polyoxyethylene surfactants, average cross-sectional headgroup areas can be calculated based on the number of ethoxylate (EO) units in the surfactant; calculated areas for C_iE_j surfactants for $j = 3$–10 are in good agreement with experimental values (25,26). In all cases, the literature values were compared with experimentally determined values at the critical micelle concentration (CMC) by the Wilhelmy plate method using a Kruss digital tensiometer K10T (Hamburg, Germany). Specifically, the breakpoint in surface tension versus log (surfactant concentration) was equated with the CMC and the limiting cross-sectional headgroup area calculated from (27)

$$\Gamma_{MAX} = -\frac{4.342 \times 10^{-3}}{nRT}\left(\frac{d\gamma}{d\log C}\right)_T \quad (3)$$

$$A_{MIN} = \frac{10^{20}}{N\Gamma_{MAX}}. \quad (4)$$

Γ is the surface excess concentration (mol/m^2), n is the number of species of which the concentration at the interface varies with the surfactant bulk phase concentration ($n = 1$ for nonionic surfactants), R is the universal gas constant, T is temperature (K), $d\gamma/d\log C$ is the maximum slope, N is Avogadro's number, and A_{MIN} is the minimum headgroup area per surfactant molecule (Å^2). Samples were prepared at a total volume of 30 mL and at surfactant concentrations spanning two orders of magnitude above and below the CMC. All measurements were made at 20°C using a temperature-controlled bath and allowed to equilibrate for 1 h after dilution.

Surfactants 1–6, 17–20, 21–24, and 33–42 (Table 1) were not included in the packing parameter calculations (Eq. 9), as adequate cross-sectional headgroup areas or definitions of chain lengths were not available. For example, sodium cholate (40), CHAPS (37), and digitonin (42) have more complicated structures; they do not have single hydrocarbon chain tails or clearly defined hydrophilic headgroups. Therefore, estimates of chain length (Eq. 4) are not possible. Likewise, because the Brij surfactants (32–36, 56), Sulfonyl 485 (51), Triton series (52–54), Tween 20 (55), and PEG distearates (57, 58) are in fact surfactant blends, individual values for the cross-sectional headgroup areas could not be determined.

RESULTS

Characterization of A_3 expression levels in HEK293

To measure total expression levels and establish a baseline for evaluation of different surfactants, we used radioligand displacement isotherms to quantify the amount of A_3 produced in HEK293 cells as well as the amount extracted using digitonin. Digitonin is a natural surfactant that has been shown previously to support activity of GPCRs, and therefore was chosen for initial purification before surfactant screening (28). However, due to the heterogeneous nature of digitonin as well as its large size relative to other single- or double-chain surfactants, it may not be suitable for other applications such as crystallization or spectroscopic studies.

We found that of the various competitor antagonists commonly used for A_3, NECA most efficiently displaced the

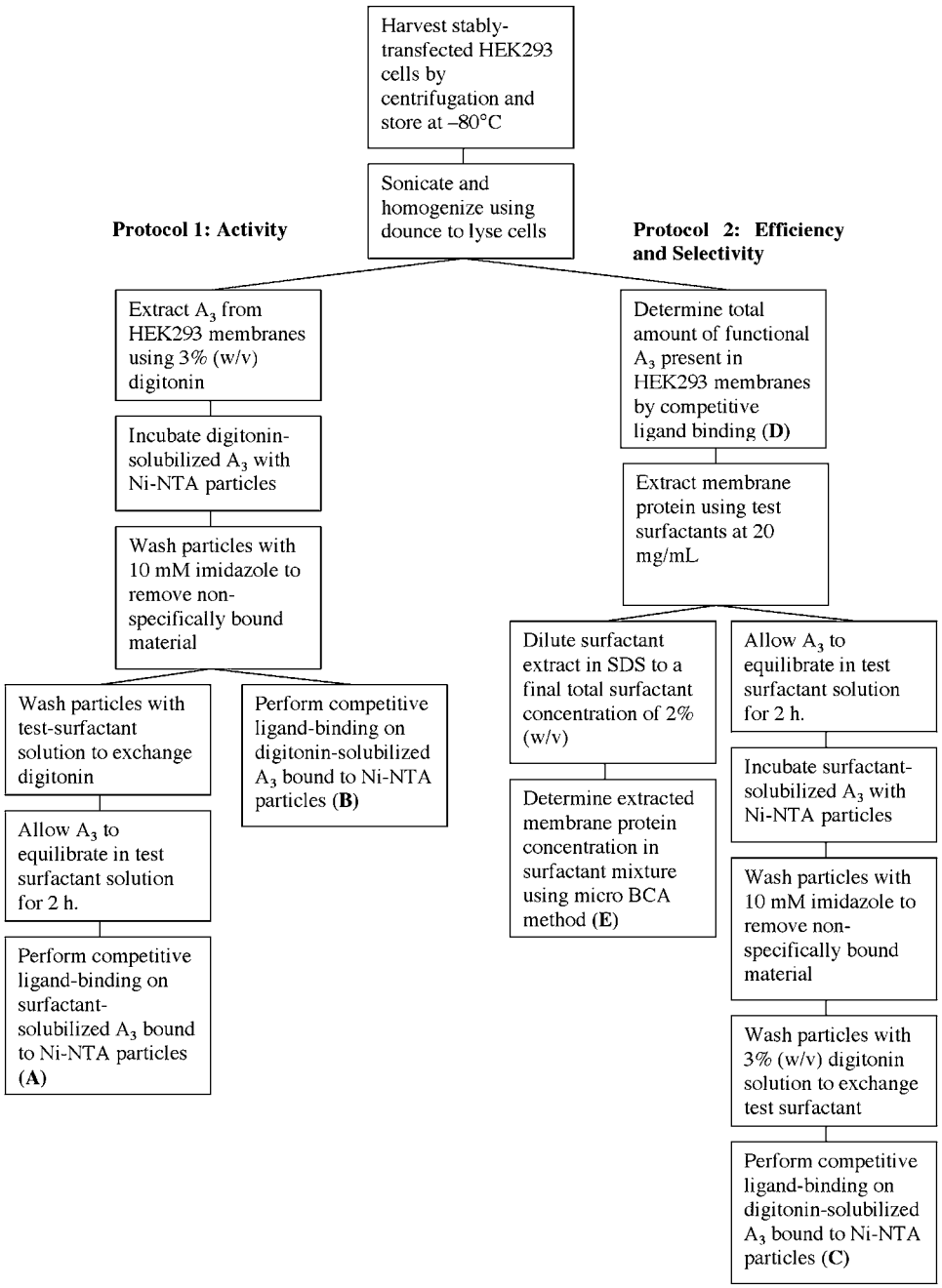

FIGURE 1 Summary of experimental methods used to measure the various parameters necessary to define activity (Protocol 1, *left column*), selectivity, and efficiency (Protocol 2, *right column*). The boldface letters refer to variables used in the definitions for activity (Eq. 7), efficiency (Eq. 5), and selectivity (Eq. 6). These protocols are described in detail in Materials and Methods.

radiolabeled I^{125}-AB-MECA. Competitive ligand-binding experiments using either digitonin-solubilized receptor bound to Ni-NTA resin or whole-cell extracts gave similar IC_{50} values (Fig. 2). From saturation binding studies the concentration of functional receptor in the whole-cell extract was determined to be 127.4 μg/L of culture, $\sim 4.3 \times 10^7$ functional receptors per cell and 44 pmol functional receptor per milligram membrane protein.

The details of the purification of A_3 are summarized in Table 2, and a SDS-PAGE gel at each stage of purification is also given for comparison (Fig. 3 *B*). Immunoblotting using A_3-specific antibodies also indicates that the major band observed after purification corresponds to A_3 (data not shown). Competitive binding experiments with the digitonin-solubilized receptor gave concentrations within 10% of those for the whole-cell extract, indicating that most of the receptor expressed can be recovered using digitonin. These concentrations are within an order of magnitude of those observed previously for A_1 in stably transfected HEK293 cells (29). Overall, these results confirm that overexpression in HEK293E cells and purification using digitonin provides a robust means by which to generate functional, purified receptor, and a benchmark against which we can compare the effects of other surfactants.

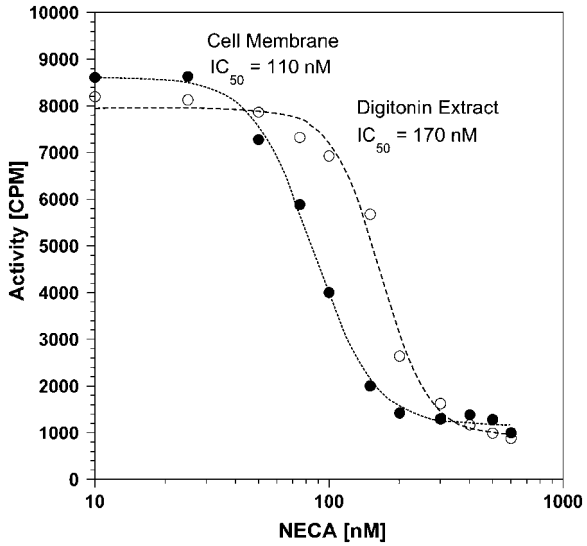

FIGURE 2 Comparison of competitive ligand binding curves for A_3 in cell membranes (●) and in digitonin extracts (○). Measurements were made at 20°C using 100 μL of membrane or resin suspension in pH 7, 50 mM potassium phosphate buffer containing 100 mM NaCl, and 50 mM EDTA. The lines indicate the best fits to a single-site, noncooperative binding isotherm (Eq. 1). Points represent the average of at least three independent measurements. Error in the measured radioactivity was <5% of the value at each data point.

Effect of surfactants on A_3 extraction efficiency, selectivity, and activity

To compare effects of different surfactant types on membrane protein properties, 56 different surfactants common to membrane protein studies were assayed for their ability to extract A_3 from the membrane and to maintain its ligand binding activity once extracted. We used the following definitions to draw comparisons:

$$Efficiency = \frac{Mass\ A_3\ extracted\ (C)}{Total\ mass\ A_3\ in\ membrane\ (D)} \quad (5)$$

$$Selectivity = \frac{Mass\ A_3\ extracted\ (C)}{Total\ mass\ membrane\ protein\ extracted\ (E)}. \quad (6)$$

Fig. 3 *A* shows a comparison of competitive binding curves for selected surfactants after exchange from digitonin. In general, IC_{50} values were essentially unchanged using the different surfactants, whereas substantial differences were seen in the maximal binding levels (B_{max}) depending on the surfactant used. Relative activity is defined as:

$$Activity = \frac{Maximal\ binding\ after\ surfactant\ exchange\ (A)}{Maximal\ binding\ in\ digitonin\ (B)}. \quad (7)$$

Thus, we define the activity of A_3 in the different surfactants relative to the activity when solubilized in digitonin, and we define efficiency and selectivity of the surfactants based on the amount of A_3 present in the HEK membranes.

TABLE 2 Summary of digitonin-solubilized A_3 purification using Ni-NTA affinity chromatography

	Mass A_3 (μg)	Mass total protein (μg)	Selectivity*	K_i NECA (nM)	K_i Cl-IB-MECA (nM)
Initial	12.64	70120	–	120	3.3
Digitonin extract	5.32	80	0.06	174	3.1
Wash	3.56	4.57	0.78	150	3.1
Eluate	3.04	3.15	0.76	120	2.9

*Selectivity is defined in Eq. 6.

The variables in Eqs. 5–7 refer to Fig. 1, which summarizes the experimental procedures used to purify A_3 receptor and to measure activity (Protocol 1), efficiency (Protocol 2), and selectivity (Protocol 2) in each surfactant. Table 1 lists the surfactants used and their corresponding activities, efficiencies, and selectivities; in all cases, the surfactant concentration used was 20 mg/mL, which ensured that at least 500 surfactant molecules were present per A_3 receptor. The number in the first column is used to identify individual surfactants on subsequent figures.

Fig. 4 compares differences in efficiency and selectivity for selected surfactants, and emphasizes that surfactants with different headgroups have very different effects on the recovery of A_3. Even within a homologous series of surfactants, increasing chain length also produces varying effects depending on the surfactant family (Fig. 5). As an example, for the glucoside family, increasing the carbon chain length yields decreasing efficiency, whereas for the HEGA and maltoside family, efficiency increases with carbon chain length. This lack of a systematic trend illustrates the difficulty in identifying guidelines for selecting optimal surfactants for membrane protein purification.

Interestingly, for the entire surfactant set, a strong correlation exists between efficiency and selectivity; in other words, increasing efficiency is accompanied by an increase in selectivity (Fig. 6 *A*). Surfactants that are effective in removing A_3 from the membrane also enrich those extracts in A_3. Similarly, activity also correlates with selectivity, although not as strongly (Fig. 6 *B*). This result implies that surfactants that are effective in the purification of A_3 are also likely to promote activity of A_3.

Use of ligands to enhance purification

It has been suggested that addition of ligands before extraction can enhance the amount of receptor recovered, and help to retain receptor activity (30). Specifically, purification of rat m3 muscarine receptor using CHAPS, digitonin, or decyl-β-D-maltoside (DM) in conjunction with the agonist *N*-[^{3}H]methylscopolamine was found to increase recoveries considerably, although this effect was surfactant specific (31). We sought to test whether this strategy is effective for A_3. We added R-PIA, Cl-IB-MECA, and NECA to the cell homogenate solution before extraction

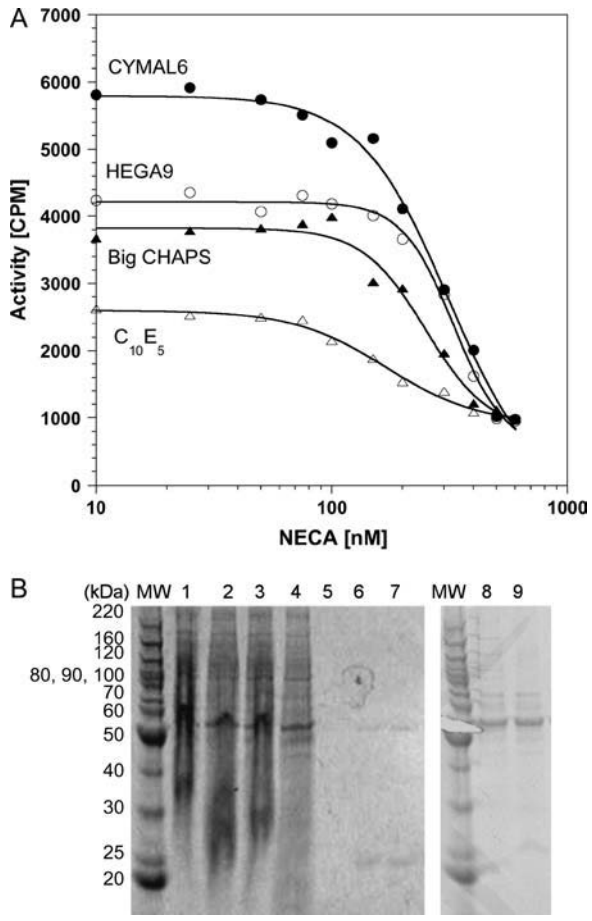

FIGURE 3 (*A*) Competitive ligand binding results for selected surfactants CYMAL6 (●), HEGA9 (○), Big CHAPS (▲), and $C_{10}E_5$ (△). Measurements were made at 20°C using 100 μL of resin suspension in pH 7, 50 mM potassium phosphate buffer containing 100 mM NaCl, and 50 mM EDTA. The lines indicate the best fits to a single-site, noncooperative binding isotherm (Eq. 1). Points represent the average of at least three independent measurements. Error was <5% of the value at each data point. (*B*) SDS-PAGE illustrating the purification of A_3 using Ni-NTA resin and surfactant exchange. Lanes are as follows: MW, molecular weight ladder (in kDa); 1, lysate from mock transfection; 2, lysate from stable transfection with A3; 3, insoluble fraction using 3% (w/v) digitonin; 4, soluble fraction using 3% (w/v) digitonin; 5, supernatant after binding to Ni-NTA resin; 6 and 7, fractions collected during elution with 0.2 M imidazole from Ni-NTA resin in 3% (w/v) digitonin; 8, purified A_3 after exchange into unidecyl-β-D-maltoside; 9, purified A_3 after exchange into HEGA 10. A summary of the amounts of A_3 recovered during each step in the purification is given in Table 2.

and compared the amount of A_3 recovered for various representative surfactants. The specificity of the ligand for the receptor dictated its effectiveness in enhancing A_3 recovery; Cl-IB-MECA was most effective (not shown). Although, in general, adding Cl-IB-MECA did enhance recovery for all surfactants, this was most pronounced for surfactants that were otherwise poor in maintaining activity or efficiency (such as CHAPS). When more effective surfactants such as digitonin or DM were used, addition of ligand provided little improvement. Overall, the effects of ligand were fairly small, amounting to <10% change in recovery, whereas changing the surfactant type could alter recovery by an order of magnitude or more.

Surfactant properties and A_3 extraction efficiency, selectivity, and activity

Although the correlations between selectivity, efficiency, and activity are useful in guiding purification, they give little insight into why certain surfactants are effective for a given membrane protein. As a result, membrane protein purification methods rely on extensive screening with many nonionic surfactants, as has been described for the neurokinin-1 and chemokine 5 receptors as well as for rhodopsin. To date, there is no consensus on which aspects of the surfactants used dictate their effectiveness in purifying receptors or maintaining their activity (4,32,33). This uncertainty arises in part because the large differences in the chemical structure of various headgroups (alkyl polyglucosides or polyoxyethylenes) or overall structure (CHAPS, sodium cholate, or digitonin) make quantitative comparison difficult. We sought to address this problem by comparing various surfactant properties with efficiency, selectivity, and activity of A_3. Our goal was to find whether a general property exists that reflects these functional properties of surfactants across various families with different chemical structures.

One obvious candidate is the critical micelle concentration. CMC is a well-known surfactant property that reflects the concentration at which the air-water interface of a given solution has become saturated, so that additional monomers associate in solution to form micelles (34). Comparison of activity, efficiency, or selectivity with CMC indicates that no general correlation or trend exists (Fig. 7); clearly CMC is not a good predictor of whether a given surfactant will be useful for purification or handling of A_3. Comparison with surfactant aggregation number also did not yield any correlation with activity, selectivity, or efficiency (not shown).

However, within individual surfactant families, increases in efficiency, selectivity, or activity often do correlate with increasing hydrocarbon tail length. This effect can be understood in terms of an increasing hydrophobicity of the surfactant, which may reflect its improved ability to partition into the membrane and solubilize hydrophobic material. Increasing chain length may also provide an improved registry with the transmembrane region of the membrane protein. Therefore, we hypothesized that a parameter that reflects the relative solubility of both the hydrophobic and hydrophilic portions of a surfactant would be more indicative of its ability to purify and retain activity of a particular membrane protein.

In this sense, the hydrophile-lipophile balance (HLB) is a more useful quantitative, though empirical, parameter. The HLB was originally developed as a scale to classify the relative effectiveness of nonionic surfactants at forming stable emulsions at room temperature (35). Surfactants with

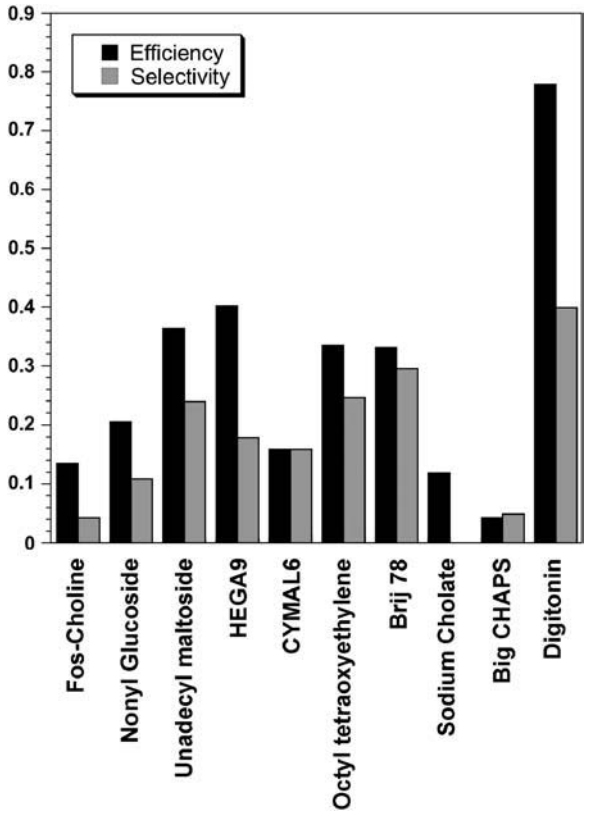

FIGURE 4 Comparison of efficiency and selectivity for selected surfactants. Efficiency is defined in Eq. 5 and selectivity in Eq. 6.

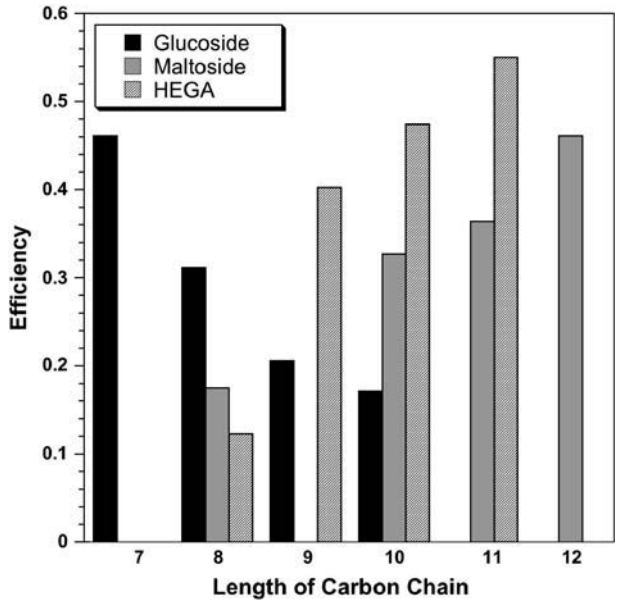

FIGURE 5 Comparison of efficiency for selected surfactant groups glucosides, maltosides, and HEGA as a function of chain length. Efficiency is defined in Eq. 5.

HLB values between 10 and 20 are water soluble and generally form stable, oil-in-water emulsions, whereas surfactants with HLB values below 10 are oil soluble and generally form water-in-oil emulsions (22).

In the original definition, HLB was meant to be a specific property based solely on the chemical structure of a surfactant. Each functional group was assigned a specific value based on correlation of emulsion properties for various surfactants. Thus, HLB was thought to be independent of intensive variables such as temperature or ionic strength (36,37). However, it has been shown that heating an oil-in-water emulsion will lead to inversion, or formation of a water-in-oil emulsion; the temperature at which this occurs is the phase inversion temperature, and is common to many nonionic surfactant emulsions (38). This inversion has been interpreted as a consequence of the increase in relative hydrophobicity of nonionic surfactants with temperature or ionic strength, which in turn leads to a change in micelle curvature and ultimately packing about the oil-water interface (22). So, although HLB may include specific structural information about a given surfactant, its interpretation in terms of other physical properties is somewhat limited. However, one advantage is that HLB numbers for single-chain surfactants with relatively simple structures can be measured experimentally or estimated based on correlations (27):

$$HLB = \sum H - \sum L + 7. \quad (8)$$

H represents group contributions for the hydrophilic portions of the surfactant, and L the contribution from the lipophilic portions. Experimentally determined HLB values for several nonionic surfactants are available, as are tabulated data for H and L for various hydrophilic and lipophilic groups (27).

We evaluated the relationships between A_3 activity, efficiency, and selectivity and the HLB values for the surfactants used in this study (Fig. 8). Strikingly, we find that an apparent maximum value for A_3 activity occurs near an HLB value of 15. Surfactants with HLB values higher or lower than 15 supported lower levels of A_3 activity. Note that the surfactants used in identifying the HLB optima for A_3 represent a subset of the overall group of surfactants examined (Table 1), because the necessary information used to estimate HLB values (Eq. 8) was unavailable in several cases, such as the CYMAL, HEGA, and Fos-choline surfactants. Because activity correlates well with selectivity, we observe a similar profile for selectivity of A_3 extraction; optimal selectivity also occurs for surfactants with HLB values around 15 (not shown). For efficiency of A_3 extraction, the HLB profile is similar but broader, with the optimum efficiency between HLB values of 13 and 16 (not shown). These exciting results suggest that HLB can be a useful parameter in guiding selection of optimal surfactants for integral membrane protein purification.

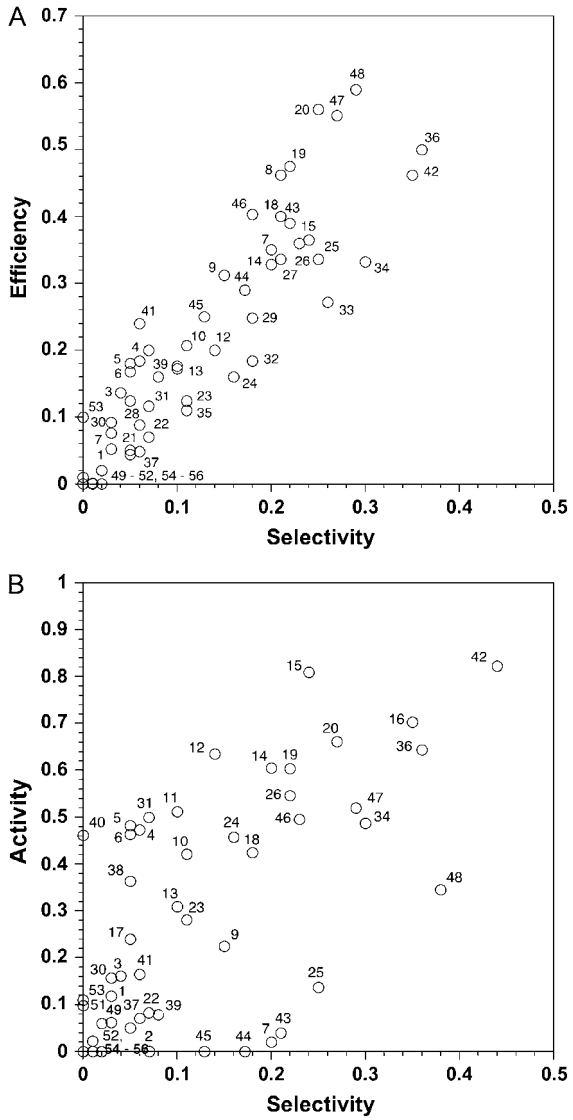

FIGURE 6 (*A*) Correlation between efficiency and selectivity for all surfactants. Numbers refer to individual surfactants listed in Table 1. Efficiency is defined in Eq. 5 and selectivity in Eq. 6. (*B*) Correlation between activity and selectivity for all surfactants. Numbers identify the surfactants (Table 1). Selectivity is defined in Eq. 6 and activity in Eq. 7.

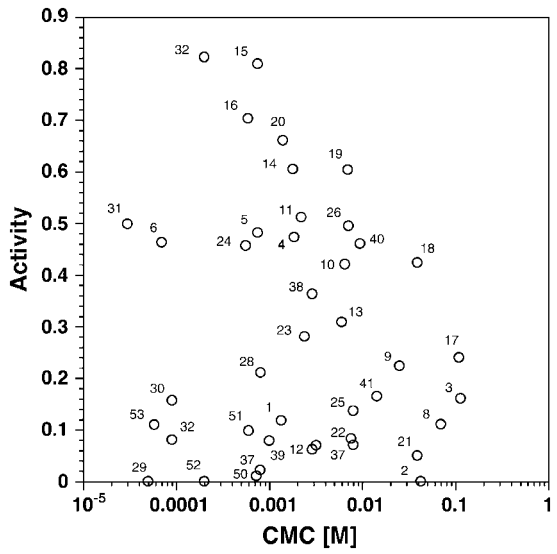

FIGURE 7 Comparison of CMC values and relative activity for all surfactants. Numbers identify the surfactants (Table 1). Activity is defined in Eq. 7.

DISCUSSION

HLB and surfactant structure

The observation that A_3 activity and selectivity are optimized in a window of HLB values offers promise for understanding membrane protein-surfactant interactions, and for the development of systematic approaches to surfactant selection. Previous observations have also suggested that HLB may be a useful parameter in membrane protein extraction (9,39–44). The HLB optimum for A_3 falls within a similar range of 12–15 found for previous studies of membrane protein purification from prokaryotic and eukaryotic sources. Specifically, D-alanine carboxypeptidase from *Bacillus subtilis* was extracted at an HLB number of ~12.8; maximal activity also occurred near this value with Triton X-114, Triton N-101, or Brij 56 (43). Similar values were also found for the mitochondrial porin from bovine heart with Triton X-114, octyl-β-D-glucoside (OG), or lauryl dimethylamine oxide. It is noteworthy that such different membrane compositions, protein types, and experimental methods all lead to similar trends with HLB, especially when considering that the current results include a much wider range of surfactant types such as alkyl polyglucosides, glucamides,

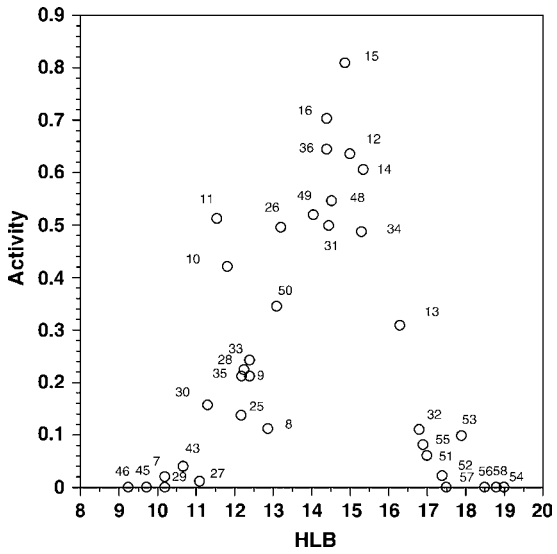

FIGURE 8 Comparison of HLB values and relative activity for all surfactants. Numbers identify the surfactants (Table 1). Activity is defined in Eq. 7 and HLB in Eq. 8.

and polyoxyethylene ethers. Furthermore, the apparent correlation with activity and selectivity is also conserved, suggesting that promoting solubility of a given membrane protein may be a generally effective strategy in maintaining their activity, because both of these are a direct result of the properties of the surfactant used. Interestingly, the HLB optimum for A_3 is shifted relative to that of either mitochondrial porin or D-alanine carboxypeptidase; A_3 favors more hydrophilic nonionic surfactants such as DM, DDM, or HEGA10 rather than Triton X-100 or OG. Understanding the relationship between the HLB optimum and the properties of the protein and its native membrane is the subject of our current investigations.

We sought to interpret this apparent trend in terms of micelle structure and its relationship to membrane protein solubilization. For nonionic emulsions at room temperature, the HLB scale reflects the relative packing at the oil-water interface, which in turn dictates its ability to disperse oil droplets in water effectively (35,45). Therefore, it is not altogether unexpected that HLB should correlate with the preference for a surfactant to assume a particular geometry at a hydrophilic-lipophilic interface. Such suggestions have been made based on estimates of effective chain or headgroup size, though for a limited range of surfactant types, and in general are difficult to extend to more complex structures such as the alkyl polyglucosides (46).

One parameter that has been successfully used to categorize surfactant self-assembly and resultant phase structure for various systems is the molecular packing parameter (47):

$$P = \frac{v}{al}, \tag{9}$$

where v is the volume of the chain, l the length of the chain, and a the cross-sectional area of the headgroup. Cross-sectional headgroup areas were determined from CMC measurements as discussed in Materials and Methods. For single-chain hydrocarbon tails, one can estimate the length of the chain as well as its volume from Israelachvili (48):

$$v = 27.4 + 26.9n \tag{10}$$

$$l = 1.5 + 1.255n, \tag{11}$$

where l is the length of the chain in Å, v the volume of the chain in Å^3, and n the number of hydrocarbon units in the chain. Equations 10 and 11 are valid under the assumption that the chain is at maximal extension for which it remains fluid; this is different from the maximal extension the chain can assume, but is of similar magnitude. However, the properties of the chain are not greatly influenced by the type of headgroup for a given surfactant, and thus these correlations hold reasonably well for a wide range of ionic, zwitterionic, and nonionic surfactants (34). Packing parameter values can be used to estimate the aggregate shape based on the geometry of the monomer, such as spherical (0–1/3), cylindrical (1/3–1/2), and lamellar (1/2–1) (48).

Comparing results for the alkyl polyglucoside and polyoxyethylene surfactants, we find that a linear correlation exists between the HLB and packing parameter with a correlation coefficient >0.85 (Fig. 9). In other words, a decreasing HLB value leads to a more hydrophobic surfactant and thus a preference for assuming a cylindrical or lamellar structure. This effect is similar to the behavior of double-chain surfactants such as lipids, where hydrophobic interactions between the chains are dominant. Furthermore, the different slopes for the alkyl polyglucosides and polyoxyethylene families are not unexpected, because glucose is much larger than an ethylene oxide headgroup, and would therefore tend to influence the packing much more strongly. This difference may explain the shallow slope for the alkyl polyglucoside relative to the polyoxyethylene surfactants; the dominant contribution of the glucose headgroup is evident in the narrow range of packing parameters.

Comparing the activity of A_3 with packing parameter, we find a similar optimum as we observed for HLB with a maximum near packing parameter values of 0.39 (Fig. 10), which corresponds to a cylindrical micelle for surfactants such as unidecyl-β-D-maltoside or $C_{12}E_{10}$. Shorter chain surfactants common for membrane protein studies, such as OG or Triton X-100, have higher packing parameter values near the limit of 1/2 for cylindrical micelles; these surfactants lead to lower A_3 activity. Likewise, the longer chain Brij surfactants show lower activity but are relatively efficient at removing A_3 from the membrane. These surfactants have packing parameters near 0.33, which is the upper limit for spherical micelles. Therefore, it might be the case that the

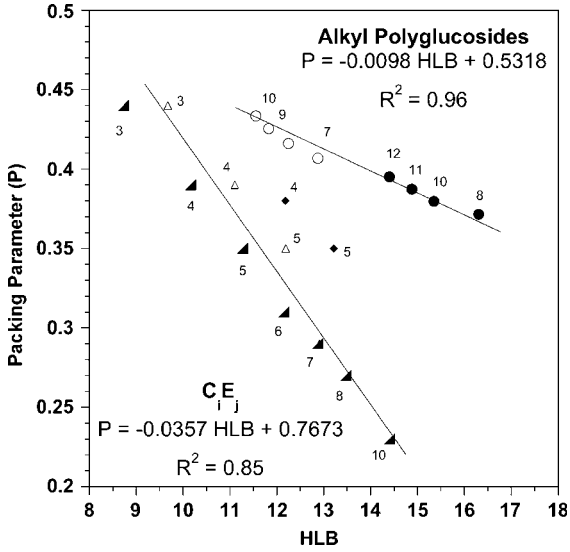

FIGURE 9 Correlation between HLB and packing parameter for alkyl glucoside (○, n-alkyl-β-D-glucosides; ●, n-alkyl-β-D-maltosides) and C_iE_j (▲, $C_{12}E_j$; △, $C_{10}E_j$; ♦, C_8E_j) surfactants. Numbers refer to the number of ethoxylate groups (j) for polyoxyethylene surfactants and chain length (n) for alkyl glucosides. Lines represent best fits to the data. Packing parameter is defined in Eq. 9.

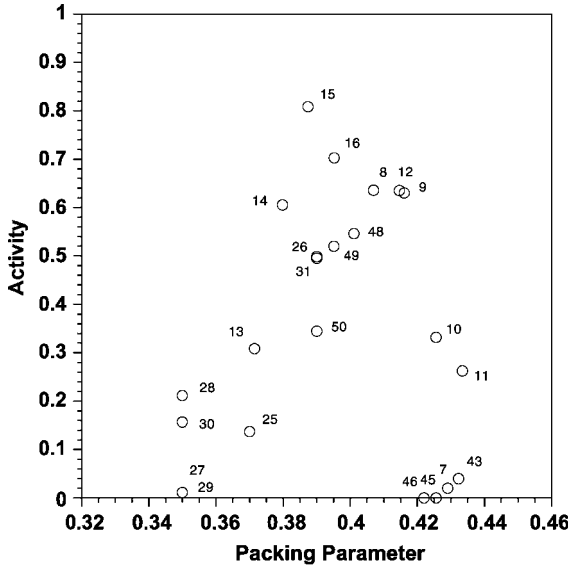

FIGURE 10 Comparison between activity and packing parameter for alkyl polyglucoside and C_iE_j surfactants. Numbers identify the surfactants (Table 2). Activity is defined in Eq. 7 and packing parameter in Eq. 9.

relative effectiveness of the long-chain maltosides (such as unidecyl-β-D-maltoside) and polyoxyethylene surfactants (such as $C_{12}E_{10}$, with larger proportions of ethylene oxide subunits in their headgroup) arises from the fact that they can form cylindrical micelles. This property could be favorable for packing around the hydrophobic regions for less spherical membrane proteins such as A_3. Thus, the optimum HLB values observed for various membrane proteins may in effect be a consequence of such geometric constraints.

Purification and surfactant properties

When designing a purification strategy for a given membrane protein, accounting for the effects of detergents on activity and recovery is a major challenge. An important result of the studies presented here is the strong positive correlation between efficiency and selectivity (Fig. 6 *A*) and between selectivity and activity (Fig. 6 *B*). Thus, detergents that are optimal for isolation of the protein also maintain its activity (and presumably its structure). This finding simplifies the choice of detergents.

The correlations observed involving efficiency and selectivity may be related to lipid concentration and specificity during surfactant solubilization. Lipids are known to play a major role in membrane protein stability, function, and structure determination, especially after solubilization with a given surfactant (49–53). We are currently investigating the effects of detergent/lipid ratio, and the extent to which it influences properties such as efficiency and selectivity, as well as membrane protein activity.

Use of ligands to enhance recovery

In certain cases, it may be necessary or advantageous to use a particular surfactant even though it partially inactivates the receptor. This loss of activity may be due to incompatibilities with materials used in purification or reagents used in activity assays. We find that some of the effects of various surfactants can be offset by the use of a ligand specific to the receptor of interest. For A_3, the improvement in recovery varied considerably across the different surfactant families studied. Surfactants such as CHAPS or sodium cholate, which tended to inactivate A_3, showed the greatest improvement with addition of NECA. However, the maximal recovery obtained in these cases was still considerably less than that of more effective surfactants such as digitonin or DDM. Furthermore, recoveries using digitonin and DDM did not improve with added NECA. Therefore, it is likely that for A_3 the surfactant provides the primary influence on recovery, whereas ligands play a lesser role in cases where the surfactant is less effective.

Implications

Characterizing the recovery and activity of A_3 in various nonionic surfactants during purification allowed us to identify relationships among efficiency, selectivity, and receptor activity. These relationships will allow one to choose surfactants that are efficient in removing A_3 from the membrane, with the likelihood that they will selectively remove A_3 relative to other membrane proteins and maintain receptor activity. In cases where a less suitable surfactant must be used, addition of specific agonists can also assist in enhancing the recovery of A_3. Likewise, surfactants such as digitonin, which are unsuitable for crystallography or spectroscopy due to their heterogeneous nature, can be replaced with other surfactants that maintain selectivity or activity during purification. Overall, these results allow for flexibility in choosing particular surfactants as well as a means to enhance the purity of the initial extract, both of which are important when working with receptors that express at low levels.

Strikingly, the activity and solubility of A_3 are optimized over a fairly narrow window of surfactant HLB values. Similar results have been observed previously for eukaryotic and prokaryotic membrane proteins, although the range of surfactant types tested in those studies was limited to mainly polyoxyethylene surfactants. Nevertheless, the relationship between HLB and membrane protein activity may be a general feature of surfactant-solubilized membrane proteins. If so, identification of optimal surfactants could be simplified to an initial screen of a set of surfactants that span a wide range of HLB values, perhaps followed by a more focused screen around a narrow HLB range.

The maximal HLB value of 15 for A_3 is somewhat higher than reported for other membrane proteins, which may

reflect differences in the relative hydrophobicity or structure of A_3. Although HLB is an empirical parameter, comparing such values against the molecular packing parameter for a given surfactant may suggest a reason that certain HLB values are favored. Surfactants that support activity, such as unidecyl-β-D-maltoside, have packing parameter values that correspond to a cylindrical micellar shape, whereas other surfactants that cause much higher levels of inactivation tend to favor more spherical or lamellar geometries. Therefore, the existence of an HLB optimum for A_3 solubility and activity probably reflects the ability of the surfactants to pack effectively around the hydrophobic transmembrane region of the protein. It will be interesting to determine whether these findings can be generalized to other integral membrane proteins, and which specific structural features of membrane proteins influence the optimal HLB values. The ability to predict which surfactants will be most useful for a given membrane protein will be a valuable tool in the isolation and characterization of these important and challenging proteins.

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