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PRESS

Journal of Structural Biology 137 (2002) 322–332

Journal of
**Structural
Biology**

www.academicpress.com

Importance of detergent and phospholipid in the crystallization of the human erythrocyte anion-exchanger membrane domain

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Received 25 January 2002; and in revised form 28 March 2002

Abstract

Three-dimensional crystals were obtained for the membrane domain of the human erythrocyte anion exchanger (AE1, Band 3). Protein homogeneity and stability and the delicate balance between the detergent used and the amount of phospholipids copurifying are critical to the formation of three-dimensional crystals of the AE1 membrane domain. While deglycosylation improved the protein homogeneity, its stability was significantly increased by inhibitor binding. Size-exclusion chromatography showed that the protein was monodisperse in detergents with acyl chains of 10–12 carbons over a pH range of 5.5–10.0. This pH range and the detergents that retained the protein's monodispersity were used for crystallization screening. Crystals were obtained with the protein purified in C₁₂E₈, dodecylmaltoside, decylthiomaltoside, and cyclohexyl-hexylmaltoside. Five to 13 lipid molecules per protein were required for the protein crystal formation. Those crystals grown in dodecylmaltoside diffracted X-rays to 14 Å. With these factors taken into consideration, ways to further improve the crystal quality are suggested. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Anion exchanger 1; Band 3; Membrane protein; Crystallization; Detergent; Lipid

1. Introduction

The human erythrocyte anion exchanger (AE1),¹ also known as Band 3, is a 95-kDa integral membrane protein with multiple functions (Jay and Cantley, 1986; Jennings, 1989; Passow, 1986; Reithmeier, 1994; Tanner, 1993; Wang, 1994). AE1 links the red cell membrane to the cytoskeleton. It also responsible for the electro-neutral exchange of HCO₃⁻ and Cl⁻ across the erythrocyte membrane, thereby facilitating CO₂ transport by the blood. In addition, the protein acts as a senescent agent assisting in clearance of aged red cells from the blood. Defects in the AE1 gene/protein result in inherited red cell abnormalities, including the common condition Hereditary Spherocytosis (HS), the less common acanthocytosis, and Southeast Asian Ovalocytosis (SAO). A large number of HS-associated mutations have been reported, including missense, nonsense, duplication, insertion, deletion, and RNA processing mutations (Gallagher and Forget, 1997). These mutations

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¹ **Abbreviations used:** 2D, two-dimensional; 3D, three-dimensional; AE1, erythrocyte anion exchanger; AE1MD, erythrocyte anion-exchanger membrane domain; BCA, bichinchonic acid; C₈E₅, pentyloxyethylene octylether; C₁₀E₆, hexyloxyethylene decylether; C₁₂E₈, octyloxyethylene dodecylether; CMC, critical micellar concentration; Cymal-3, cyclohexyl-propylmaltoside; Cymal-4, cyclohexyl-butylmaltoside; Cymal-5, cyclohexyl-pentylmaltoside; Cymal-6, cyclohexyl-hexylmaltoside; DDM, dodecylmaltoside; DHPC, diheptanoyl phosphatidylcholine; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DM, decylmaltoside; DTM, decylthiomaltoside; EDTA, ethylenediamine tetraacetic acid; H₂DIDS, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid; HPLC, high-performance liquid chromatography; MME, monomethyl ether; MO, monooleoyl glycerol; MP, monopalmitoyl glycerol; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PI, phosphoinositide; PS, phosphatidylserine; PMSF, phenyl methyl sulfonyl fluoride; PNGase F, peptide:N-glycosidase F; SDS, sodium dodecyl sulfate; SE, size-exclusion; SM, sphingomyelin; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; UDM, undecylmaltoside.

occur throughout the AE1 molecule in both the cytoplasmic and the membrane-spanning regions. Homozygous mutations in AE1 may be lethal (De Franceschi et al., 1997). The mutation responsible for acanthrocytosis is found to be Pro868 → Leu (Bruce et al., 1993), and in SAO a deletion occurs in amino acids 400–408 (Jarolim et al., 1991). Both mutations take place in the membrane-spanning region of the protein.

The AE1 protein consists of two structurally distinct domains, an amino-terminal cytosolic domain (residues 1–360) and a carboxyl-terminal membrane domain (residues 361–911) (Jennings, 1989; Low, 1986; Tanner, 1993; Wang, 1994). The two domains can be generated by trypsin cleavage at residue Lys360. The cytosolic N-terminal domain has a molecular weight of approximately 43 kDa. Proteins involved in CO₂ transport such as hemoglobin, as well as cytoskeletal proteins, bind to this cytoplasmic region of AE1 (Low, 1986). Separated from the cytoplasmic domain by a flexible hinge region (Wang, 1994) is the 52-kDa membrane domain of the AE1 protein (AE1MD). The membrane domain is solely responsible for the protein's ion transport activity and retains this functionality following enzymatic removal of the cytosolic domain (Casey and Reithmeier, 1991). At 37 °C, the protein exchanges monovalent Cl⁻ anions for HCO₃⁻ across the membrane at a rate of about 5 × 10⁴ ions per molecule per second. Due to such a high flux rate, other ions, e.g., iodide, thiocyanate, and phosphate, are also transported by AE1, though at slower rates (Jennings, 1989).

Its natural abundance has allowed AE1 to be extensively characterized, both biochemically and functionally. The association behaviors of the entire 95-kDa AE1 protein in detergent solution have been studied, and it can exist as a monomer, dimer, tetramer or in aggregated forms in solution, depending on the detergent present (Casey and Reithmeier, 1991; Schubert, 1988; Vince et al., 1997). The protein is heterogeneously glycosylated at a single asparagine residue, Asn642 (Fukuda et al., 1984). Treatment with peptide:N-glycosidase F (PNGase F) removes the polysaccharide at the peptide-sugar bond (Casey et al., 1992). AE1's transport activity can be inhibited by a variety of stilbene disulphonates through covalent or noncovalent interactions (Landolt-Marticorena et al., 1995). Two of them, DIDS and H₂DIDS, inhibit ion transport of AE1 by cross-linking Lys539 and 851 on the same AE1 molecule in a pH-dependent manner (Jennings and Passow, 1979; Okubo et al., 1994). Such inhibitor binding stabilizes AE1, resulting in a 10 °C increase in the denaturing temperature of the protein (Taylor et al., 1999; Van Dort et al., 1994). A ping-pong mechanism model has been suggested for the AE1 exchange function (Jay and Cantley, 1986). The model predicts that the transporter protein exists as two major conformations. DIDS preferentially binds to AE1 from the extracellular side of the

membrane, and AE1-DIDS complex has been shown to adopt its outward-facing conformation (Jennings et al., 1998). Following the addition of DIDS, a small conformational change has been detected in AE1 using tryptophan fluorescence energy transfer (Jay and Cantley, 1986).

Structural information will facilitate our understanding of AE1's anion transport mechanism. Two-dimensional (2D) crystals of the entire AE1 were reported (Dolder et al., 1993). The structure of the cytoplasmic N-terminal 43-kDa fragment has been determined at 2.6 Å from three-dimensional crystals by X-ray crystallography (Zhang et al., 2000). Furthermore, the membrane domain (Wang et al., 1993) was crystallized in two-dimensions by reconstituting the protein into lipid bilayers. Subsequently structure determination by electron microscopy at 20 Å resolution depicts a AE1MD dimer with a transmembrane pore at the monomer-monomer interface and a large cytoplasmic protrusion (Wang et al., 1994). Although the structure revealed the general architecture of the transporter, its structural details remain unknown.

In this work, we present an optimized purification method for the human AE1 membrane domain suitable for three-dimensional crystallization studies. Factors critical for 3D crystal formation of the protein have been identified, and crystals diffracting to 14 Å are reported.

2. Materials and methods

Materials. Detergents were purchased from Anatrace (Maumee, OH), synthetic lipids from Avanti Polar Lipids (Alabaster, AL), PNGase F from New England BioLabs (Beverly, MA), PEG from Fluka (Buchs, Switzerland), and chromatography columns from Amersham Pharmacia (Piscataway, NJ). All other reagents were from Sigma (St. Louis, MO) and were of analytical grade or higher.

Preparation of erythrocyte membrane. Human erythrocyte "ghost" membrane was prepared from packed outdated red cells (Tisch Hospital Bloodbank, NYU Medical Center) or freshly donated human blood. Cells were either directly lysed in hemolysis buffer (0.1 M phosphate buffer, pH 7.4, 0.5 mM EDTA, 1 mM PMSF) or incubated with DIDS prior to cell lysis (Landolt-Marticorena et al., 1995). Following lysis, cells were continuously washed in hemolysis buffer and centrifuged at 9000g for 15 min at 4 °C. This was repeated approximately 10 times until membrane appeared white. The membrane was then stripped of cytoskeletal and peripheral membrane proteins with 10 vol of stripping buffer (15 mM NaOH, pH 12, 2 mM EDTA, and 0.2 mM PMSF), followed by centrifugation at 16000g for 20 min (Casey et al., 1989). This stripping process

was repeated once more and aliquoted membrane was stored at -20°C . To generate AE1 membrane domain, ghost membrane was diluted twofold with hemolysis buffer lacking PMSF. Trypsin (Worthington Chemicals, Toronto, Canada) was then added at a concentration of $5\ \mu\text{g}/\text{ml}$ of diluted ghost membrane. The ghosts were stirred at 4°C for 1 h, followed by the addition of PMSF to a final concentration of 2 mM to stop the trypsin digestion. The ghost membrane was centrifuged at $35\ 000g$ for 15 min, washed with 10 vol of hemolysis buffer, and centrifuged again.

AE1MD purification. The AE1 membrane domain was purified using a modified protocol according to Casey et al. (1989). Stripped and trypsinized ghost membrane was solubilized by the addition of C_{12}E_8 or dodecylmaltoside (DDM) to a final concentration of 1%. Solubilized membrane was loaded onto a 5-ml HiTrap Q anion chromatography column on FPLC (Amersham Pharmacia), preequilibrated with loading buffer (20 mM imidazole, pH 7.0, 1 mM EDTA, 0.5 mM PMSF, 1 mM NaN_3 , 10% glycerol, 0.1% DDM, or C_{12}E_8), at a rate of 0.5 ml/min. After washing with 15 ml loading buffer, the column was eluted with a 15 ml linear 0–1 M NaCl gradient at 0.25 ml/min.

AE1MD eluted from the first Q column was deglycosylated with 5 U/mg PNGase F at 20°C overnight (Casey et al., 1992). Completeness of the deglycosylation was analyzed by Coomassie-stained SDS-PAGE. Following dialysis against the column buffer containing 50 mM NaCl, the deglycosylated protein was loaded onto a 1-ml HiTrap Q anion-exchange chromatography column, in the same loading buffer used for the first column, to further purify the protein and to exchange detergent. The detergent used for the second Q column was DDM (0.1%), UDM (0.1%), DM (0.1%), DTM (0.1%), Cymal-6 (0.25%), Cymal-5 (0.5%), C_{12}E_8 (0.1%), C_{10}E_6 (0.1%), or C_8E_5 (0.7%). Protein was then eluted from the column with a steep NaCl gradient (0–2 M) to ensure high concentration fractions. Fractions (0.5 ml) with UV absorption above 0.5 were collected for further experiments. Protein concentration was measured using the Micro-BCA assay (Pierce Chemical Co., Rockford, IL).

Determination of AE1MD monodispersity and stability. Monodispersity of purified AE1MD protein in various detergent solutions and at different pH ranges, with and without DIDS bound, was measured by analytical size-exclusion chromatography (Boulter and Wang, 2001; Casey and Reithmeier, 1993; Harlan et al., 1995). Protein monodispersity under different conditions was monitored and compared to control samples. The following parameters were screened. (A) DIDS binding: Monodispersity and stability of purified AE1MD samples of $50\ \mu\text{g}$, with or without DIDS bound, were compared. (B) Detergent: Purified AE1MD in 0.1% C_{12}E_8 was incubated at 37°C for 1 h in the presence of a

second detergent at a concentration 0.1–0.2% above its critical-micellar concentration (CMC). More than 20 detergents with various head groups and acyl chain lengths were tested. (C) pH: $50\ \mu\text{g}$ samples of purified AE1MD (10 mM imidazole, pH 7.0) were titrated to the relevant pH by addition of $5\ \mu\text{l}$ of 1 M buffer of the desired pH and then incubated at 25°C for 30 min. The following buffers were used: pH 4.5, acetate/acetic acid; pH 5.5, acetate/acetic acid; pH 6.0, Bis-Tris/HCl; pH 7.0, Bis-Tris/HCl or phosphate; pH 7.5, Tris/HCl or Hepes; pH 8.5, Taps/HCl; pH 9.0, glycine/HCl; pH 10.0, glycine/HCl.

Samples prepared accordingly were loaded onto a Shodex KW804 analytical size-exclusion column equilibrated with buffer (50 mM Tris, pH 8.0, 200 mM Na_2SO_4 , 0.1% C_{12}E_8 , and 3 mM NaN_3) and developed at 0.5 ml/min. The system was powered with a Waters 600S solvent delivery system controlled by the Millennium software (Waters, Milford, MA). UV absorption spectra at 280 nm were integrated to determine the proportion of AE1MD in monodisperse and aggregated forms. Retention times of a set of soluble proteins with known Stokes radius (R_s) (thyroglobulin, 86 Å; apo-ferritin, 63 Å; catalase, 52 Å, and aldolase, 46 Å) (Le Maire et al., 1986) were used to determine the AE1MD Stokes radius and subsequently its oligomeric state.

Phospholipid analysis. The types of phospholipids copurified with AE1MD in the first and second Q columns were analyzed by two-dimensional thin-layer chromatography (TLC) (Boulter and Wang, 2001; Kates, 1972). Lipid was extracted from the purified AE1MD with 10 vol of chloroform:methanol (3:1) (Folch et al., 1957). This sample was dried to a film under nitrogen gas and then redissolved in 100 μl chloroform:methanol. The dissolved lipid mixture was spotted at the bottom right-hand corner of a $20 \times 20\ \text{cm}$ TLC plate (250 μm , silica gel G, Fisher Scientific, Pittsburgh, PA) and allowed to dry thoroughly. TLC plates were then placed in a sealed glass chromatography tank saturated with solvent vapor, until the solvent migrated to $\sim 1\ \text{cm}$ from the top of the plate. The solvent for the first dimension (basic) was chloroform:methanol:ammonia at 65:25:5, and for the second dimension (acidic) chloroform:acetone:methanol:acetic acid:water at 3:4:1:1:0.5. Plates were dried thoroughly after each development and were finally stained with iodide (Sigma) vapor in the sealed chromatography tank. Phospholipids were identified by their mobility in the two solvents according to Kates (1972), using synthetic phospholipids as standards.

The total amount of phospholipids copurified with AE1MD was determined using samples directly extracted from the purified protein, with a phosphorus assay (Boulter and Wang, 2001; Chen et al., 1956). The amounts of cholesterol copurified with AE1MD was not analyzed.

MALDI-TOF mass spectrometry. The molecular mass and homogeneity of purified AE1 membrane domain were measured by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Cohen and Chait, 1997), in Dr. B. Chait's laboratory at Rockefeller University. To avoid interference caused by detergent or lipid, the sample was extracted using 0.1% TFA (Cadene and Chait, 2000). Internal protein calibrants were used to obtain an accurate molecular weight, and MALDI-TOF data were interpreted using the PAWS program from ProteoMetrics (New York, NY).

Three-dimensional crystallization. FPLC fractions containing AE1MD were combined and concentrated to 5–15 mg/ml using an Ultrafree-0.5 Biomax concentrator with a 10,000 MW cutoff (Millipore, Bedford, MA). Following 30 min dialysis against a buffer (10 mM imidazole, pH 7.0, and 5% glycerol), the concentrated protein was filtered using a 0.2- μ m syringe filter (Millipore). Next, 3D crystallization experiments were carried out in a sitting-drop experimental setup using 24-well crystallization plates (Hampton Research, Laguna Niguel, CA). Concentrated AE1MD sample of 1 μ l was mixed with an equal volume of reservoir solution, placed over 1 ml reservoir solution, and sealed with transparent sealing tape. The reservoir solution contained 10–40% PEG, 0.1–0.4 M NaCl, pH 5–10. PEG200, 400, 550, 2000, 4000, 8000, and PEG5000MME (monomethyl ether) were screened as precipitants. The refinement of crystallization condition was aided by the Additive Screen Kits from Hampton Research.

X-ray diffraction. Crystals of AE1 membrane domain were analyzed using X-ray diffraction techniques at room temperature and under cryo conditions (95 K) at laboratory X-ray source (Rigaku stage with R-axis II image plate detector). The crystal quality was also checked using synchrotron radiation (Beamline X12B) at the National Synchrotron Light Source in the Brookhaven National Laboratory.

3. Results

Optimization of AE1MD purification protocol. AE1 membrane domain was purified from outdated human red blood cells, using a modified version of a published protocol (Casey et al., 1989). Erythrocyte membranes were treated with DIDS, which bound to AE1 covalently and locked the transporter protein into a fixed conformation. Mild trypsinization cleaved off the cytosolic 42-kDa domain from the membrane domain. Repeating the stripping step twice removed a large proportion of glycoporphin A, a single-span membrane protein that otherwise contaminated the preparation. This was particularly important not only because glycoporphin A also bound to the Q anion-

exchange columns in later purification steps, but also because its presence was difficult to detect by Coomassie blue-stained SDS-PAGE. Detergents C₁₂E₈ and dodecylmaltoside both solubilized the stripped red cell membrane completely and effectively extracted AE1MD from the membrane. The first Hi-Trap Q anion-exchange column produced 90% pure AE1 membrane domain, as determined by scanning of the Coomassie blue-stained SDS-PAGE (Fig. 1). Deglycosylation of AE1MD with PNGase F, as shown by the SDS-PAGE, removed the oligosaccharide from the protein completely. The cleaved sugar residues and the glycosidase were subsequently separated from AE1MD with a newly introduced, second Q anion-exchange column. AE1MD eluted from the second Q column as a sharp peak at 0.8 M NaCl. The steep NaCl gradient resulted in concentrated AE1MD elutions at a relatively low (0.1%) detergent concentrations, which was desirable for later crystallization experiments. The final protein purity was close to 95%, at a concentration of 1–2 mg/ml in the peak fractions. The second anion-exchange chromatography column was also used to exchange detergent for crystallization experiments when desired.

Stabilization of dimeric AE1 membrane domain by DIDS binding. For protein crystallization experiments, a basic requirement to the protein sample is for it to stay in a single oligomeric state over a sufficient period of time, often several days to weeks, at 4 or 20 °C (Ferre-D'Amare and Burley, 1994; Garavito et al., 1996;

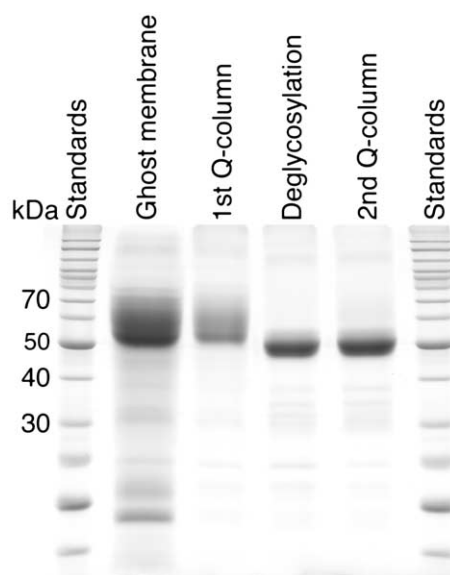


Fig. 1. Coomassie-stained SDS-PAGE showing purification and deglycosylation of human erythrocyte anion-exchanger membrane domain. After the first Q anion-exchange chromatography column as described in Casey et al. (1989), a second Q column was introduced to further purify and delipidate the protein.

Rosenbusch et al., 2001). The association states of the AE1 membrane domain were analyzed based on its Stokes radius under different conditions. Using size-exclusion chromatography, the Stokes radius of AE1MD in C₁₂E₈ was determined to be 66 Å (Fig. 2), in agreement with previous measurements (Casey and Reithmeier, 1991; Salhany et al., 1997). Upon prolonged incubation, the protein either stayed as a dimer or became aggregated, rather than forming other types of oligomers. DIDS binding did not change AE1MD's Stokes radius and, hence, the oligomeric state. Dimeric AE1 membrane domain in complex with DIDS was significantly more stable to incubation at 37 °C than the protein without the inhibitor bound (Table 1). The DIDS-bound dimeric AE1 membrane domain was monodisperse after incubation over a wide pH range (Table 2), from 5.5 to 10.0, providing ample room for screening crystallization conditions.

Mass spectrometry of AE1 membrane domain. The homogeneity of the AE1MD-DIDS preparation was analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Fig. 3). Washing the specimens with 0.1% trifluoroacetic acid (Cadene and Chait, 2000) removed all lipid and detergent copurified with AE1MD, ensuring accurate measurements of the protein molecular mass. Except the two proteins used as internal calibrants, AE1MD was the only protein detected, indicating a highly purified preparation. Two doubly charged peaks were observed around 30 kDa in the spectrum, labeled *a* and *b*. Peak *a*, with mass of 61814.2 Da, corresponded to the membrane domain of the AE1 protein (amino acids 361–911, 61479.9 Da, including 238.2 Da for DIDS). The differ-

ence of 334.3 Da was probably a result of posttranslational modifications typically occurring to AE1MD, such as fatty acylation and methylation. It has been shown previously that AE1 protein is covalently modified by fatty acids at Cys843 at a molar ratio of 1:1 (Okubo et al., 1991). The fatty acids involved in the modification are myristate, palmitate, oleate and stearate, with palmitate being the major component, which adds 238.5 Da to the protein. The remaining 98.5 Da of the 334.3 Da are probably from methylation. Based on this assumption, the high molecular weight species at 63585.2 Da, peak *b*, might represent a larger tryptic fragment, amino acids 346–911 (63222.8 Da + 334.3 Da = 63557.1 Da). The broadness of peak *b* is most likely due to the presence of multiple trypsin cleavage sites, at Arg344, Arg345, and Arg346.

Monodispersity of AE1 membrane domain in different detergents. We surveyed a panel of 20 detergents to assess their ability to preserve the monodispersity of AE1MD dimers (Table 1). The protein was particularly stable in maltoside detergents, including dodecylmaltoside, undecylmaltoside (UDM), decylmaltoside (DM), decylthiomaltoside (DTM), Cymal-6, Cymal-5, and Cymal-4. It also remained monodisperse in long-chain C_mE_n and glucoside detergents, like C₁₀E₆, decylthiogluco- side (DTG), and nonylthiogluco- side (NTG) (Fig. 2). Short-chain detergents, including C₈E₅, octylthiogluco- side (OTG) and heptathiogluco- side (HTG), however, destabilized the protein. Furthermore, lipid-like detergents, diheptanoyl phosphatidylcholine (DHC), mono- oleoyl glycerol (MO), and monopalmitoyl glycerol (MP) maintained the monodispersity of the AE1 membrane domain.

Analysis of phospholipids copurified with AE1MD in various detergents. The phospholipids copurified with the AE1 membrane domain were identified by chloro- form/methanol extraction followed by two-dimensional thin-layer chromatography and quantified using phosphorous assays. Importantly, the amount of phospho- lipid copurifying with the protein depended on the detergent used for solubilization and purification. The total amounts of phospholipids after the first anion exchange column were measured by phosphorous assays to be 26 and 36 mol per mole protein for C₁₂E₈ and DDM, respectively (Table 3). As expected, the second anion exchange column further delipidated AE1MD. Samples purified using this column in the presence of C₁₂E₈, C₁₀E₆ and C₈E₅, respectively, contained 13, 7, and 7 mol phospholipids per mole of AE1MD (Table 3). With acyl chains of 10–12 carbons, C₁₂E₈ and C₁₀E₆ retained slightly more phospholipids than the shorter and harsher C₈E₅ detergent, and they were also able to preserve AE1MD monodispersity. A lipid amount of 5 mol per mole of protein was produced when DDM, another C12 acyl chain detergent, was used in the both anion-exchange chromatography steps. Though sub-

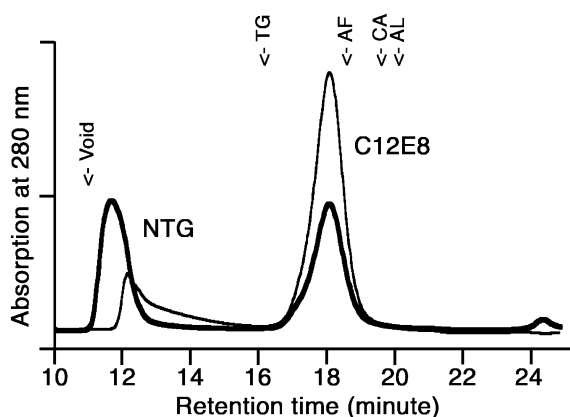


Fig. 2. Size-exclusion HPLC chromatograms of AE1MD in C₁₂E₈ (thin line) and NTG (thick line) solution. The samples were run on a Shodex KW-804 column, equilibrated with 50 mM Tris, pH 8.0, 200 mM Na₂SO₄, 3 mM NaN₃, and 0.1% C₁₂E₈. Peaks were identified by their retention time. AE1MD in complex with detergent had a Stokes radius of 66 Å, corresponding to a protein dimer. Elution times of standard proteins of known Stokes radius are indicated: TG, thyroglobulin (86 Å); AF, apoferritin (63 Å); CA, catalase (52 Å); AL, aldolase (46 Å).

Table 1
Effects of detergents on monodispersity of AE1 membrane domain with or without DIDS bound

Detergent	CMC (%)	Protein without DIDS		Protein with DIDS bound	
		% Detergent	Dimer remaining ^b	% Detergent	% Dimer remaining
C ₁₂ E ₈ ^a	0.0038	0.2	33	0.2	77
C ₁₀ E ₆	0.038	0.1	0	0.2	50
C ₈ E ₅	0.21	—	—	1.5	0
DDM	0.008	0.2	17	0.2	79
UDM	0.029	0.2	24	0.2	77
DM	0.8	0.2	6	0.2	71
DTM	0.045	0.2	12	0.2	70
Cymal-6	0.028	—	—	0.1	78
Cymal-5	0.118	—	—	0.3	76
Cymal-4	0.36	—	—	0.7	50
Cymal-3	1.6	2.6	7	—	—
DTG	0.032	—	—	0.1	73
NTG	0.093	—	—	0.1	50
MEGA-9	0.83	—	5	2.0	17
DHPC	0.067	—	—	0.2	73
MO	—	—	—	0.1	74
MP	—	—	—	0.1	76

^a C₈E₅, pentyloxyethylene octylcylether; C₁₀E₆, hexyloxyethylene decylether; C₁₂E₈, octyloxyethylene dodecylether; Cymal-3, cyclohexyl-propylmaltoside; Cymal-4, cyclohexyl-butylmaltoside; Cymal-5, Cyclohexyl-pentylmaltoside; Cymal-6, cyclohexyl-hexylmaltoside; DDM, dodecylmaltoside; DHPC, diheptanoyl phosphatidylcholine; DM, decylmaltoside; DTM, decylthiomaltoside; MO, monooleoyl glycerol; MP, monopalmitoyl glycerol; UDM, undecylmaltoside.

^b Purified AE1MD in 0.1% C₁₂E₈ was incubated at 37 °C for 1 h in the presence of a second detergent at a concentration 0.1–0.2% above its critical micellar concentration and analyzed by size-exclusion chromatography on HPLC.

Table 2
Effects of pH on monodispersity of AE1 membrane domain with DIDS bound

PH	Buffer ^a	% Dimer remaining ^b
4.5	Acetate	3
5.5	Citrate	72
6.0	Bis-Tris	72
7.0	Imidazole	80
7.0	Phosphate	80
7.5	HEPES	80
7.5	Tris-HCl	82
8.5	TAPS	82
9.0	Glycine	81
10.0	Glycine	81

^a Buffers were prepared at a concentration of 1.0 M.

^b Samples 50 µg of purified AE1MD (10 mM imidazole, pH 7.0) were titrated to the relevant pH by addition of 5 µl of 1 M buffer of the desired pH and then incubated at 25 °C for 30 min, followed by analysis using size-exclusion chromatography on HPLC.

stantially less phospholipid was present, the protein remained monodisperse and stable in DDM, as analyzed by size-exclusion HPLC. In general, detergents with an acyl chains shorter than 10 carbons caused aggregation of the AE1 membrane domain, probably because too much lipid was striped from the protein. Using 2D-TLC, four major types of endogeneous erythrocyte phospholipids were detected in the sample extracted from the AE1MD samples purified in DDM using organic solvent: phosphatidylserine (PS), sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE).

Crystallization of DIDS-bound AE1 membrane domain. DIDS-labeled AE1MD purified from human red cells with two consecutive anion-exchange columns was subjected to sitting-drop vapor diffusion crystallization. Crystal screening was carried out at pH 5–10, using PEG200, 400, 550, 1000, 2000, 4000, 8000, and PEG5000MME as precipitants. The following detergents were used for AE1MD crystallization because of their capability of retaining the protein's monodispersity: DDM, UDM, DM, DTM, Cymal-6, Cymal-5, C₁₂E₈, C₁₀E₆, and C₈E₅. Following detergent exchange on the second chromatography column, crystals of various morphologies were obtained from AE1MD purified in DDM, Cymal-6, DTM, and C₁₂E₈ (Table 4). Crystals had shapes of plates or bars. Thin sheets with 0.1 × 0.1 × 0.01-mm dimensions were obtained in Cymal-6, DTM, or C₁₂E₈. Finally, AE1MD purified using one or three consecutive chromatography Q columns, in either DDM or C₁₂E₈, did not produce any crystals.

We further optimized crystallization conditions by varying PEG, salt, and pH and by including small organic molecules such as dioxane and isopropanol. The best crystals, measuring 0.5 × 0.25 × 0.1 mm, however, were grown with protein purified in DDM at a concentration of 14 mg/ml (Fig. 4). They were obtained with reservoir solution containing 25% PEG400, 0.2 M NaCl, 0.1 M HEPES, pH 7.8, plus 10 mM guanidium hydrochloride, or 10 mM trimethylamine-HCl. Diffraction was observed at 14 Å from crystals mounted at room temperature on our laboratory X-ray source.

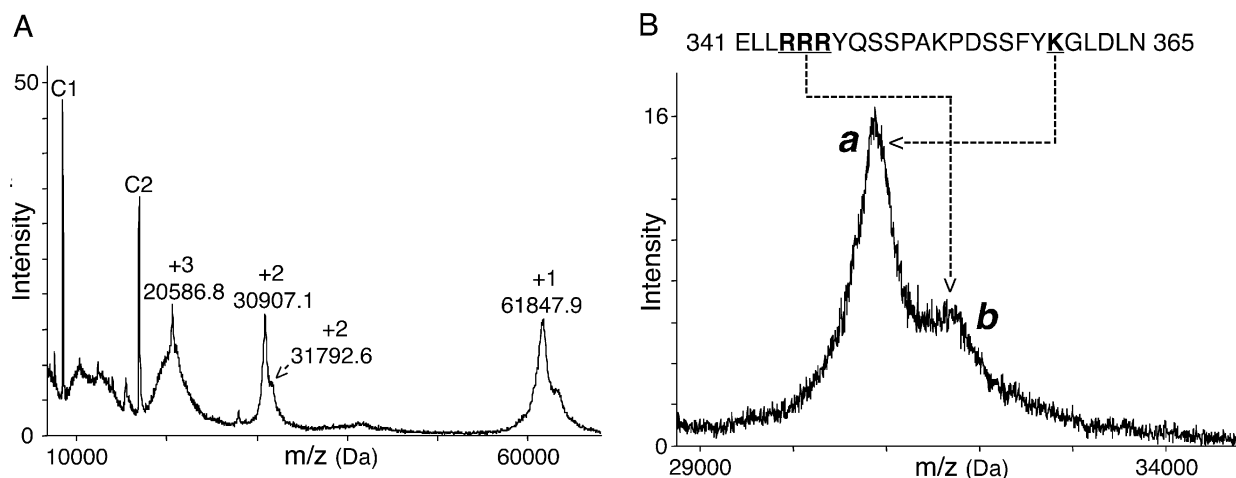


Fig. 3. Mass spectrometry measurement of purified AE1MD. DIDS-bound, deglycosylated protein was subjected to MALDI-TOF mass spectrometry. Detergent and lipid were completely removed by washing with 0.1% trifluoroacetic acid. Two major doubly charged peaks were observed in the 29–34 kDa range, *a* at 61 814.2 Da and *b* at 63 585.2 Da. The peak *a* corresponded to AE1 membrane domain (amino acids 361–911) covalently bound to a DIDS molecule. The broad peak *b* was interpreted as a larger tryptic fragment, amino acids 345–911, resulted from cleavage at multiple sites. (A) The whole spectrum. (B) Details of the doubly charged AE1MD peak, shown together with AE1 protein sequence near the tryptic sites. C1 and C2 were internal calibrants.

Table 3
Endogenous phospholipid copurified with AE1 membrane domain

First anion-exchange column				Second anion-exchange column			
Detergent	Protein monodispersity	P_i /protein	Crystal formation	Detergent	Protein monodispersity	P_i /protein	Crystal formation
C ₁₂ E ₈	Yes	26	No	C ₁₂ E ₈	Yes	13	Yes
C ₁₂ E ₈	—	—	—	C ₁₀ E ₆	Yes	7	No
C ₁₂ E ₈	—	—	—	C ₈ E ₅	No	7	No
DDM	Yes	36	No	DDM	Yes	5	Yes
DDM	—	—	—	Cymal-6	Yes	—	Yes
DDM	—	—	—	DTM	Yes	—	Yes

The crystals diffracted to a similar resolution under cryo conditions at a synchrotron radiation source. The crystal symmetry appeared to be orthorhombic. The diffraction pattern could be indexed as a C-centered orthorhombic lattice, with, $a = 426.35 \text{ \AA}$, $b = 115.14 \text{ \AA}$, and $c = 104.66 \text{ \AA}$, at a distortion index of 0.43%. Its fit to a primitive orthorhombic unit cell ($a = 220.76 \text{ \AA}$, $b = 115.14 \text{ \AA}$, and $c = 104.66 \text{ \AA}$), however, had a distortion of 6.63%. With the current crystal quality, it was not possible to determine the crystal symmetry and unit cell dimensions with more certainty.

4. Discussion

Several factors critical to the formation of three-dimensional crystals of the human erythrocyte anion-exchanger membrane domain have been identified. They include protein homogeneity and stability, detergent type, and amount of phospholipids copurifying. Some of these factors are interrelated; for example, both the

protein monodispersity and the amounts of lipid copurified depend on the detergent used for purification. A delicate balance between the type of the detergent used and the amount of endogenous phospholipids copurifying is required for the formation of three-dimensional crystals of the human erythrocyte anion-exchanger membrane domain.

Deglycosylation enhances the AE1MD's homogeneity and is critical for the formation of 3D crystals of the protein. AE1 from human erythrocyte is heterogeneously glycosylated (Fig. 1), with 3–8 kDa of sugar bound per protein molecule (Fukuda et al., 1984). PNGase F cleaves the oligosaccharide off from the polypeptide at Asn462. Treatment of AE1MD using the glycosidase with 5 U/mg protein at 20 °C overnight removed the oligosaccharide completely (Casey et al., 1992) and markedly improved the homogeneity of the AE1MD sample (Fig. 1). The detached oligosaccharide was separated from AE1MD by the second Q chromatography column. This deglycosylation step is essential for 3D crystallization of the AE1 membrane domain.

Table 4
Nucleation of AE1 membrane domain crystals

PEG ^a	DDM	Cymal-6	DTM	C ₁₂ E ₈
400	28%, pH 7.5	22%, pH 7.5 24%, pH 7.5 26%, pH 7.5	9%, pH 9.0	—
550	—	—	30%, pH 9.0	25%, pH 6.5
1000	20%, pH 9.0	17–19%, pH 8.5 19–20%, pH 9.0 19%, pH 9.5 19%, pH 10.0	—	—
2000	—	—	—	29%, pH 7.0
3350	25%, pH 9.0	—	—	29%, pH 7.0 29%, pH 7.5 29%, pH 8.0 29%, pH 9.0
4000	16%, pH 9.0 28%, pH 9.0	—	—	25%, pH 7.0
5000MME ^b	—	—	—	25%, pH 9.0
8000	—	—	8%, pH 6.0 6%, pH 7.0 15%, pH 7.0 6%, pH 9.0	18%, pH 6.5 30%, pH 7.0 8%, pH 8.5
10 000	—	—	20%, pH 7.5	—

^a PEG, polyethylene glycol.

^b MME, monomethyl ether.

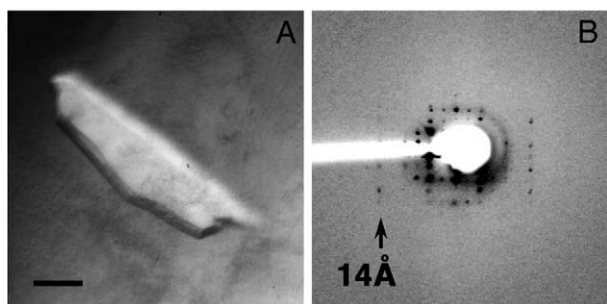


Fig. 4. Crystal of AE1 membrane domain and corresponding X-ray diffraction pattern. (A) Crystal was grown from DIDS-bound, deglycosylated AE1MD purified in DDM. (B) Diffraction pattern recorded from an AE1MD crystal at room temperature on the home X-ray source. A reflection at 14 Å resolution is indicated. The bar represents 100 μm.

This is similar to 2D crystallization of AE1MD for which deglycosylation was also needed (Wang et al., 1993). Likewise, it has been reported that deglycosylation also improved the 3D crystal quality of AQP1, the bovine erythrocyte water channel protein (Sui et al., 2000).

Chemical microheterogeneity still exists in the AE1MD preparation, which we were able to detect only by mass spectrometry. In addition to heterogeneous fatty acylation of the protein (Okubo et al., 1991), the carboxyl groups of a number of aspartate residues on the inner surface of AE1 are methylated (Lou and Clarke, 1986), and such methylation is presumably heterogeneous, since the AE1 membrane domain here is purified from aged human blood. Furthermore, the linker between the cytosolic and the membrane domains

of AE1 is known to be flexible and hence susceptible to proteolysis (Wang, 1994). The existence of multiple tryptic sites in this region therefore also contributes to the heterogeneity of the protein, resulting in broad peaks in the MALDI-TOF spectrum. Such microheterogeneity is in contrast to that of recombinant bacterial membrane transporters of γ -aminobutyric acid (Li et al., 2001) and of glycerol 3-phosphate (Auer et al., 2001).

The conformational heterogeneity of AE1MD is greatly reduced by DIDS binding. As an ion transporter, AE1 protein adopts multiple conformations in detergent solution, and DIDS binding locks the protein in its inward-facing state. Such a uniform conformation of AE1MD-DIDS complex clearly favors crystallization, since we were not able to grow 3D crystals of the protein without DIDS bound. In addition, the binding DIDS significantly increased the stability of the dimeric AE1 membrane domain. This is in agreement with previous studies that DIDS binding was found to increase the denaturing temperature of the AE1 membrane domain (Davio and Low, 1982). The use of appropriate pH range also improves AE1MD's stability, and therefore facilitates the formation of 3D crystals of the protein.

Besides protein homogeneity and stability, the choice of detergent is critical for the crystallization of the AE1 membrane domain. Detergent is important for membrane protein crystallization for three reasons: (i) only those detergents that preserve the protein monodispersity and stability are likely to allow the protein to crystallize (Garavito et al., 1996; Rosenbusch et al., 2001); (ii) the size of the detergent micelles needs to be proper for the protein molecules to make lattice contacts (Michel,

1991); (iii) The detergent used for purification partially determines the amounts of lipids copurifying with the protein, which in turn decides the crystallizability of the protein. AE1MD-DIDS complex showed monodispersity in a number of non-ionic detergents with acyl chains of 10 carbons or longer. Detergents of short carbon chains, however, were unable to preserve AE1MD monodispersity in solution. This agrees with the AE1 stability measured by differential scanning calorimetry where detergents with a relatively long acyl chain (e.g., Triton X-100 and C₁₂E₈) are required for optimal thermal stability of AE1 (Sami et al., 1992) and previous results using OG (Werner and Reithmeier, 1985). Perhaps not unexpected, the association behavior of AE1 membrane domain in detergent resembles that of the entire AE1 (Casey and Reithmeier, 1993; Vince et al., 1997), only with better monodispersity and stability observed.

The amount of phospholipids is also critical for AE1MD crystallization. AE1MD purified with either one or three consecutive chromatography columns, in either DDM or C₁₂E₈, did not produce crystals. Only the protein samples subjected to two successive chromatography steps formed crystals. The use of two consecutive chromatography columns resulted in a total of 13, 7, and 5 mol copurified phospholipids per mole of AE1MD in the presence of C₁₂E₈, C₁₀E₆ and DDM, respectively. Further delipidation of AE1MD with a third chromatography column in any of the detergents above resulted in protein aggregation. This suggests that 5–13 erythrocyte phospholipid molecules per AE1MD are required for its stability and crystallization. With such amount of lipids, however, only C₁₂E₈ DDM, Cymal-6, and DDM, but not C₁₀E₆, or C₈E₅, yielded crystals, even though the amounts of phospholipids copurified in these detergents were similar and C₁₀E₆ was also able to preserve AE1MD's monodispersity. C₈E₅, though, retained amounts of phospholipids comparable to those of C₁₀E₆ and DDM and did not allow AE1MD to remain monodisperse and stable. A suitable detergent, in the presence of 5–10 phospholipids per protein molecule, is therefore sufficient and necessary to maintain AE1MD monodisperse and stable. Readdition of phospholipid can restore the stability of AE1 (Schopfer and Salhany, 1992; Vince et al., 1997). The right type of detergent stabilizes the protein and provides the appropriate matrix needed to make protein-protein contacts in the crystal. The most noticeable detergent being DDM, which delipidated AE1MD to a greater level than C_mE_n detergents, also produced the best diffracting crystals. Other membrane protein crystals have been generated using DDM. While it is often thought as a mild detergent, we have shown that it does play a key role in delipidation. We therefore conclude that a delicate balance between the detergent type used for purification and the amount of endogenous phospholipids copurifying is required for the formation of

three-dimensional crystals of the human erythrocyte anion-exchanger membrane domain.

It is yet to be determined if a particular type of phospholipid is essential for 3D AE1MD crystal formation. Human erythrocyte membrane contains endogenous phospholipids PC, PE, PS, and SM at 33%, 30%, 13%, and 24%, respectively, in dry weight (Devaux and Seigneuret, 1985; Manku et al., 1983). DDM delipidated the AE1 membrane domain in the proportions to their native contents. Thus, the type and amount of copurifying lipid depend on both the detergent used and the protein itself. Phospholipid preserves the integrity of membrane proteins and therefore helps with crystallizing membrane proteins. This observation agrees with previous studies on AE1MD stability by differential scanning calorimetry. The denaturing temperature of AE1 membrane domain was increased by 19 degrees when long-chain PC molecules were present (Maneri and Low, 1988). Five phospholipid molecules were needed to preserve the protein's integrity, and their removal resulted in aggregation of the protein (Maneri and Low, 1989).

Whereas the detergent's importance was recognized right from the start of membrane protein crystallography (Garavito and Rosenbusch, 1980; Michel, 1982), the significance of lipid for the crystallization of membrane proteins was only realized much later. Indeed, a specific galactolipid was needed for 3D crystal formation of the plant light-harvesting complex (Nussberger et al., 1993). The well-diffracting crystals of cytochrome *b_c1* complex from bovine heart were produced in the presence of 0.1 mg of phospholipid per milligram of protein (Yu et al., 1996). Similarly, lipid was added to the crystallization drops of Ca²⁺-ATPase from skeletal muscle sarcoplasmic reticulum, whose structure was recently solved at 2.6 Å resolution (Toyoshima et al., 2000). Here we have shown the necessity of phospholipids for the crystallization of human erythrocyte anion-exchanger membrane domain. Similar requirements will likely be needed for 3D crystallization of other eukaryotic, particularly mammalian, membrane proteins.

We are still faced with the major task of improving the resolution of the AE1 crystals to a level to allow structure determination by X-ray analysis. One critical step requires further increase of the protein's homogeneity. Despite improvements made in purification and characterization of the human AE1 membrane domain, microheterogeneity due to proteolysis, methylation, and fatty acylation still exists in the AE1MD preparation, which we are able to detect only by mass spectrometry. Therefore, using recombinant AE1MD protein is clearly the method of choice to further improve its crystal quality. Overexpression of mammalian membrane proteins for structural studies remains a challenge (Grisshammer and Tate, 1995), but progress has been made with the AE1 protein. The entire AE1 has been

expressed in the yeast *Saccharomyces cerevisiae* at a level 0.7 mg AE1 per liter culture (Sekler et al., 1995). The expressed protein was purified and shown to be active following reconstitution into proteoliposomes. Similarly, the 95-kDa AE1 has been expressed in Sf9 cells using a recombinant baculovirus system, at a density of 0.5×10^6 copies/cell. A similar expression system for the AE1 membrane domain has been developed in *S. cerevisiae*, and at least some of the overexpressed protein was functional (Groves et al., 1996). Such an expression system with higher yields will be ideal for crystallization of AE1 membrane domain. Another strategy is to crystallize the yeast homologue of AE1MD, which has recently been characterized functionally and biochemically (Zhao and Reithmeier, 2001).

The conditions for growing crystals of the erythrocyte anion-exchanger membrane domain reported can be used as a starting point for searching for crystallization conditions once an overexpression system becomes available. The detergents and pH range that retain AE1MD monodispersity, for example, can be screened for crystallization. In addition, since the protein is stable in monooleoyl glycerol and monopalmitoyl glycerol, the cubic lipid phase crystallization method can be tested (Landau and Rosenbusch, 1996).

Acknowledgments

We thank Dr. Gene Scarborough for advice on crystallization, Drs. Xiao-Dan Li and Jonathan Boulter for helpful discussions, Dr. Manfred Auer for donating blood, Dr. Martine Cadene for mass spectrometry measurements, and Ms. Heather Griffith for critical reading of the manuscript. The staff of Beamline X12B at the National Synchrotron Light Source in the Brookhaven National Laboratory is acknowledged for their assistance in X-ray diffraction experiments. The research was financially supported by NIH (DK-53973).

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