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Purification of functional human Cl^-/HCO_3^- exchanger, AE1, over-expressed in *Saccharomyces cerevisiae*

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ABSTRACT

There is no high-resolution structure for the membrane domain of the human erythrocyte anion exchanger, AE1 (Band 3). In this report, we have developed an expression and purification strategy for AE1 to be used in crystallization trials. *Saccharomyces cerevisiae* strain BJ5457 was transformed with an expression vector encoding the AE1 membrane domain (AE1MD, amino acids 388–911), fused C-terminally to an epitope tag, corresponding to the nine C-terminal amino acids of rhodopsin. The fusion protein, AE1MD-Rho, was expressed at a concentration of 0.3 mg/l of culture. Confocal immunofluorescence microscopy and sucrose gradient ultracentrifugation revealed that AE1MD-Rho did not process to the plasma membrane of *S. cerevisiae*, but was retained in an intracellular membrane fraction. Treatment with the endoglycosidase, PNGase F, showed that AE1MD-Rho is not N-glycosylated. AE1MD-Rho solubilized from yeast membranes, with Fos-choline detergent, was purified to 93% homogeneity in a single-step, using a 1D4 antibody affinity resin, in amounts up to 2.5 mg from 18 l of culture. The ability of purified AE1MD-Rho to transport sulfate was examined in reconstituted vesicles. The rate of sulfate efflux mediated by vesicles reconstituted with AE1MD-Rho was indistinguishable from vesicles with purified erythrocyte-source AE1. Using this purification strategy, sufficient amounts of functional, homogeneous AE1MD-Rho can be purified to enable crystallization trials.

Introduction

AE1, also known as Band 3 or SLC4A1, belongs to the SLC4A subfamily of electroneutral Cl^-/HCO_3^- exchangers, which includes AE2 and AE3. Anion exchangers catalyze the exchange of Cl^- for HCO_3^- either into or out of the cell, causing cytosolic alkalinization or acidification, respectively [1]. The bicarbonate transport function of anion exchange proteins is required for pH homeostasis, CO_2 metabolism and volume regulation [2]. Members of the anion exchanger family share a common protein structure [3], composed of two major domains: a cytosolic N-terminal domain, and a transmembrane domain. The focus of this study is the approximately 500 residue long transmembrane domain of anion exchangers, which share 80% sequence similarity [4], and contain 12–14 transmembrane segments. The membrane domain of the anion exchangers is alone responsible for the Cl^-/HCO_3^- transport function of the proteins [5].

Two different transcripts arise from the AE1 (SLC4A1) gene: the 911 amino acid full length erythrocyte variant (eAE1)[1] and the kidney variant (kAE1) that lacks the first 65 N-terminal amino acids of the erythrocyte isoform. In type A intercalated cells of the renal collecting duct, kAE1 reabsorbs HCO_3^- into the blood to prevent systemic acidosis [1]. eAE1 maximizes the blood's carrying capacity of HCO_3^- and anchors the biconcave-shaped red blood cell cytoskeleton to the plasma membrane [6]. Mutations in AE1 can cause hereditary spherocytosis, the most common erythrocyte membrane disorder, and distal renal tubular acidosis [1].

Most of the structural information known about the anion exchanger family arose from studies conducted on AE1, due to its abundance in the erythrocyte (approximately one million copies), and ease of AE1 purification [7]. The topology of AE1 [8], possible residues involved in the dimer interface of the protein [9], and the translocation pore of the protein [10,11] have been the subject

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[1] *Abbreviations used:* AE1-Ct, GST and AE1 C-terminus fusion protein; AE1MD, AE1 membrane domain; AE1MD-Rho, AE1 membrane domain and Rho epitope fusion protein; BSA, bovine serum albumin; $C_{12}E_8$, octaethyleneglycol mono dodecyl ether; DDM, dodecyl maltoside; eAE1, erythrocyte AE1 varient; FC, Fos-choline; IMAC, immobilized metal affinity chromatography; kAE1, kidney AE1 varient; LPC, lysophophatidyl choline; OG, octyl glucoside; Rho, nine C-terminal amino acids of rhodopsin; SDS, sodium dodecyl sulfate.

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of study. The crystal structure of the N-terminal cytoplasmic domain of eAE1 (residues 1–379) has been solved to a resolution of 2.6 Å [12]. Three-dimensional crystals grown from the membrane domain of AE1 isolated from erythrocytes have diffracted X-rays to 14 Å, but a corresponding crystal structure was not determined [13]. Two-dimensional crystals of the eAE1 membrane domain have yielded structures at 20, 18, and 7.5–16 Å resolution [14–17]. Still, none of these structures is of sufficient resolution to determine the helical packing of the entire AE1 membrane domain, the location of amino acid residues, or provides insight into its mechanism of action.

Attempts to obtain a high-resolution structure of the membrane domain of AE1 purified from erythrocytes have been unsuccessful, possibly arising from heterogeneity of the protein caused by various degrees of glycosylation, proteolysis, and other modifications [13]. AE1 has been expressed and partially purified from yeast, using a six histidine purification tag and immobilized metal affinity chromatography (IMAC) [18,19]. The maximum protein purity achieved (35%) was possibly limited by the presence of metal-binding proteins, endogenous to yeast, that co-purified with AE1 during IMAC. To obtain the pure population of AE1 needed for crystallization we have prepared an expression construct corresponding to amino acids 388–911 of AE1 (AE1MD, the membrane domain) with a C-terminal purification tag, corresponding to the nine C-terminal residues of rhodopsin. The fusion protein, called AE1MD-Rho, binds the 1D4 monoclonal antibody that can be immobilized to resin to form an affinity purification matrix. AE1MD-Rho is eluted with a peptide, corresponding to the nine C-terminal residues of rhodopsin. In this report, we have successfully used this purification strategy to produce a highly pure source of AE1MD-Rho, which is structurally and functionally indistinguishable from erythrocyte AE1.

Materials and methods

Materials

DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Pfx DNA polymerase, some restriction enzymes, ProLong® Gold antifade reagent with DAPI, and 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) were purchased from Invitrogen (Carlsbad, CA). T4 DNA ligase, some restriction enzymes, and PNGase F were from New England Biolabs (Ipswich, MA). ECL chemiluminescent reagent and radioactive [^{35}S]Na_2SO_4 were from Perkin–Elmer Life Sciences (Waltham, MA). Anti-AE1 antibody IVF12 was a gift from Dr. Mike Jennings (University of Arkansas) [20]. Anti-Pma1 antibody was a gift from Dr. Gary Eitzen (University of Alberta) [21]. Anti-Sec61 antibody was a gift from Dr. Randy Schekman (Berkeley, University of California) [22]. The anti-rhodopsin antibody, Rho 1D4, was purchased from the University of British Columbia's University–Industry Liaison Office (www.flintbox.com) [23]. Donkey anti-mouse IgG conjugated to horseradish peroxidase and Glutathione Sepharose 4B were from GE Healthcare Bio-Sciences (Piscattaway, NJ). Donkey anti-rabbit IgG conjugated to horseradish peroxidase was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit Alexa 484 antibody and anti-mouse Alexa 544 antibody were from Molecular Probes (Carlsbad, CA). The 1D4 peptide (TETSQVAPA) was synthesized by GenScript (Piscataway, NJ). Fos-choline 14 (FC), *n*-dodecyl-β-D-maltopyranoside (DDM), and *n*-octyl-β-D-glycopyranoside (OG) were from Anatrace (Maumee, OH). Soybean lysophosphatidylcholine (LPC) was from Avanti Polar Lipids (Alabaster, AL). CSM Leu$^-$ amino acids and zymolyase 100T were from MP Biomedical (Solon, OH). Sephadex G-50, Sepharose 2B, egg yolk phosphatidylcholine (EYPC) and octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) were from Sigma–Aldrich (Oakville, ON, Canada). Affi-gel 102 and Bio-beads SM2 were from Biorad (Hercules, CA). Complete Mini Protease Inhibitor Cocktail was from Roche Applied Science (Indianapolis, IN). Outdated red cells were from University of Alberta Blood Bank, and erythrocyte AE1 was purified as described previously [7].

Molecular biology

The yeast expression vector YEpM [24] was a gift from Dr. Theodore Wensel (Baylor College of Medicine). The construct corresponds to the vector called YEpPMA1MDR1 [24], but with the MDR1 sequence (between the BamHI and XhoI sites) replaced by the polylinker sequence, ACTAGTGTTTAAACACGCGT, that contains a 5′ Spe1 site and a 3′ MluI site. The yeast expression plasmid, pPB1, which encodes for the protein AE1MD-Rho, was constructed using pJRC9 [25] as a PCR template for AE1 and YEpM as a vector. The forward primer (5′-GGCCACTAGTTTTAATTATCAAACAATATCAATATG*TCTAGATATCCATATTATTTGTCTGATATT*ACAGATGCATTCAGCCCCCAGG-3′) contains a SpeI restriction site, the upstream sequence of Pma1 needed for expression (underlined), a start codon, codon preferencing in yeast for the sequence of the first 10 amino acids of AE1MD-Rho (italics), and an annealing sequence. The reverse primer (5′-GGGACGCGTTTA*CGCAGGCGCGACTTGGCTGGTCTCTGT*CACAGGCATGGCCACTTCGTCG-3′) contains a MluI restriction site, a stop codon, the sequence for the nine C-terminal amino acids of rhodopsin (italics), and an annealing sequence. The PCR product and YEpM vector were digested with SpeI and MluI, and ligated to create pPB1.

pHJC1 encodes, AE1-Ct, a GST fusion protein containing the last 40 amino acids of AE1 (residues 872–911). The coding sequence for the last 40 amino acids of AE1-Ct was amplified using pJRC9 [25] as a PCR template, and inserted into the GST expression plasmid pGEX-6p-1 (GE Healthcare Life Sciences). The forward primer sequence is 5′-CGCGGATCCGTCCTGCTGCCGCTCATCTTC-3′. The reverse primer sequence is 5′-CGCGGATCCTCACACAGGCATGGCCACTTCGT-3′. The PCR product and pGEX-6p-1 vector were digested with BamHI and ligated together to create pHJC1.

DNA sequences generated by PCR were confirmed by DNA sequencing to ensure no sequence errors were introduced (DNA Core Services Laboratory, Department of Biochemistry, University of Alberta).

Purification of AE1-Ct

AE1-Ct transformed BL21 codon plus *Escherichia coli* cells were used to inoculate LB medium containing 0.1 mg/ml ampicillin and grown at 37 °C until an A_{600} of 0.6–0.8 was reached. Protein expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 1 mM to the culture and incubation for 3 h at 37 °C. Cells were harvested by centrifugation at 7500*g* for 10 min at 4 °C. Cell pellets were resuspended in 4 °C PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), containing Complete Mini Protease Inhibitor Cocktail. Cells were disrupted by sonication (four times for 60 s) using a W185 probe sonifier (Heat systems-Ultrasonic Inc., Plainview, NY), and stirred slowly for 30 min, following the addition of Triton X-100 (1% v/v). Disrupted cells were centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was collected and incubated with 1.2 ml GSH-Sepharose 4B at room temperature with rotation for 1–2 h. The resin was washed six times with PBS and AE1-Ct was eluted from the GSH-Sepharose 4B three times with 100 μl glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl, pH 8.0). Total protein concentration was determined by a Bradford protein assay [26] and protein purity was assessed by SDS–PAGE.