The electrogenic sodium bicarbonate cotransporter NBCe1-A mediates the basolateral absorption of sodium and bicarbonate in the proximal tubule. In this study the oligomeric state and minimal functional unit of NBCe1-A were investigated. Wild-type (wt) NBCe1-A isolated from mouse kidney or heterologously expressed in HEK293 cells was predominantly in a dimeric state as was shown using fluorescence energy transfer, pulldown, immunoprecipitation, cross-linking experiments, and nondenaturing perfluorooctanoate-PAGE. NBCe1-A monomers were found to be covalently linked by S–S bonds. When each of the 15 native cysteine residues were individually removed on a wt-NBCe1-A backbone, dimerization of the cotransporter was not affected. In experiments involving multiple native cysteine residue removal, both Cys630 and Cys642 in extracellular loop 3 were shown to mediate S–S bond formation between NBCe1-A monomers. When native NBCe1-A cysteine residues were individually reintroduced into a cysteineless NBCe1-A mutant backbone, the finding that a Cys992 construct that lacked S–S bonds functioned normally indicated that stable covalent linkage of NBCe1-A monomers was not a necessary requirement for functional activity of the cotransporter. Studies using concatameric constructs of wt-NBCe1-A, whose activity is resistant to methanesulfonate reagents, confirmed that NBCe1-A monomers are functional. Our results demonstrate that wt-NBCe1-A is predominantly a homodimer, dependent on S–S bond formation that is composed of functionally active monomers.

Bicarbonate absorption by the proximal tubule of the kidney plays an important role in the regulation of the plasma bicarbonate concentration and in systemic acid-base balance. Decreased proximal tubule bicarbonate absorption causes proximal renal tubular acidosis (1–4). Familial autosomal recessive proximal renal tubular acidosis results from mutations in the electrogenic sodium bicarbonate cotransporter NBCe1-A encoded by the SLC4A4 gene (5, 6). Mice lacking NBCe1 also have severe proximal renal tubular acidosis (7). These findings confirm that flux through the cotransporter is the predominant process mediating bicarbonate absorption across the basolateral membrane of the proximal tubule (8).

In addition to NBCe1, other SLC4 proteins are known to be associated with various diseases/phenotypes. Specifically, abnormalities in the function of the anion exchanger AE1 (SLC4A1 gene) cause distal renal tubular acidosis and red blood cell abnormalities (9–12). Polymorphisms in the anion exchanger AE3 (SLC4A3) and transgenic mice studies indicate a role in preventing seizures (13, 14). Mice with loss of NBCn1 (SLC4A7) exhibit combined blindness and abnormal hearing and are a model for Usher syndrome 2B in humans (15). Polymorphisms in the electrogenic sodium bicarbonate cotransporter NBCe2 (SLC4A5) are associated with hypertension (16). Finally, mutations in NaBC1, the electrogenic sodium borate cotransporter encoded by SLC4A11, cause CHED2 disease, Harboyan syndrome, and Fuchs endothelial corneal dystrophy (17–20).

NBCe1-A is composed of 1035 amino acids and has a predicted minimum molecular mass of 116 kDa (6). The estimated molecular mass of glycosylated NBCe1-A is ~130–145 kDa (21). According to NBCe1-A topology models based on hydropathy analysis, the cotransporter is predicted to have between 10 and 14 transmembrane segments (22). Tatishchev et al. (22) have shown that the cotransporter spans the membrane a minimum 10 times with the N and C termini of NBCe1-A localized intracellularly. Based on AE1 topology (23) the cotransporter may have up to 13 transmembrane regions with two re-entrant loops (24). Studies of the oligomeric structure of AE1 have shown that it forms dimers and tetramers under nondenaturing conditions (25–27), and tetramers of AE3 have been isolated from rabbit kidney membranes (28). Preliminary studies showed that NBCe1-A forms oligomers (29) and that the cytoplasmic N terminus of NBCe1-A is dimeric (30).

Given its importance in proximal tubule bicarbonate absorption, systemic acid-base balance, and in human disease, in this study we characterized the oligomeric structure of NBCe1-A and determined the minimal functional unit of the cotransporter. The following questions were addressed in our experiments. 1) Is NBCe1-A an oligomer? 2) What is the oligomeric state of NBCe1-A? 3) What is the role of S–S bonds in the...
oligomerization of NBCe1-A? 4) What is the minimal functional unit of NBCe1-A?

EXPERIMENTAL PROCEDURES

Membrane Isolation from Mouse Kidney—All animal procedures were approved by the Institutional Animal Care and Use Committee of UCLA. All procedures were performed at 4 °C. Mouse kidney (~0.5 g) was disrupted in a glass homogenizer in 600 μl of cell lysis solution (200 mM Tris-HCl, pH 7.5), containing 1 μg/ml pepstatin and 1 tablet of Complete protease inhibitor mixture (Roche Applied Science) per 2 ml of solution. The tissue homogenate was homogenized initially and then passed 40 times through a 25-gauge needle (BD Biosciences). Tissue homogenates were centrifuged at 600 × g for 10 min and then at 18,000 × g for 10 min. The resulting supernatant was centrifuged at 200,000 × g for 1 h. The precipitate of mouse kidney membranes was used for analysis.

Transfection of Cells and Cell Culture—HEK293 cells (American Type Culture Collection) were grown at 37 °C, 5% CO₂, in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 200 mg/liter L-glutamine, and penicillin/streptomycin mixture (Gemini Bio-Products, West Sacramento, CA). For immunoblot expression experiments, HEK293 cells growing on 10-cm plates at 80–90% confluency were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. 2 h later the transfection medium was replaced with fresh DMEM containing 10% fetal bovine serum. Plates were incubated at 37 °C (5% CO₂) for 24 h prior to use for immunoblot analysis. For immunohistochemistry and experiments involving the study of NBCe1-A transport, the cells were split onto glass coverslips coated with fibronectin in a 6-well plate and transfected the following day at 80–90% confluency with Lipofectamine 2000. Immunohistochemical and functional studies were performed 24 h later.

Membrane Isolation from Cultured Cells—HEK293 cells were collected from 10-cm cultured plate, washed three times with ice-cold phosphate-buffered saline (PBS, Invitrogen), and suspended at 4 °C in 250 μl of 200 mM Tris-HCl, pH 7.5, containing 1 μg/ml pepstatin and Complete protease inhibitor mixture (1 table/2 ml). Cells were then homogenized by passing 10 times through a 25-gauge needle, centrifuged at 600 × g for 10 min, then at 18,000 × g for 10 min, and finally at 200,000 × g for 1 h. The resulting pellet was used for analysis. In some studies, the total cell lysate was analyzed where indicated.

SDS-PAGE—50 μl of membrane pellet was mixed with an equal volume of 2× SDS sample buffer containing 0.125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, with or without 0.2 mM dithiothreitol (DTT; final concentration 0.1 mM), and 0.005% bromphenol blue and incubated at 95 °C for 5 min. Proteins were separated on 4–8 or 7.5% polyacrylamide gels (Jule, Milford, CT) at 100 V.

Nondenaturing PFO-PAGE—All experiments were performed using either 7.5 or 4–8% gradient precast Tris-glycine mini gels (Jule, Milford, CT). Running Tris-glycine buffer contained PFO (Oakwood Products, West Columbia, SC). PFO-PAGE was performed as described previously (31). Briefly, 50 μl of crude membranes was mixed with an equal volume of sample buffer containing 125 mM Tris, 8% PFO, 20% glycerol, with or without 0.2 mM DTT, and 0.005% bromphenol blue, pH 8.0. The sample was vortexed and incubated at room temperature for 30 min. Proteins were resolved by PFO-PAGE at 100 V.

Immunoblotting—Following PFO- or SDS-PAGE, the proteins were electrotransferred onto Hybond-P polyvinylidene difluoride membranes (GE Healthcare) as described (22). Non-specific binding was blocked by incubation for 1 h in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 140 mM NaCl) that contained 5% dry milk and 0.05% Tween 20 (Bio-Rad). The following primary antibodies were used: rabbit anti-human N-terminal NBCe1-A-specific (32) and C-terminal NBC1-specific (NBC1-4B2), (22) antibodies at 1:1,000 dilution, mouse anti-His antibody (Invitrogen), and rabbit anti-GFP (Invitrogen) antibodies at a dilution of 1:5,000. Secondary horseradish peroxidase-conjugated species-specific antibodies from Jackson ImmunoResearch (West Grove, PA) were used at 1:10,000 dilution. Primary and secondary antibodies were incubated with the membranes for 1 h at room temperature. The bands were visualized using an ECL kit and Hyperfilm ECL (GE Healthcare). The apparent molecular masses were determined by log-linear curve fitting of the molecular weight standards indicated for each gel. Immunoblotting was performed four times for each experimental protocol.

Pulldown and Coimmunoprecipitation Experiments—NBCe1-A was subcloned into pEGFP-C3 (BD Biosciences) using the EcoRI and Apal sites to create an N-terminal EGFP tag (EGFP-NBCe1-A). NBCe1-A containing an N-terminal His₆ tag (His₆-NBCe1-A) was also generated in the pcDNA3.1 vector. Both constructs were expressed individually or together in HEK293 cells. Crude membranes were isolated from the cells as described above and mixed with equal volume of 200 mM Tris-HCl, pH 7.5, containing 1 μg/ml pepstatin and Complete protease inhibitor mixture (1 table/2 ml) and 1% n-dodecyl-β-D-maltoside. Samples were then incubated at 4 °C for 30 min with gentle agitation, mixed with either Ni²⁺-NTA Superflow agarose beads (Qiagen) or the anti-GFP antibody (Invitrogen) pre-coupled to protein A-Sepharose (GE Healthcare), and incubated at 4 °C while rotating overnight. Proteins bound to Ni²⁺-NTA Superflow agarose beads were eluted with 300 mM imidazole in binding buffer. The samples were mixed with an equal volume of 2× SDS sample buffer containing DTT (final concentration 0.1 mM) and analyzed by SDS-PAGE. Agarose beads alone were used as a negative control in the Ni²⁺-NTA Superflow agarose beads experiments. Proteins bound to protein A-Sepharose beads were eluted with 0.1 M glycine, pH 3, and the samples were neutralized by adding 1 M Tris-HCl, pH 9, mixed with sample buffer as described, and analyzed by SDS-PAGE. Nonspecific IgG was used as a negative control.
Oligomeric Structure and Functional Unit of NBCe1-A

**Immunocytochemistry**—The coverslips were rinsed twice with 1× PBS and then incubated with 1 ml of cold methanol for 2 min. Methanol was then removed by rinsing with 1× PBS. The well characterized NBCe1-A-specific N-terminal antibody (32) was applied at 1:100 dilution in PBS for 1 h at room temperature. After several washes in PBS, goat anti-rabbit IgG conjugated with Cy3 (1:500 dilution; Jackson ImmunoResearch) was applied for 1 h at room temperature. The slides were rinsed in PBS and mounted in Crystal/Mount (Biomedia, Foster City, CA). A PXL charge-coupled device camera (model CH1; Photometrics), coupled to a Nikon Microphot-FXA epifluorescence microscope, was used to capture and digitize the fluorescence images for image acquisition.

**Molecular Mass of NBCe1-A**—Protein standards and purified NBCe1-A were mixed with PFO sample buffer to a final concentration of 1% PFO, and loaded onto 7.5% Tris-Gly gel (Jule, Milford, CT). PFO-PAGE was performed as described before, and the proteins were analyzed by either silver staining or immunoblotting. The protein standards (Sigma) used were as follows: 1) urease (monomer, 91 kDa; dimer, 182 kDa; trimer, 273 kDa); 2) albumin (monomer, 66 kDa; dimer, 132 kDa; trimer, 198 kDa; tetramer, 264 kDa); 3) apoferritin (443 kDa); 4) alcohol dehydrogenase (150 kDa); and 5) β-amylase (200 kDa). 50 ng of each protein standard was loaded onto the gel, and a silver stain was done (Silver Stain Plus kit, Bio-Rad).

**Purification of AE1 Protein**—The accuracy of the protein standard calibration curve discussed in the previous protocol was confirmed using AE1 protein because it is a well-characterized membrane protein and shares sequence homology with NBCe1-A. Bovine red blood cell membranes were prepared from fresh bovine blood as described previously (33). The red blood cell ghosts (5 ml) were then extracted with 50 mM Tris-HCl, pH 7.5, containing 1% C12E8 (Anatrace, Maumee, OH) and 1 mM phenylmethylsulfonyl fluoride for 1 h at 4°C, and insoluble membranes were precipitated by ultracentrifugation at 150,000 g for 45 min. The supernatant was then loaded on a 2 × 8-cm column of diethylaminoethylcellulose (DE-52, Whatman) pre-equilibrated with the 50 mM Tris-HCl, pH 7.5. The column was washed with the same buffer and then with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. The fraction eluted with 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, was loaded onto a 1 × 5-cm hydroxyapatite (Sigma) column equilibrated with 10 mM sodium phosphate, pH 8.0. AE1 was eluted with 100 mM sodium phosphate and concentrated using Microcon YM-100 filter units (Millipore, Temecula, CA).

**NBCe1-A Cross-linking**—To covalently cross-link membrane-embedded NBCe1-A proteins extracellularly, a water-soluble homobifunctional N-hydroxysuccinimide ester BS3 (Pierce) was used. BS3 is a water-soluble homobifunctional N-hydroxysuccinimide ester with an arm length of 11.4 Å that reacts with primary amino groups. BS3 is not membrane-permeable and is used to form covalent bonds only between external parts of membrane-embedded proteins. NBCe1-A was transiently expressed in HEK293 cells. 24 h after transfection, the cells were harvested from a 10-cm cultured plate, suspended, and washed three times in ice-cold PBS. BS3 was added to a final concentration of 5 mM, and the cell suspension was incubated at room temperature for 30 min. Control samples were incubated with PBS alone. The reaction was stopped by adding 1 M Tris-HCl, pH 8.0, to a final concentration 100 mM, and incubation was at room temperature for 15 min. The cells were then centrifuged at 600 × g for 2 min and washed twice with ice-cold PBS. The pellet was resuspended in 250 ml of lysis solution (200 mM Tris-HCl, pH 7.5, 1 mg/ml pepstatin, and 1 tablet of Complete Mini protease inhibitor mixture per 2 ml (Roche Applied Science)). The cells were then lysed; the membranes were isolated, and membrane proteins were analyzed by SDS-PAGE and immunoblotting as described above.

**Fluorescence Resonance Energy Transfer (FRET)**—HEK293 cells (American Type Culture Collection) were grown at 37 °C, 5% CO2, in DMEM supplemented with 10% fetal bovine serum, 200 mg/liter 1-glutamine, and penicillin/streptomycin mixture (Gemini Bio-Products). 24 h before transfection, a fully confluent 10-cm polystyrene plate (BD Biosciences) of cells was split 1:3 onto a plate with 12 ml of medium that was then immediately divided into a 6-well plate (2 ml/well) containing coated coverslips. 24 h later, the 80% confluent coverslips were transfected with NBCe1 constructs. Two NBCe1 constructs were used in these studies. The first construct consisted of NBCe1-A tagged on its N terminus with Cerulean fluorescent protein (34) (Cer-NBCe1-A). NBCe1-A was subcloned into the Cerulean vector between BglII and Apal sites. In the second construct, NBCe1-A was tagged on its N terminus with EYFP (EYPN-BCE1-A) between EcoRI-Apal site in the pEYFP-C1 vector (BD Biosciences). The plasmids containing these constructs were transfected into HEK293 cells grown in coated coverslips in 6-well plates using Lipofectamine 2000 (Invitrogen). The transfection medium was removed after 2 h and replaced with fresh DMEM with 10% fetal bovine serum without antibiotics until the following day when the FRET experiments were performed.

FRET between Cerulean–NBCe1-A and EYFP–NBCe1-A was assessed by calculating the sensitized emission (the EYFP emission upon Cerulean excitation) from confocal donor and acceptor images, acquired separately using a multiphoton fluorescence microscope. The imaging equipment consisted of a Leica TCS SP2 confocal microscope system (Leica Microsystems, Heidelberg, Germany), a DM IRE2 inverted microscope powered by a wide band, fully automated, infrared (710–920 nm) combined photo-diode pump laser and mode-locked titanium: sapphire laser (Mai–Tai, Spectra-Physics, Mountain View, CA), and a blue argon 514 nm/20-milliwatt laser.

Cerulean fluorescence protein was excited using optimized donor excitation with the multiphoton laser at 820 nm (34). Spectral detection bandwidth of the Leica SP2 channels was set up to balance minimal cross-talk with optimal collection efficiency (35). Cerulean fluorescence protein emission was detected between 460 and 490 nm; EYFP was excited with a 514 nm argon laser, and emission was detected between 528 and 603 nm. FRET between Cerulean fluorescent protein and EYFP was calculated using the FRET sensitized emission module of the Leica confocal software (LCS 2.61.1537) applying the equation: FRET = B − bA − (c − ab)b, where A = Cerulean emission (by Cerulean excitation); B = FRET emission (by Cerulean excitation); C = EYFP emission (by EYFP excitation); a = correction factor of EYFP only measurement (A/C when only EYFP is expressed); b = correction factor of Cerulean only
shown.

In contrast to the absence of interaction between NBCe1-A-linked Cerulean and EYFP (left upper panel), NBCe1-A dimerization results in FRET between Cerulean and EYFP (right upper panel). Cerulean and EYFP images (lower panel) of HEK293 cells expressing Cerulean-NBCe1-A and EYFP-NBCe1-A were acquired on a confocal microscope and total donor (Cerulean), total acceptor (EYFP), and sensitized FRET emission (NET FRET) were calculated as described under "Experimental Procedures." Both Cerulean, EYFP, and NET FRET signals were found mainly in the cell membrane, whereas no significant FRET was observed intracellularly. Cerulean and EYFP images were 0.038, 0.427, and 0.055, respectively.

**Construction of NBCe1-A Cysteine Mutants**—Mutations in the full-length human NBCe1-A were performed using a QuickChange site-directed mutagenesis kit (Invitrogen). For certain protocols, a cysteineless mutant was created by replacing all 15 native cysteines with serine residues. The cysteineless mutant was used as a backbone for generating additional constructs where in each of the positions, where cysteine residues were normally located, a native cysteine residue was replaced individually in separate constructs one at a time. A total of 15 additional constructs was generated in this fashion. In other experiments, the wt-NBCe1-A backbone was used to generate cysteine mutants in which individual native cysteine residues were removed one at a time leaving the remaining native cysteines in place. In total, 15 separate mutants were generated. Finally, additional mutants were generated where multiple cysteine residues were removed on the wt-NBCe1-A backbone from extracellular loop 3.

**Construction of NBCe1-A Concatamers**—In experiments involving the use of concatameric constructs, we took advantage of our recent finding that the NBCe1-A<sup>T442C</sup> mutant functions normally but can be completely inhibited by MTS reagents (36). In contrast, these agents have no effect of wt-NBCe1-A. The following concatameric constructs of wt-NBCe1-A and NBCe1-A<sup>T442C</sup> were studied: wt-NBCe1-A-linker-wt-NBCe1-A; wt-NBCe1-A-linker-NBCe1-A<sup>T442C</sup>; NBCe1-A<sup>T442C</sup>-linker-wt-NBCe1-A; and NBCe1-A<sup>T442C</sup>-linker-NBCe1-A<sup>T442C</sup>. The two individual units were linked through a linker peptide (Thr-Gly) in pcDNA3.1/neo vector (Invitrogen). To insert the linker peptide, the first unit (either wt-NBCe1-A or the T442C mutant) was cloned in pcDNA 3.1 vector (Invitrogen) between EcoRI and XhoI sites. An Agel (Thr-Gly) site was introduced at the 3’ end of the first unit by site-directed mutagenesis (Stratagene) instead of the stop codon. The second unit (either the T442C mutant or wt-NBCe1-A) was made using the PCR and inserted between Agel and XhoI sites in the first construct. Immunoblot analysis of the concatameric constructs was performed as described above. Flux through the four concatamers was measured using BCECF-AM as discussed below. MTSes was stored under argon at −20 °C and dissolved in water to a final concentration immediately before each experiment. The cells were exposed to measurement (EYFP emission by Cerulean excitation/A when only Cerulean is expressed), and c = correction factor of EYFP only measurement (EYFP emission by Cerulean excitation/C when only EYFP is expressed). Factors a, b, and c in the acquired images were 0.038, 0.427, and 0.055, respectively.

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FIGURE 2. Pulldown and immunoprecipitation studies. His<sub>6</sub>-NBCe1-A and EGFP-NBCe1-A constructs were coexpressed in HEK293 cells. The cells were lysed as described under “Experimental Procedures.” Ni<sup>2+</sup>-NTA Superflow agarose beads were used to pull down the His<sub>6</sub>-tagged cotransporter. Proteins bound to Ni<sup>2+</sup>-NTA Superflow agarose beads were eluted with 300 mM imidazole in binding buffer. The His<sub>6</sub>-NBCe1-A construct was detected with an anti-His<sub>6</sub> antibody, and EGFP-NBCe1-A was detected with an anti-GFP antibody using SDS-PAGE. The cell lysate was assayed using both antibodies. Agarose beads alone were used as a negative control in the Ni<sup>2+</sup>-NTA Superflow agarose beads experiments. The converse experiment was performed wherein an anti-GFP antibody was used to immunoprecipitate the EGFP-NBCe1-A construct, and the anti-His<sub>6</sub> and anti-GFP antibodies were used to detect both constructs in the immunoprecipitate using SDS-PAGE. The cell lysate was assayed using both antibodies. Nonspecific IgG was used as a negative control.

FIGURE 3. Determination of the oligomeric structure of NBCe1-A in mouse kidney using non-denaturing conditions. A, mouse renal cortex membrane fractions were run on PFO-PAGE in the absence of DTT, and immunoblots were probed with the following: lane 1, anti-NBCe1-A antibody; lane 2, immunizing peptide-blocked NBCe1-A. B, relative abundance of monomeric and oligomeric forms of NBCe1-A. Immunoblots were scanned, and the bands were compared using the Adobe Photoshop 9 software (Adobe Systems, San Jose, CA). The dimeric variant was assigned an arbitrary value of 1. Note that the band referred to as a tetramer could represent a dimer of NBCe1-A dimers or an NBCe1-A hetero-complex.

RESULTS

FRET between Cerulean-NBCe1-A and EYFP-NBCe1-A—As an initial approach to investigate whether NBCe1-A monomers interact directly in living cells, we measured FRET between NBCe1-A monomers labeled in their N termini with Cerulean fluorescence protein and extended yellow fluorescence protein (EYFP) coexpressed in HEK293 cells. Both constructs were expressed on the plasma membrane of the cells, and a FRET signal was detected in the cells coexpressing both constructs (Fig. 1). The positive FRET results indicated that the distance between the two fluorophores is <10 nm and suggested that NBCe1-A monomers interact to form oligomers on the plasma membrane of HEK293 cells.

Pulldown and Immunoprecipitation Studies—To determine whether the results of the FRET experiments suggesting that NBCe1-A monomers interact in vivo could be confirmed, as a separate approach, pulldown and immunoprecipitation studies were done. For this purpose, two tagged NBCe1-A constructs, e.g. EGFP-NBCe1-A and His<sub>6</sub>-NBCe1-A, were coexpressed in HEK293 cells. In the first series of experiments, we used Ni<sup>2+</sup>-beads to pull down the His<sub>6</sub>-NBCe1-A construct and determined whether the EGFP-NBCe1-A could be pulled down simultaneously. As shown in Fig. 2, both constructs were expressed, and the cell lysate contained the His<sub>6</sub>-NBCe1-A construct as detected using an anti-His<sub>6</sub> antibody, and EGFP-NBCe1-A was detected with an anti-GFP antibody. In pulldown experiments using Ni<sup>2+</sup>-NTA Superflow agarose beads, both the His<sub>6</sub>-NBCe1-A construct as well as EGFP-NBCe1-A were detected. These findings suggest that the two tagged constructs interact in HEK293 cells. In separate experiments, we used an anti-GFP antibody to immunoprecipitate the EGFP-NBCe1-A construct. As shown in Fig. 2, the immunoprecipitate contained both the EGFP- and His<sub>6</sub>-tagged constructs. Therefore, the results of the pulldown and immunoprecipitation experiments complement the data obtained in the FRET studies and indicate that NBCe1-A monomers interact to form oligomers in vivo.
NBCe1-A Oligomerization in Mouse Kidney—Previous experiments done in cells heterologously expressing NBCe1-A have reported that the apparent monomer molecular mass of NBCe1-A is \(130-145 \text{kDa}\). To determine whether native NBCe1-A is an oligomer as suggested by the results in HEK293 cells, the cotransporter was isolated from mouse kidney using the nondenaturing detergent PFO (39) in the absence of DTT and analyzed by PFO-PAGE. As shown in Fig. 3A, unlike previous studies in the literature that utilized denaturing conditions, a major band was detected that corresponded with the expected dimeric size and a less intense band of higher molecular weight was detected that likely represents a tetramer. Only a weak band corresponding to the monomeric form of the cotransporter was detected. The relative amount of dimeric versus tetrameric and monomeric forms of the cotransporter is shown in Fig. 3B. These results indicate that NBCe1-A is expressed in the kidney as an oligomer and that the cotransporter is predominantly a dimer.

NBCe1-A Oligomerization in HEK293 Cells—The experiments in mouse kidney using nondenaturing conditions suggested that NBCe1-A forms oligomers that consist predominantly of dimers. Further studies were done in HEK293 cells as a model system to investigate the mechanism of stable oligomerization of the cotransporter. In the presence of the nondenaturing detergent PFO in the absence of DTT as shown in Fig. 4A, a major band was detected at \(290 \text{kDa}\) that likely represents a dimer, and a less intense band of higher molecular weight was detected that could represent a tetramer, a dimer of

![FIGURE 4. Determination of the oligomeric structure of NBCe1-A expressed in HEK293 cells using nondenaturing conditions. A, samples were subjected to PFO-PAGE as follows: lane 1, without DTT; lane 2, plus DTT; lane 3, mock-transfected cells and immunoblots were probed with an anti-NBCe1-A antibody. B, relative abundance of monomeric and oligomeric forms of NBCe1-A. Immunoblots were scanned, and the bands were compared using the Adobe Photoshop 9 software. The dimeric variant was assigned an arbitrary value of 1.](image)

![FIGURE 5. Covalent cross-linking of membrane-embedded NBCe1-A proteins expressed in HEK293 cells with N-hydroxysuccinimide ester (BS\(^3\)). Membrane proteins cross-linked with BS\(^3\) (lane 1) and not cross-linked (lane 2) were analyzed by SDS-PAGE and immunoblotting in the presence of DTT. The immunoblots were probed with an anti-NBCe1-A antibody.](image)

![FIGURE 6. Determination of the molecular mass of NBCe1-A by 7.5% PFO-PAGE. A, calibration curve was generated using standards of known molecular mass (closed circles). To confirm the fidelity of the standardized PFO-polyacrylamide gel to estimate the molecular mass of membrane proteins, the curve was used to predict the molecular mass of AE1 monomer (open triangle) and dimer (closed triangle). The estimated molecular mass of AE1 monomer and dimer was 98.6 \(\pm 0.5 \text{kDa}\) (n = 4) and 198.0 \(\pm 3.0 \text{kDa}\) (n = 4), respectively. The molecular mass of NBCe1-A monomer and dimer was 143.2 \(\pm 2.1 \text{kDa}\) (n = 4) and 291.4 \(\pm 1.3 \text{kDa}\) (n = 4), respectively. B, bovine red blood cell ghost sample run on PFO-PAGE in the presence of DTT, and immunoblots were probed with a previously characterized anti-AE1 antibody kindly provided by Dr. Inaba (81). Note that unlike NBCe1-A, AE1 retains its oligomeric structure in the presence of DTT using PFO-PAGE analysis.](image)
Lack of a Role of GXXXG Motif in Interhelical Hydrogen Bonding of NBCe1-A—NBCe1-A possesses 15 cysteine residues, which could form S–S bonds that play a role in the oligomerization of the cotransporter. As shown earlier, wt-NBCe1-A oligomers are split into monomers by DTT suggesting that S–S bonds play an important role in its stabilizing NBCe1-A oligomers. Accordingly, it would be predicted that in the absence of native cysteine residues, NBCe1-A would not form stable oligomers. To address this question, we determined whether a cysteineless NBCe1-A mutant could form dimers and/or higher order oligomers. Under non-denaturing conditions as shown in Fig. 7B in the absence of DTT, the cysteineless mutant was essentially undetectable. As shown in Fig. 5 unlike cells not exposed to BS⁵, following cross-linking of NBCe1-A with BS⁵, the oligomers were resistant to DTT. These results confirmed the data obtained using PFO and indicate that NBCe1-A and monomers are a maximum distance of 11.4 Å based on the length of the cross-linker.

**Determination of the Molecular Mass of NBCe1-A**—The molecular mass of the NBCe1-A oligomer was deduced under non-denaturing conditions (Fig. 6). A molecular mass calibration curve for a 7.5% PFO-polyacrylamide gel was constructed from migration distances of specific proteins with known molecular masses as described previously (40). As shown in Fig. 6A, the migration of the protein standards fit a log-linear molecular mass- \( R_f \) relation indicating that the proteins are adequately separated based on their molecular mass. To confirm the fidelity of the standardized PFO-polyacrylamide gel to estimate the molecular mass of membrane proteins, we further assessed the mobility of AE1, given that it is the most structurally characterized member of the SLC4 family and shares a sequence homology to NBCe1-A (Fig. 6B) (41–46). Using the standard curve in Fig. 6A, the value of the molecular mass of the monomeric form of AE1 was 98.6 ± 0.5 kDa (\( n = 4 \)) and dimeric form was 198.0 ± 3.0 kDa (\( n = 4 \)) as reported previously (43, 45, 46). As shown in Fig. 6, the estimated apparent molecular mass of the NBCe1-A monomer and dimer (the predominant oligomeric form of the cotransporter) were predicted to be 143.2 ± 2.1 kDa (\( n = 4 \)) and 291.4 ± 1.3 kDa (\( n = 4 \)), respectively.

The results suggest that S–S bonds are involved in the oligomerization of the cotransporter. We also performed cross-linking experiments on plasma membrane NBCe1-A expressed in HEK293 cells to capture the oligomeric state of the cotransporter in living cells prior to performing the immunoblotting experiments (40). In these experiments, covalent cross-linking of NBCe1-A was performed using extracellular BS⁵. A major band likely corresponding to a dimer and a less intense higher order oligomer that likely corresponds to the tetrameric form were detected after cross-linking (Fig. 5). Monomers were detected after cross-linking (Fig. 5). Monomers were...
Oligomeric Structure and Functional Unit of NBCe1-A

FIGURE 9. A, oligomeric structure of 15 NBCe1-A mutants containing single individual native cysteine residues added to a cysteineless NBCe1-A backbone analyzed under non-denaturing conditions in the absence of DTT. B, oligomeric structure of mutants NBCe1-A-Cys583, NBCe1-A-Cys585, NBCe1-A-Cys630, and NBCe1-A-Cys642 under non-denaturing conditions in the presence of DTT. A and B, number at the top of each lane refers to the position of cysteine residue remaining in the construct.

FIGURE 10. Representative experiment showing the function of NBCe1-A-Cys992 mutant. A, mock-transfected cells; B, wt-NBCe1-A; and C, NBCe1-A-Cys992 mutant. A–C, cells were initially bathed in a HEPES-buffered solution in the absence of Na⁺. Where indicated, the solution was switched to an HCO₃⁻-buffered zero-Na⁺ containing solution. The flux through NBCe1-A (wild-type and mutant constructs) was assayed following the addition of a Na⁺-containing HCO₃⁻-buffered solution. Separate experiments using the identical protocol were performed in the presence of the NBCe1-A inhibitor Tenidap (0.2 mM; kindly provided by Vladimir Maslenko). See Table 1 for a summary of all cysteine mutants generated on a cysteineless backbone.

TABLE 1

| Cysteine residue | pH₇ (Na⁺ addition) | Equivalent base flux | Equivalent base flux (% wt-NBCe1-A) |
|------------------|-------------------|----------------------|-----------------------------------|
| Cys120           | 6.84 ± 0.03       | 13 ± 1*              | 66 ± 5*                           |
| Cys122           | 6.84 ± 0.03       | 12 ± 2*              | 59 ± 9*                           |
| Cys125           | 6.86 ± 0.03       | 17 ± 2*              | 79 ± 7*                           |
| Cys126           | 6.81 ± 0.02       | 16 ± 2*              | 76 ± 7*                           |
| Cys118           | 6.85 ± 0.02       | 10 ± 1*              | 49 ± 3*                           |
| Cys123           | 6.78 ± 0.03       | 0 ± 0*               | 0*                                |
| Cys124           | 6.76 ± 0.01       | 2.0 ± 0.2*           | 9 ± 1*                            |
| Cys125           | 6.82 ± 0.01       | 6.1 ± 1*             | 29 ± 5*                           |
| Cys126           | 6.79 ± 0.02       | 9.1 ± 2*             | 44 ± 7*                           |
| Cys127           | 6.84 ± 0.02       | 18 ± 2*              | 86 ± 9*                           |
| Cys128           | 6.77 ± 0.01       | 8.5 ± 1*             | 40 ± 3*                           |
| Cys129           | 6.79 ± 0.04       | 5.5 ± 1*             | 25 ± 3*                           |
| Cys130           | 6.81 ± 0.04       | 3.4 ± 0.2*           | 16 ± 1*                           |
| Cys131           | 6.83 ± 0.03       | 20 ± 2*              | 95 ± 6*                           |
| Cys132           | 6.82 ± 0.05       | 15 ± 2*              | 70 ± 7*                           |

*p < 0.05 versus wt-NBCe1-A flux (equivalent base flux) of 21 ± 2 mm/min (Dunnett’s t test). Five or more experiments were done for each construct.

helix dimerization of glycophorin A and the oligomerization of other proteins (47, 48). Interaction between glycine residues stabilizes oligomers by forming interhelical hydrogen bond. Interhelical hydrogen bonding between NBCe1-A monomers could therefore play an important role in stabilizing the NBCe1-A monomers prior to S–S bond formation in the endoplasmic reticulum lumen. To determine whether the GXXXG motif in NBCe1-A is a necessary requirement for stabilizing NBCe1-A oligomers, each of the residues in the GGLLG sequence was mutated individually to a serine residue, and their ability to oligomerize was examined. As shown in Fig. 8, all constructs were able to form stable oligomers under non-denaturing conditions. These results indicate that stable oligomerization of the cotransporter is not dependent on the GG4 motif.

Cysteine Mutants—NBCe1-A has 15 native cysteine residues (Fig. 7A) that could contribute to S–S bond formation (a) within a given monomer and/or (b) between individual monomers. Only the latter process can contribute to the formation of stable NBCe1-A oligomers via S–S bond formation. We generated 15 NBCe1-A mutants containing single individual native cysteine residues that were added onto a cysteineless backbone, and each construct was expressed in HEK293 cells. Of the constructs studied, four mutants were able to form S–S bonded dimers (Fig. 9) as follows: NBCe1-A-Cys583, NBCe1-A-Cys585, NBCe1-A-Cys630, and NBCe1-A-Cys642. All four cysteine residues are in putative extracellular loop 3 (Fig. 7). NBCe1-A-Cys992 mutant was also of particular interest because although stable monomers linked by an S–S bond were not formed, the function of this construct was not different from that of wt-NBCe1-A (Fig. 10 and Table 1). Representative experiments are shown in Fig. 10 where the mean functional activity of the NBCe1-A-Cys992 mutant was 20 ± 2 mm/min, n = 9 (p = not significant versus the wt-NBCe1-A). These data indicate the following: 1) S–S bonds between NBCe1-A monomers are not a necessary requirement for functional activity, and 2) the cysteine residues in extracellular loop 3 do not play an essential functional role.

Given that one or more of the cysteine residues in extracellular loop 3 is capable of mediating the formation of stable oligomers in the context of an otherwise cysteineless backbone, in separate experiments, individual cysteine residues were removed from a wt-NBCe1-A backbone to determine whether a single S–S bond mediated by one of the four cysteine residues in extracellular loop 3 was involved in intermonomeric S–S bonding. As shown in Fig. 11A, none of the individual cysteine
residues when removed from a wt-NBCe1-A backbone prevented stable dimerization of the cotransporter. These data indicate that more than one S–S bond must mediate the stable oligomerization of the cotransporter likely involving two or more cysteine residues in putative extracellular loop 3. Therefore, additional experiments were done where cysteine residues in extracellular loop 3 were mutated. The results shown in Fig. 11B indicated that Cys630 and Cys642 form S–S bonds between NBCe1-A monomers.

**NBCe1-A Minimal Functional Unit—**The previous data indicated that stable S–S bonded NBCe1-A is not a requirement for cotransporter function. In the following experiments, we therefore addressed the question as to whether individual monomers within the NBCe1-A oligomer are functional. We recently reported that an NBCe1-A T442C mutant functions normally and that the flux through the mutant cotransporter unlike wt-NBCe1-A is completely inhibited by MTS reagents (49). This mutant could therefore be used in concert with wt-NBCe1-A to help identify the functional unit of the cotransporter. One approach to identifying the functional unit of NBCe1-A is to coexpress plasmid DNA encoding wt-NBCe1-A and the T442C mutant and to determine the percent functional inhibition using MTS reagents. This approach requires the following: 1) both wt-NBCe1-A and the T442C mutant are expressed independently, and 2) the membrane expression levels correspond to a given quantity of plasmid DNA transfected and expressed. If the two cotransporters associate randomly to form functional oligomeric complexes, using the binomial theorem, one can predict the magnitude of functional inhibition with MTS reagents and the proportion of each cotransporter that is expressed. However, given the aforementioned assumptions and uncertainties involved, we elected to utilize a concatameric approach. This method was first utilized with voltage-gated K⁺/H⁺ channels (50) and has since been applied to analyze other membrane proteins, including aquaporins (51), the Na⁺/K⁺-ATPase (52), ligand-gated ion channels (53), lactose permease (54), Na₃P₂ (55), KAAT1 (56), and CAATCH1 (56). The following concatameric constructs of wt-NBCe1-A and NBCe1-A T442C were analyzed by immunoblotting, immunocytochemistry, and functionally the following: 1) wt-NBCe1-A-linker-wt-NBCe1-A used as a negative control.
trol; 2) wt-NBCe1-A-linker-NBCe1-A T442C; 3) NBCe1-
AT442C-linker-wt-NBCe1-A; and 4) NBCe1-A T442C-linker-
NBCe1-A T442C used as a positive control. As shown in Fig.
12A, under nondenaturing conditions, each construct was
expressed at the predicted size of dimers of NBCe1-A demo-
strating that both halves of each concatamer were
expressed properly. In addition as shown in Fig. 12,
B–E, each concatamer was targeted to the plasma membrane. As
shown in Fig. 13 and Fig. 14, the function of the four con-
catamers was similar to wt-NBCe1-A and NBCe1-A T442C.
In the presence of the MTS reagent MTSES (4 mM), the function
of both mixed concatamers (wt-NBCe1-A-linker-NBCe1-
A T442C and NBCe1-A T442C-linker-wt-NBCe1-A) was decreased to
~50% of normal. The function of the wt-NBCe1-A homo-con-
catamer was unaffected, whereas MTSES blocked the function of the
mutant NBCe1-A T442C homo-concatamer. The results indicate that
individually NBCe1-A monomers are functional.

DISCUSSION

In this study, we report for the first time studies that have charac-
terized the oligomeric state of wt-NBCe1-A and have deter-
mmined the minimal functional unit of the cotransporter. Our ex-
periments show that native NBCe1-A is an oligomer in mouse
kidney and also when the cotransporter is expressed heterologously
in HEK293 cells. The predominant oligomeric state of the cotrans-
porter is dimeric, although higher order oligomers (dimers of dimers
or tetramers) are also present to a lesser extent. The oligomerization
of NBCe1-A is mediated by covalent S–S bonds that are formed between
monomeric subunits. NBCe1-A therefore differs structurally from
AE1, which forms stable dimers in the absence of S–S bond formation.
Importantly, although NBCe1-A forms a structural oligomer, each
monomeric subunit maintains its own independent transport activity.

The oligomerization state of most transporters in the SLC4 family is
currently unknown, with the excep-
tion of AE1 (8, 57) and brain AE3
(28). Depending on the detergent
used for solubilization and temper-
ature, AE1 was shown to be pre-
dominantly a monomer, dimer, or
tetramer (26, 41, 42, 45). AE1 dimers were shown to be
arranged in two-dimensional crystals in higher order units
characterized by 3-fold symmetry (57). Transmission elec-
tron microscopy studies of bAE3 purified by immunoaffinity
chromatography from rabbit kidney revealed predominantly
AE3 tetramers (28). Because both AE1 and AE3 transport
chloride in exchange for bicarbonate, our current results
represent the first detailed study of the oligomerization of a
sodium-dependent bicarbonate cotransport of the SLC4
family. Moreover, NBCe1-A is the first member of the SLC4
family whose stable oligomerization has been shown to be
dependent on disulfide bond formation. In this way, the

FIGURE 13. Representative experiments depicting the following function. A, wt-NBCe1-A; B, NBCe1-A T442C
and the four concatamers (C–F). C, wt-NBCe1-A-linker-wt-NBCe1-A; D, wt-NBCe1-A-linker-NBCe1-A T442C;
E, NBCe1-A T442C-linker-wt-NBCe1-A; and F NBCe1-A T442C-linker-NBCe1-A T442C in the presence or absence of
MTSES.
cotransporter differs from AE1 whose oligomerization occurs independent of the potential reduction of S–S bonds (44).

In this study, we used the nondenaturing detergent PFO for determining the oligomeric state of NBCe1-A. PFO is a novel detergent that is known to protect the interactions between monomers in oligomeric membrane proteins and has been used in various studies to evaluate the mass and quaternary structure of membrane proteins such as claudin-4, ABCG2, the vanilloid receptor, and prestin (40, 58–60). Our experiments under denaturing and nondenaturing conditions demonstrate that both the dimeric and tetrameric (or dimer of dimer) variants of NBCe1-A are converted into monomers in the presence of DTT. Given that DTT was capable of completely splitting all NBCe1-A oligomers into monomers, other than S–S bonds, there does not appear to be any other significant mechanism for covalent bond formation between NBCe1-A monomers as has been reported, for example, in regard to the AT1 receptor (61).

In addition, our findings suggest that NBCe1-A monomers in the absence of S–S bonding lack strong noncovalent electrostatic/hydrophobic interactions under nondenaturing conditions that can maintain their initial oligomeric state. Finally, our results demonstrate that the interaction between NBCe1-A and AE1 monomers is fundamentally different in that AE1 monomers form stable oligomers in a nondenaturing detergent under reducing conditions. These findings suggest that AE1 oligomerization is dependent on strong noncovalent electrostatic/hydrophobic interactions rather than covalent S–S bond formation (26).

The apparent molecular mass of the NBCe1-A monomer and dimer was \( \sim 143 \) and \( \sim 291 \) kDa using PFO-PAGE methodology (31, 40). Because soluble proteins and membrane proteins can differ in their mobility in the PFO-PAGE system, the molecular masses obtained by PFO-PAGE analysis must be regarded as relative values rather than absolute. The errors introduced by this approach do not appear to be too great, however, given that by using the calibration curve shown in Fig. 6A, we were able to accurately estimate the molecular mass of the related SLC4 membrane protein AE1 monomer as \( \sim 99 \) kDa and the dimer as \( \sim 198 \) kDa.

Cysteine residues are known to form intra-monomeric disulfide and/or inter-monomeric S–S bonds thereby stabilizing a protein’s folded confirmation (62–68). In the absence of all native cysteine residues, NBCe1-A monomers are retained intracellularly presumably because of abnormal folding of the protein (63, 64, 69, 70). In addition, the importance of S–S bond formation in mediating the oligomerization of membrane proteins as a requirement for normal transport function has been shown for sodium-dependent neurotransmitter transporters (71), SGLT1 (72), and for the oligomerization of mouse and human resistins (73), E-cadherin (74), and hyaluronan-binding protein 1 (75).

Our data rule out a requirement for intramonomeric disulfide and/or inter-monomeric S–S bonds as being an absolute requirement for normal NBCe1-A function. Specifically, when Cys\(^{992}\) was introduced into the cysteineless mutant, functional activity was normal. In these experiments, because all the native cysteine residues, NBCe1-A monomers are retained intracellularly presumably because of abnormal folding of the protein (63, 64, 69, 70). In addition, the importance of S–S bond formation in mediating the oligomerization of membrane proteins as a requirement for normal transport function has been shown for sodium-dependent neurotransmitter transporters (71), SGLT1 (72), and for the oligomerization of mouse and human resistins (73), E-cadherin (74), and hyaluronan-binding protein 1 (75).

Our data rule out a requirement for intramonomeric S–S bond formation as being an absolute requirement for normal NBCe1-A function. Specifically, when Cys\(^{992}\) was introduced into the cysteineless mutant, functional activity was normal. In these experiments, because all the native cysteine residues other than Cys\(^{992}\) were absent, Cys\(^{992}\) in a given monomer could theoretically only form a disulfide bond with a second monomer. However, the Cys\(^{992}\) mutant lacked an S–S bond unlike the wild-type transporter, which is presumably because of the cytoplasmic localization Cys\(^{992}\) that precludes Cys\(^{992}\) from forming a disulfide bond.
from mediating S–S bonding in either the mutant or wild-type transporter.

Of the 15 native cysteine residues, our data suggest that Cys630 and Cys842 in extracellular loop 3 are involved in the formation of S–S bonds between NBCe1-A monomers. None of the cysteine residues outside of extracellular loop 3 formed S–S bonded oligomers. These findings are compatible with the fact that intracellular cysteine residues (N-terminal Cys120, Cys389, and Cys399, and C-terminal Cys992 and Cys1035) would not be expected to form S–S bonds because of the reducing environment in the cytoplasm (76, 77). Moreover, cysteine residues in transmembrane regions are in general in a nonaqueous environment (unless a given residue resides in an aqueous pore) and would also not be expected to be involved in S–S bond formation.

Although NBCe1-A is structurally an oligomer dependent on S–S bond formation, our results provide the first demonstration that individual NBCe1-A monomers are functional. Taking advantage of the specific inhibition of the NBCe1-A-T442C by MTS reagents (49), we used a concatameric approach to investigate the minimal functional unit of the cotransporter. These studies confirmed that individual NBCe1-A monomers in the cotransporter dimer are functional. From our data, we cannot rule out, whether additional ion permeation pathways are created by the formation of NBCe1-A dimers and tetramers. This possibility is suggested by the recent preliminary data of Chang et al. (78) who reported that in oocytes transfected with nonfunctional NBCe1-A mutants, functional cotransporter activity can be detected. The latter findings suggest that oligomerization of nonfunctional mutant monomers can create a functional ion permeation pathway. Whether these findings hold true in the context of wild-type monomer oligomerization is unknown. The results we obtained using NBCe1-A concatamers suggest that the function of monomeric NBCe1-A is similar to the wild-type cotransporter. Whether more subtle differences in ion affinity and permeation characterize the different oligomeric states of the cotransporter requires further studies.

Both in kidney and in HEK293 cells expressing the cotransporter, the ratio of NBCe1-A dimers in comparison with monomers and higher order oligomers (consisting of tetramers or dimers of dimers) is favored thermodynamically by a factor of 5:1 and 2.5:1, respectively. Other transport proteins known to be oligomers whose transport behavior is consistent with activity as monomers include lactose, permease (54), AE1 (79), aquaporin-1 (51), NKCC2 (80), KAA1 (56), and CAATCH1 (56). An obvious question then arises from these studies and our data. Given that monomers are functional in NBCe1-A and the aforementioned transporters, what are the selective biological advantages for functional monomers to assemble as oligomers? Although conjectural given our present state of knowledge, oligomerization may provide a selective advantage in altering plasma membrane half-life and stability and modulating the dynamics of protein/protein interactions in the plasma membrane.

Currently, all mutations in NBCe1 are inherited in an autosomal recessive pattern. In contrast, mutations in AE1 cause distal renal tubular acidosis that can be inherited in a dominant or recessive manner (9–12). In autosomal dominant AE1 mutations, dimers containing a mixed wild-type and mutant monomer are targeted to the endoplasmic reticulum-associated degradation pathway presumably because of lack of acquisition of a native conformation (12). Mutations in AE1 inherited in a recessive manner are expressed normally on the plasma membrane with the functional monomer in the mixed dimer still able to transport normally (12). The finding that NBCe1-A is predominantly a dimer composed of functional monomers leads to the interesting prediction that in the known mixed mutant-wild-type dimers (heterozygotes; parents of patients with autosomal recessive NBCe1-A-mediated proximal renal tubular acidosis), the wild-type monomer is functional, and the mutant monomer is not capable of targeting the mixed dimer to the endoplasmic reticulum-associated degradation pathway resulting in the lack of a significant phenotype. To address this issue more precisely, an understanding of the specific residues involved in coordinating the electrostatic/hydrophobic interaction between monomers prior to S–S bond formation will play an important role in future studies involving the biosynthesis of NBCe1-A.

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