Interplay between Disulfide Bonding and N-Glycosylation Defines SLC4 Na\(^+\)-coupled Transporter Extracellular Topography*

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Background: Cysteines in extracellular loop 3 (EL-3) are highly conserved in SLC4 Na\(^+\)-coupled transporters. Results: Disulfide bonding and N-glycosylation uniquely determine the topography of NBCe1-A EL-3. Conclusion: A domain-like structure formed by EL-3 is present at the NBCe1-A dimeric interface. Significance: The interplay between disulfide bonding and N-glycosylation may play an important role in transmembrane protein maturation.

The extracellular loop 3 (EL-3) of SLC4 Na\(^+\)-coupled transporters contains 4 highly conserved cysteines and multiple N-glycosylation consensus sites. In the electrogenic Na\(^+\)-HCO\(_3\)\(^-\) cotransporter NBCe1-A, EL-3 is the largest extracellular loop and is predicted to consist of 82 amino acids. To determine the structural-functional importance of the conserved cysteines and the N-glycosylation sites in NBCe1-A EL-3, we analyzed the potential interplay between EL-3 disulfide bonding and N-glycosylation and their roles in EL-3 topological folding. Our results demonstrate that the 4 highly conserved cysteines form two intramolecular disulfide bonds, Cys\(^{S53}\)-Cys\(^{S85}\) and Cys\(^{617}\)-Cys\(^{642}\), respectively, that constrain EL-3 in a folded conformation. The formation of the second disulfide bond is spontaneous and unaffected by the N-glycosylation state of EL-3 or the first disulfide bond, whereas formation of the first disulfide bond relies on the presence of the second disulfide bond and is affected by N-glycosylation. Importantly, EL-3 from each monomer is adjacent located at the NBCe1-A dimeric interface. When the two disulfide bonds are missing, EL-3 adopts an extended conformation highly accessible to protease digestion. This unique adjacent parallel location of two symmetrically folded EL-3 loops from each monomer resembles a domain-like structure that is potentially important for NBCe1-A function in vivo. Moreover, the formation of this unique structure is critically dependent on the finely tuned interplay between disulfide bonding and N-glycosylation in the membrane processed NBCe1-A dimer.

SLC4 transporters represent a family of membrane proteins that transport HCO\(_3\)\(^-\) in various cell types (1–3). These transporters are widely expressed in the brain, heart, kidney, pancreas, intestine, muscle, teeth, and eye where they play critical roles in intracellular pH regulation, transepithelial transport, and systemic acid-base balance. SLC4 transporters have high amino acid sequence homology but vary in their Na\(^+\)-dependence, stoichiometry (electroneutral/electrogenic), and mechanisms of ion transport (exchanger/cotransporter) (1–3).

Of all the SLC4 Na\(^+\)-coupled transporters, the electrogenic Na\(^+\)-base cotransporter NBCe1 has been studied extensively because of its essential role in mediating HCO\(_3\)\(^-\) transport in multiple organs including kidney, brain, eye, and tooth ameloblasts (1–3). Eight missense mutations in NBCe1 have thus far been reported to cause a severe form of human proximal renal tubular acidosis with extrarenal manifestations (1, 4). These mutations affect NBCe1 structure-function properties and membrane expression (1, 4, 5). A naturally occurring missense mutation, T485S, was recently shown to affect the electrogenic nature of NBCe1, switching the mode of transport from Na\(^+\)-HCO\(_3\)\(^-\) to Na\(^+\)-CO\(_3\)\(^2-\) cotransport (6).

The human SLC4A4 gene encodes three NBCe1 protein variants (-A, -B, and -C), and additional transcripts (-D and -E) have been reported in mouse (7–9). These variants differ in their N- and C-terminal regions but have the identical transmembrane region (8). NBCe1-A exists as a homodimer in the plasma membrane with each monomer functioning independently (10). Each monomer consists of an N-terminal cytoplasmic region, a C-terminal transmembrane region, and a short C-terminal cytoplasmic tail. The N-terminal cytoplasmic region is in a folded conformation and interacts tightly with the transmembrane region (11, 12), and the C-terminal cytoplasmic tail contains motifs that are critical for NBCe1-A membrane trafficking (13–15). Interestingly, the C-terminal tails from each monomer are closely associated in the cytosol, suggesting that they may have additional roles for NBCe1-A function (16). The transmembrane region of NBCe1-A contains 14 transmembrane segments (TMs) with TM1, -3, and -8 involved in forming the
ion permeation pathway (6, 17, 18) and TM10–14 forming a scaffold structure to accommodate the ion interaction site (12) (see Fig. 1A).

Sequence alignment analysis reveals that a common structural feature exists among all SLC4 Na+-coupled transporters: a large extracellular loop 3 (EL-3) with multiple N-glycosylation consensus sites and 4 highly conserved cysteines (see Fig. 1B). In NBCe1-A, this loop is predicted to contain 82 amino acids (5) with three N-glycosylation consensus sites (Asn592, Asn597, and Asn617) of which only two (Asn597 and Asn617) are glycosylated (19). The N-glycosylation of EL-3 is predicted to be important for NBCe1-A plasma membrane trafficking, whereas the role of the 4 highly conserved cysteines in EL-3 has remained unclear. In the human organic cation transporter 2, cysteines in the extracellular loops play a crucial role in protein plasma membrane trafficking and are involved in transporter oligomerization (20). Moreover, in Cys-loop ligand-gated ion channel receptors, the disulfide bonds in a large extracellular loop are involved in forming the substrate interaction site (21). The TM prior to NBCe1-A EL-3 (TM5) contains a charged peptide motif 558KKMIK562 that forms one of the DIDS (an NBCe1-A functional inhibitor) binding sites (22). A single amino acid substitution, D555E in TM5, was shown to convert NBCe1-A from a cotransporter to an anion channel (23). Taken together, EL-3 may have an important structure-function role in NBCe1-A ion transport.

In the present study, we determined that the 4 cysteines in NBCe1-A EL-3 form two intramolecular disulfide bonds that constrain EL-3 in a folded conformation. Our results also demonstrated that EL-3 from each monomer resides closely at the NBCe1-A dimeric interface, forming a unique topological structure. Moreover, our data have shown for the first time that in a transmembrane protein, a unique interplay exists between disulfide bonding and N-glycosylation that governs the topological folding of a large extracellular loop.

EXPERIMENTAL PROCEDURES

Materials—Site-directed mutagenesis kits were from Stratagene. Biotin maleimide, BCECF-AM, DIDS, DMEM, and all cell culture reagents were from Invitrogen. Protein A-Sepharose, streptavidin-biotinylated horseradish peroxidase (HRP) complex and goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP) were from GE Healthcare. Endo-F (peptide-N-glycosidase F) and Endo-H were from New England Biolabs. NHS-SS-maleimide, BCECF-AM, DIDS, DMEM, and all other reagents were from Sigma. Biotin maleimide, BCECF-AM, Protein samples were eluted in 2× SDS sample buffer containing 2% β-ME, resolved on 7.5% polyacrylamide gels, and transferred to PVDF membranes. Biotinylated proteins were detected by incubation of blots with 1:10,000 diluted streptavidin-biotinylated horseradish peroxidase (GE Healthcare) in TBSTB buffer (TBST buffer (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5) containing 0.5% (w/v) bovine serum albumin). The protein expression level was determined by probing the stripped blots with an anti-NBCe1 C terminus polyclonal antibody (12) at 1:3000 dilutions in TBSTB buffer (TBST buffer containing 5% (w/v) non-fat milk).

Plasma Membrane Stripping—Cell membranes were isolated in hypotonic solution (10 mM Tris, 2 mM EDTA, pH 7.5). Membrane treatment with Na2CO3 was performed as described previously (5, 12). Briefly, isolated cell membranes were resuspended in 100 μl of 1% sucrose solution and incubated with 2

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ml of 0.1 or 1 M of Na₂CO₃ at 4 °C on a rotating shaker for 30 min. Membranes were then pelleted, washed three times with PBS, pH 7.0 and equilibrated in PBSCM, pH 7.0 prior to biotin maleimide (BM) labeling.

**NBCe1-A Membrane Stability Assay**—24 h post-transfection, cells expressing WT-NBCe1-A or NBCe1-A lacking the 4 cysteines in EL-3 were exposed to cycloheximide (10 μM) (to suppress protein synthesis) (25) in fresh cell culture medium. At 0, 24, 48, 72, and 96 h post-cycloheximide treatment, the cells were rinsed with PBS and incubated with sulfo-NHS-SS-biotin for 30 min at 4 °C following the manufacturer’s protocol. The cells were then collected, lysed, and immunoprecipitated with streptavidin-agarose beads. Protein samples were eluted with SDS sample buffer containing 2% β-ME, resolved by SDS-PAGE, and detected with a rabbit anti-human NBCe1-A antibody.

**NBCe1-A Deglycosylation Analysis**—48 h post-transfection, cells expressing various NBCe1-A constructs were collected, and cell membranes were isolated as described above. The isolated cell membranes were then resuspended in Endo-F or Endo-H digestion buffer and processed following the manufacturer’s protocol. Protein samples were resolved by SDS-PAGE and detected with a rabbit anti-human NBCe1-A antibody.

**Image and Data Analysis**—Films from immunoblots and biotinylation blots were scanned with a Hewlett-Packard Scanjet 5590. Scanned images were quantified with UN-SCAN-IT gel™ Version 6.1 software. Biotinylation levels were calculated according to Ref. 26.

**Statistical Analysis**—Means ± S.E. were calculated with SigmaPlot 10 software. Statistical analysis was performed using SigmaPlot 10 software. Dunnett’s t test was used to assess statistical significance with p < 0.05 considered significant.

**RESULTS**

**The Cysteines in NBCe1-A EL-3 Form Intramolecular Disulfide Bonds**

Identification of Aqueous Accessible Cysteines in NBCe1-A—Wild-type NBCe1-A (WT-NBCe1-A) contains 15 endogenous cysteines of which 5 are located in the cytoplasmic regions and 4 are located in EL-3 that are potentially exposed to the aqueous environment (Fig. 1A). To determine whether one or more of these cysteines are accessible to the intracellular or extracellular medium, we engineered a construct with the 5 cytoplasmic cysteines substituted with serines (NBCe1-A-5C). By using this construct as a template, we individually reintroduced each of the 5 substituted cysteines. Aqueous accessibility of the reintroduced cysteines was assessed by BM labeling; BM is a reagent that only reacts with aqueous exposed sulfhydryl groups on the surface of a protein (27). Fig. 2A shows that WT-NBCe1-A was strongly labeled, whereas NBCe1-A-5C had no labeling. Cys389, Cys992, and Cys1035 were labeled, whereas Cys120 and Cys399 were not labeled, indicating that the latter two are buried in the N-terminal cytoplasmic region of NBCe1-A. The lack of BM labeling of NBCe1-A-5C suggests that the 4 cysteines in EL-3 either form intramolecular disulfide bonds or are tightly folded in NBCe1-A EL-3.

**Cysteines in EL-3 Form Intramolecular Disulfide Bonds**—To determine this, we expressed NBCe1-A-5C in HEK 293 cells and compared protein migration on SDS-PAGE under nonreducing and reducing conditions. Fig. 2B shows that the nonreduced NBCe1-A-5C protein migrated as a protein band around 140 kDa on SDS-PAGE, ruling out that the 4 cysteines in EL-3 do not form intermolecular disulfide bonds. When NBCe1-A-5C was reduced, the protein migrated as a lower molecular weight band, indicating that a

**FIGURE 1. Topology of NBCe1-A and sequence alignment of the predicted EL-3 among SLC4 Na⁺–HCO₃⁻ cotransporters.** A, the NBCe1-A topology was proposed based on our recent and previous reports (5, 11, 12). The branched structures at Asn597 and Asn617 represent N-linked glycosylation. Locations of the 15 endogenous cysteines are indicated as filled red circles. Square marks the 4 highly conserved cysteines in EL-3. The N (Nt) and C (Ct) termini are marked. TMs are numbered 1–14. B, amino acid sequences of NBCe1-A and SLC4 Na⁺–HCO₃⁻ transporters were aligned using the ClustalW program. The 4 highly conserved cysteines are colored in red. Asterisks indicate the potential N-glycosylation sites in NBCe1-A EL-3.
structural constraint in NBC1-A-5C mediated by intramolecular disulfide bond(s) was removed. To determine whether the 4 cysteines in EL-3 are responsible for the increased migration of reduced NBCe1-A, we substituted all 4 cysteines with serines (NBCe1-A-9C) and resolved the protein by SDS-PAGE. Fig. 2B shows that β-ME treatment had no effect on NBCe1-A-9C migration in contrast to NBCe1-A-5C, indicating that the 4 cysteines in EL-3 form intramolecular disulfide bonds. Interestingly, NBCe1-A-9C migrated as a high molecular weight band compared with NBCe1-A-5C, suggesting that the normally unglycosylated site in EL-3 (Asn592) became glycosylated when disulfide bonds were absent.

The Disulfide Bonds in EL-3 Are Buried in the NBCe1-A Protein Complex, Constraining EL-3 in a Folded Conformation

We next tested whether the disulfide bonds in EL-3 could be freed by DTT treatment. Plasma membranes from HEK 293 cells expressing NBCe1-A-5C (5C−), NBCe1-A-9C− (9C−), or NBCe1-A-C1035 (C1035) were isolated and treated with 50 mM DTT prior to BM labeling. Fig. 3A shows the positive control C1035 strongly labeled; the negative control 9C− had no labeling. Surprisingly, 5C− also was unlabeled after the treatment, indicating that the reducing reagents failed to free the cysteines in EL-3. We then attempted to expose the disulfide bonds in EL-3 by stripping the isolated membranes with 0.1M or 1M Na2CO3, pH 12 followed with 50 mM DTT treatment and BM labeling. Again, the result showed that none of the 4 cysteines in EL-3 were freed (Fig. 3A). To further confirm that the disulfide bonds in EL-3 remained intact after these treatments, we resolved the proteins by SDS-PAGE under nonreducing conditions. Fig. 3B shows that the disulfide bonds in 5C− were not reduced even after 1M Na2CO3 treatment in contrast to the positive control (Cys642) whose intermolecular disulfide bond was completely reduced. Taken together, our data indicate that the disulfide bonds in EL-3 are not exposed on the extracellular surface of NBCe1-A.

To determine how the disulfide bonds shape the EL-3 conformation, we tested the accessibility of EL-3 to chymotrypsin digestion in the presence or absence of the 4 cysteines. Transfected HEK 293 cells were incubated briefly with chymotrypsin (5 min) and subsequently stained with an antibody (Ab-162) that specifically recognizes the peptide between the two N-glycosylated sites, Asn597 and Asn617, in EL-3. Fig. 4A shows that chymotrypsin treatment completely eliminated the epitope for Ab-162 staining in 9C− but had no effect on Ab-162 staining of 5C−, indicating that in the absence of the disulfide bonds, EL-3 adopts an extended conformation highly accessible to protease digestion. Chymotryptic digestion was then verified by immunoblotting (Fig. 4B), which showed that a distinct N-terminal peptide and a C-terminal peptide were released from 9C− but
not from 5C/H11002, indicating that cleavage only occurred in EL-3 when the cysteines were absent. These results demonstrate clearly that the disulfide bonds in EL-3 constrain the loop in a folded conformation on the NBCe1-A extracellular surface.

The Pattern of Disulfide Bonding among the 4 Cysteines in NBCe1-A EL-3

To determine how the disulfide bonds are formed among the 4 cysteines in EL-3, we engineered 12 constructs with different cysteine combinations in EL-3 using 5C/H11002 as the template (Table 1). We predicted that if one or more of the cysteines in EL-3 are substituted with serines, then the unpaired cysteines would be freely available for BM labeling. Surprisingly, after a 10-min incubation with BM, only two of the cysteine substituted combinations were moderately labeled (Fig. 5A). The negative data may have resulted from intermolecular cross-linking of the unpaired cysteines on each of the NBCe1-A monomers and therefore treated the transfected HEK 293 with 5 mM DTT followed with BM labeling. Indeed, Fig. 5B shows that DTT treatment freed the cross-linked cysteines, resulting in their subsequent labeling by BM.

To further determine whether intermolecular cross-linking occurred between the unpaired EL-3 cysteines in the NBCe1-A dimer, we resolved each of the cysteine substituted combinations by SDS-PAGE under both nonreducing and reducing conditions. Fig. 5C shows that all the cysteine combinations formed β-ME-sensitive dimers on SDS-PAGE except the Cys630/642 combination, indicating that the unpaired cysteines are intermolecularly cross-linked and that the Cys630 and Cys642 pair normally forms a native disulfide bond. Fig. 5D summarizes the results of BM labeling of all the cysteine combinations without or with DTT pretreatment and the efficiency of cross-linking. The native disulfide bonding between Cys630 and Cys642 indicates that Cys583 and Cys585 form a disulfide bond in wild-type NBCe1-A. Noticeably, when Cys583/642 or Cys585/642 were present, the cysteines were moderately labeled by BM, and fewer dimers were formed, further supporting that Cys630 and Cys642 and that Cys583 and Cys585 form disulfide bonds, respectively.

Interestingly, we observed that NBCe1-A lacking Cys583 and Cys585 migrated significantly more slowly compared with 5C/H11002 on SDS-PAGE under the reducing condition (Fig. 5B). To further confirm this, we resolved the NBCe1-A constructs with either single or double cysteines in EL-3 by 7.5% SDS-PAGE for an extended period of time. Fig. 5E shows that when Cys583/642 or Cys585/642 were present, the cysteines were moderately labeled by BM, and fewer dimers were formed, further supporting that Cys630 and Cys642 and that Cys583 and Cys585 form disulfide bonds, respectively.

Effect of N-Glycosylation on Disulfide Bonding in NBCe1-A EL-3

NBCe1-A EL-3 contains three N-glycosylation consensus sites, but only two were reported to be glycosylated in Xenopus oocytes, and removal of all the three consensus sites had no

| Number | Cysteine substitutions | Remaining cysteine combinations |
|--------|------------------------|---------------------------------|
| 1      | C583S                  | Cys630/Cys642                  |
| 2      | C585S                  | Cys583/Cys630/Cys642           |
| 3      | C630S                  | Cys583/Cys642/Cys650           |
| 4      | C642S                  | Cys583/Cys642/Cys630           |
| 5      | C583S/C585S            | Cys630/Cys642                  |
| 6      | C630S/C642S            | Cys583/Cys642                  |
| 7      | C583S/C642S            | Cys583/Cys630                  |
| 8      | C585S/C642S            | Cys583/Cys630                  |
| 9      | C583S/C630S/C642S      | Cys583                         |
| 10     | C583S/C630S/C642S      | Cys583                         |
| 11     | C583S/C588S/C642S      | Cys630                         |
| 12     | C583S/C588S/C630S      | Cys642                         |
To determine the glycosylation status of NBCe1-A in mammalian cells, we individually substituted Asn592, Asn597, or Asn617 or simultaneously substituted Asn597/617, Asn592/617, Asn592/597, or all three Asn sites with Gln in WT-NBCe1-A EL-3 and expressed these constructs in HEK 293 cells. Fig. 6A shows that NBCe1-A constructs with single glycosylation consensus site substitution all migrated at a similar molecular weight position as WT-NBCe1-A; with two consensus sites substitutions (except Asn592/617), all migrated as a lower molecular weight band; and with three consensus sites substitutions, the proteins migrated as an even lower molecular weight band. Endo-F treatment completely eliminated glycosylation.
nated the differences in protein migration of all the constructs except 9C\(^{-}\), indicating that the difference in protein migration is due to the presence of various numbers of complex glycans in NBCe1-A. This result also indicated that, in the absence of Asn\(^{597/617}\), Asn\(^{592}\) became glycosylated. To test whether high mannose glycans is responsible for the slowed protein migration of Endo-F-treated 9C\(^{-}\), cell membranes containing 9C\(^{-}\) were isolated and treated with Endo-H at 37 °C for 2 h. Fig. 6B shows that Endo-F treatment significantly increased 9C\(^{-}\) migration on SDS-PAGE; however, Endo-H did not produce any effect. This result indicates that the difference in migration of Endo-F-treated 9C\(^{-}\) compared with the other constructs is likely due to an altered protein structure induced by high exposure of Asn\(^{597/617}\) in EL-3, NBCe1-A protein was minimally expressed in the HEK 293 cells (Fig. 6A). We predict that this construct may have created a motif recognized by the endoplasmic reticulum-associated degradation pathway (28).

To determine whether N-glycosylation affects disulfide bond formation in EL-3, we compared the structural difference between 5C\(^{-}\) and the construct with N597Q/N617Q substitution (NBCe1-A-5C\(^{-}\)/N597Q/N617Q (2N\(^{-}\)-5C\(^{-}\))). Fig. 7A shows that in the absence of Asn\(^{597/617}\), glycosylation none of the 4 cysteines in EL-3 were freely available for BM labeling. Moreover, NBCe1-A protein migrated as a monomer and was sensitive to \(\beta\)-ME reduction (Fig. 7B), suggesting that the formation of intramolecular disulfide bonds was not affected.

To examine whether N-glycosylation affects EL-3 folding, we tested the accessibility of the disulfide bonds in 2N\(^{-}\)-5C\(^{-}\) to DTT reduction and chymotrypsin digestion as described above. Fig. 7, C and D, show that whole cell treatment with 5 mM DTT had minimal effect on 2N\(^{-}\)-5C\(^{-}\) migration on SDS-PAGE; however, chymotrypsin (5-min) treatment released an N-terminal 75-kDa and a C-terminal 37-kDa protein band from 2N\(^{-}\)-5C\(^{-}\), suggesting that EL-3 is less tightly folded when the two glycosylation sites are absent. The slow migration of the released N-terminal band in 2N\(^{-}\)-5C\(^{-}\) (compared with that of 9C\(^{-}\)) also suggested that Asn\(^{592}\) was glycosylated when the other two sites were absent.

We then tested whether N-glycosylation of EL-3 affects Cys\(^{583/585}\) intermolecular cross-linking (in the absence of Cys\(^{630/642}\)). Fig. 7E shows that, when Asn\(^{597/617}\) were substituted with Gln, Cys\(^{583/585}\) migrated as a monomer, and surprisingly, when only the normally unglycosylated site Asn\(^{592}\) was substituted, Cys\(^{583/585}\) migrated as a monomer. In contrast, substitution of Asn\(^{597/617}\) or Asn\(^{592}\) with Gln had no detectable effect on Cys\(^{630/642}\) disulfide bonding (in the absence of Cys\(^{583/585}\)). Probing of these constructs with BM indicated that none of the cysteines were available for labeling (Fig. 7F), indicating that Cys\(^{583/585}\) and Cys\(^{630/642}\) form intramolecular disulfide bonds, respectively, under these conditions. Further analysis showed that all these constructs remained sensitive to Endo-F digestion (Fig. 7G), suggesting that Asn\(^{597/617}\) were glycosylated in the absence of Asn\(^{592}\) and that Asn\(^{592}\) was glycosylated in the absence of Asn\(^{597/617}\). Taken together, our findings suggest that a significant interplay between N-glycosylation and disulfide bonding of Cys\(^{583/585}\) exists that determines the conformation of this very large extracellular loop in NBCe1-A dimer.

**EL-3 from Each NBCe1-A Monomer Is Closely Associated at the Dimeric Interface**

EL-3 is predicted to be the largest extracellular loop on the surface of NBCe1-A. To clearly determine the size of EL-3, we individually substituted amino acids in the regions of Ala\(^{556}\)–Ser\(^{582}\) and Asn\(^{641}\)–Ser\(^{652}\) in 9C\(^{-}\) to cover the predicted linker regions between TM5 and EL-3 and between EL-3 and TM6. Whole cell labeling with BM was performed on all the constructs expressed in HEK 293 cells. Fig. 8, A and B, shows that the substituted cysteines D565C–S571C, N643C, and F644C but not P568C could be labeled only after the cells were treated with 5 mM DTT, suggesting they form intramolecular disulfide bonds precluding BM labeling. To verify this, we resolved NBCe1-A proteins under the nonreducing condition. Fig. 8D shows that the positive control C642 but not 5C\(^{-}\) was intermolecularly cross-linked to form an NBCe1-A dimer. Substituted cysteines in the regions of Y567C–N572C and N643C–D647C were cross-linked to form dimers, and cysteines in D565C–Y566C and I648C–L650C were not cross-linked. The slight difference in BM labeling and cross-linking is likely due to local structural constraints caused by protein folding. Based on the results of BM labeling (Fig. 8C) and protein cross-linking, we conclude that EL-3 contains 83 amino acids ranging from Asp\(^{566}\) to Asp\(^{647}\) (L562C was weakly labeled irrespective of reduction). The pattern of cross-linking also suggests that the extracellular ends of TM5 and TM6 from each of the respective monomers reside adjacent to the NBCe1-A dimeric interface.
FIGURE 7. Effect of N-glycosylation on disulfide bond formation in EL-3 and on topological folding of EL-3. A, HEK 293 cells expressing 2N^-5C^- or C1035 were labeled with BM as described in Fig. 2. B, the β-ME-treated and untreated 2N^-5C^- and 5C^- protein samples were resolved by 7.5% SDS-PAGE, transferred to a PVDF membrane, and probed with an anti-NBCe1-A antibody. C, HEK cells expressing 5C^-, 2N^-5C^-, or C642 were rinsed with PBS and incubated with 5 mM DTT at 37 °C for 30 min. Plasma membranes were then isolated, lysed in 2× SDS sample buffer without β-ME, and resolved by 7.5% SDS-PAGE. D, cell membranes from chymotrypsin-treated HEK 293 cells were isolated, resolved by 7.5% SDS-PAGE, and processed as described in Fig. 4 legend. E, cell membranes from HEK 293 cells expressing various cysteine combinations were isolated and resolved by 7.5% SDS-PAGE without β-ME treatment. F, representative data of BM labeling. The cysteine combinations were labeled with BM and processed as described in Fig. 2 legend. C1035 is the positive control; 5C^- is the negative control. G, representative data of Endo-F deglycosylation assay. Protein samples were processed as described in Fig. 6 legend. Each experiment was performed at least three times.
Biological Importance of Disulfide Bonding in NBCe1-A EL-3

Lack of Disulfide Bonding in EL-3 Had No Effect on NBCe1-A Ion Transport—To assess whether the disulfide bonds in EL-3 are important for NBCe1-A ion transport, we removed the 4 cysteines in EL-3 (NBCe1-A-4C) and compared its base transport activity with WT-NBCe1-A. Transfected 293 cells were first equilibrated in a Na\(^+-\)free HEPES solution and then exposed to a Na\(^+-\)free HCO\(_3\)-buffered CO\(_2\)-equilibrated solution. The rapid influx of CO\(_2\) quickly lowered the intracellular pH (pH\(_i\)). Once pH\(_i\) had stabilized, the cells were bathed in Na\(^+-\)-containing HCO\(_3\)-buffered solution to drive NBCe1-A transport. Fig. 9, A and B, show that NBCe1-A-4C\(^-\) transports base at a similar rate to that of WT-NBCe1-A, indicating that the disulfide bonding in EL-3 has no apparent effect on NBCe1-A total base transport in the HEK 293 cells.

We next tested whether the lack of disulfide bonds in EL-3 affects DIDS (the functional inhibitor of NBCe1-A) inhibition of the transporter. Fig. 9B shows that 1 mM DIDS treatment for 1 h inhibited both WT-NBCe1-A and NBCe1-A-4C\(^-\) base transport to a similar level, suggesting that in the absence of disulfide bonds, the DIDS interaction site(s) has remained intact.

Effect of Abnormal Intermolecular Disulfide Bonding on NBCe1-A Ion Transport—To test whether proper disulfide bonding among the 4 cysteines in EL-3 is important for NBCe1-A ion transport, we analyzed the base transport of NBCe1-A constructs containing various cysteine combinations. Fig. 10A shows that substitution of any of the 4 cysteines or when only Cys\(^{581}\), Cys\(^{585}\), or both were present NBCe1-A transport function was dramatically impaired, but simultane
ous substitution of both Cys\textsuperscript{583} and Cys\textsuperscript{585} or when only Cys\textsuperscript{617}, Cys\textsuperscript{642}, or both were present, the transport function was significantly improved. These results indicate that intermolecular cross-linking between Cys\textsuperscript{583} and Cys\textsuperscript{585} on each monomer restricted the orientation or conformational changes of TM5 in NBCe1-A, which abrogated ion transport, suggesting that TM5 has an important role in NBCe1-A transport function.

**DISCUSSION**

In the present study, we scrutinized the structural importance of the 4 highly conserved cysteines to the topological folding of EL-3, the largest NBCe1-A extracellular loop. Our results demonstrated that the 4 cysteines form two intramolecular disulfide bonds constraining EL-3 in a uniquely folded conformation. This unique conformation is finely tuned by the presence of two N-glycosylations. Moreover, our findings demonstrate that the two EL-3 loops from each monomer reside closely at the NBCe1-A dimeric interface. This close association suggests that a unique domain-like structure is present on the NBCe1-A extracellular surface that potentially has an important physiological role related to NBCe1-A function.

Our results demonstrate clearly that interplay occurs between disulfide bonding and N-glycosylation in EL-3 that
determines the topological folding of this large extracellular loop. This conclusion is supported by the following findings. 1) The 4 cysteines in EL-3 form two intramolecular disulfide bonds, Cys$^{583}$-Cys$^{585}$ and Cys$^{630}$-Cys$^{642}$, respectively, restraining EL-3 in a folded conformation. 2) When one or both of the two disulfide bonds are absent, EL-3 adopts an extended conformation highly accessible to DTT reduction or chymotrypsin digestion. 3) The two disulfide bonds are folded in the NBS1-A protein, unexposed to the extracellular medium, even after 1 M Na$_2$CO$_3$ and 50 mM DTT treatment. 4) The correct formation of the first disulfide bond is dependent on the presence of the second disulfide bond. 5) When the second disulfide bond is absent, the correct formation of the first disulfide bond is suppressed by the presence of N-glycosylation at Asn$^{597}$, Asn$^{597}$, and Asn$^{617}$. 6) When the two N-glycosylation sites Asn$^{597}$ and Asn$^{617}$ were absent, EL-3 became less tightly folded and was accessible to chymotrypsin digestion. These findings indicate that a complex enzymatic orchestration must exist between N-glycosylation and disulfide bond-forming enzymes in the endoplasmic reticulum that determines the final folding of EL-3 during NBS1-A protein maturation.

Our data have also shown that EL-3 contains 83 amino acids ranging from Asp$^{565}$ to Asp$^{647}$. This large extracellular loop is in a folded conformation restrained by the two native disulfide bonds. The formation of the second disulfide bond (Cys$^{630}$-Cys$^{642}$) is spontaneous and unaffected by the N-glycosylation state of EL-3 or the first disulfide bond, whereas formation of the first disulfide bond (Cys$^{583}$-Cys$^{585}$) is dependent on the presence of the second disulfide bond. When Cys$^{630}$ and Cys$^{642}$ were absent, Cys$^{583}$ and Cys$^{585}$ became intermolecularly cross-linked, forming a reduction-sensitive NBS1-A dimer. Interestingly, when only 1 of the 4 cysteines was present in EL-3, it also cross-linked to itself on the opposite monomer to form a reduction-sensitive NBS1-A dimer. These results demonstrate that the two disulfide bonds work synergistically to control EL-3 in a folded conformation, thereby structuring the topological surface of NBS1-A dimer.

EL-3 contains three N-glycosylation consensus sites, but only two are glycosylated in the plasma membrane processed NBS1-A (19). Our data determined that Asn$^{597}$ became glycosylated when either Asn$^{597}$, Asn$^{617}$, or both were absent or when both of the cysteines at 583 and 585 were absent. The simplest explanation is that Asn$^{597}$ is initially glycosylated in the endoplasmic reticulum catalyzed by the oligosaccharyltransferase, and during protein maturation, the glycan is enzymatically removed. Indeed, the oligosaccharyltransferase subunits Ost3p and Ost6p were shown to have oxidoreductase activity (29), which would prevent the disulfide bond formation between Cys$^{583}$ and Cys$^{585}$ during protein synthesis that allows efficient glycosylation at Asn$^{597}$. Our data are compatible with the hypothesis that the first disulfide bond and glycans at Asn$^{597}$ and Asn$^{617}$ form a three-dimensional motif that is required for an endoglycosidase to recognize and remove the complex glycan at Asn$^{597}$. When any of these three requirements is missing, the glycosidase is unable to recognize this site and subsequently unable to remove the glycan at Asn$^{597}$. We predict that this process must have occurred in the second stage of membrane protein folding during NBS1-A dimerization. The detailed enzymatic mechanisms behind this phenomenon require further investigation but clearly have potential general relevance to the biosynthesis of membrane proteins that have a structural and/or functional requirement of N-glycosylation and disulfide bond formation.

One of the major findings in the present study is that each of the substituted cysteines was cross-linked to the corresponding residue on the opposite monomer. The spontaneous intermo-

![Figure 10. Biological importance of disulfide bonding in NBCe1-A EL-3.](image-url)

- **A.** Transport function of NBCe1-A with various cysteine combinations in NBCe1-A EL-3 was assayed as described in Fig. 9 legend. Transport data are expressed as a percentage of NBCe1-A-5C$^-$ function. Error bars represent mean ± S.E. ($n = 6–7$). B, representative data of NBCe1-A protein surface labeling after cycloheximide treatment. 24 h post-transfection, cells expressing WT-NBCe1-A or NBCe1-A-4C$^-$ were incubated with cycloheximide (10 μM) in fresh cell culture medium. At 0, 24, 48, 72, and 96 h post-cycloheximide treatment, the cells were rinsed with PBS and incubated with sulfo-NHS-SS-biotin for 30 min at 4 °C following the manufacturer’s protocol. GAPDH was used as an internal standard. C, summary of sulfo-NHS-SS-biotin surface labeling assay. The level of NBCe1-A protein in each sample was quantified by densitometry and compared with the protein level at 0 h post-cycloheximide treatment. Error bars represent mean ± S.E. ($n = 3$).
Disulfide Bonding in NBCe1-A EL-3

lecular cross-linking requires the 2 cysteines to be in close proximity, indicating that EL-3 from each of the monomers resides closely at the dimeric interface and that the extracellular ends of TM5 or -6 from each monomer are adjacently located. Upon closer examination, we observed an almost evenly distributed number of amino acids (~12) between TM5 and the first disulfide bond, the first disulfide bond and Asn^597, Asn^617 and the second disulfide bond, and Cys^630 and Cys^642. This unique pat-

FIGURE 11. Proposed topological folding of NBCe1-A EL-3. A, EL-3 from each NBCe1-A monomer resides closely at the dimeric interface with the extracellular ends of TM5 and TM6 located adjacently. Each EL-3 is folded in a unique conformation restricted by the two disulfide bonds (red line). The two N-glycosylation sites are indicated as brown diamonds. B, detailed topological folding of EL-3. The two N-glycosylation sites are shown as brown diamonds; two disulfide bonds are shown as red lines; strongly BM-labeled substituted cysteine residues (after DTT treatment) are shown in blue, weakly labeled residues are shown in light blue (K562C was also weakly labeled without DTT treatment), and unlabeled residues are shown in yellow; and substituted cysteines forming cross-linked NBCe1-A dimers are indicated by asterisks.
tern of amino acid distribution indicates that EL-3 folds like a "clover" on the surface of NBCe1-A at the dimeric interface (Fig. 11, A and B). The adjacent parallel location of two symmetrically folded EL-3s from each monomer resembles a domain-like structure on the NBCe1-A extracellular surface.

Disulfide bonds in a transmembrane protein are known to be important for protein structural stabilization and thermodynamics (30). However, our data indicated that NBCe1-A lacking the disulfide bonds in EL-3 had a similar membrane stability compared with WT-NBCe1-A, suggesting that the disulfide bonds in EL-3 do not contribute to the core structural folding of the transporter but rather define the extracellular surface topography.

Disulfide bonding on the surface of the human organic cation transporter 2 and the Cys-loop ligand-gated ion channel receptors was shown to have critical functional roles (20, 21). Our functional assay showed that presence or absence of the two disulfide bonds in EL-3 had no demonstrable effect on NBCe1-A base transport, indicating that in the heterologous expression system the two disulfide bonds in EL-3 do not affect the baseline NBCe1-A base transport activity. Interestingly, our functional assay also showed that intermolecular cross-linking between the mispaired cysteines at 583 or 585 but not at 630 or 642 dramatically impaired the base transport function, suggesting that restriction of the normal orientation or conformational changes of TM5 abrogates NBCe1-A ion transport. It is known that DIDS interacts with the peptide motif 558KKMIK562 in TM5 close to the beginning of EL-3 (22) and that D555E substitution in TM5 converts NBCe1-A from a transporter to an anion channel (23). Our findings provide further support that TM5 has a critical role in NBCe1-A base transport.

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