Covalent Modification of the Nucleotide Binding Domains of Cystic Fibrosis Transmembrane Conductance Regulator*

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Joseph F. Cotten and Michael J. Welsh‡
From the Howard Hughes Medical Institute and Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

The cytosolic nucleotide binding domains of cystic fibrosis transmembrane conductance regulator (NBD1 and NBD2) mediate ATP-dependent opening and closing of the Cl⁻ channel pore. To learn more about NBD structure and function, we introduced a cysteine residue into the Walker A motif or the LSGGQ motif of each NBD and examined modification by N-ethylmaleimide (NEM). Covalent modification of either Walker A motif partially inhibited cystic fibrosis transmembrane conductance regulator channel activity, decreasing the open state probability by prolonging the long closed duration. An increase in cytosolic ATP concentration slowed the rate of modification. The data suggest that both NBDs interact with ATP to influence channel opening and that inhibition by NEM modification was in part due to decreased ATP binding. When cysteine was placed in the NBD2 Walker A motif, it was modified more rapidly than when it was placed in NBD1, suggesting that the NBDs are not structurally or functionally identical. Modification of a cysteine inserted in the LSGGQ motif of either NBD1 or NBD2 also inhibited channel activity. The rate of modification was comparable with that of a thiol in free solution, suggesting that the LSGGQ motif resides in a surface-exposed position in both NBDs.

The cytosolic nucleotide binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP- and phosphorylation-regulated Cl⁻ channel defective in patients with the genetic disease cystic fibrosis (for reviews, see Refs. 1 and 2). CFTR is a member of the ATP-binding cassette (ABC) family of transport proteins. This family also includes P-glycoprotein, a drug efflux transport protein involved in multidrug resistance in tumor cells (for a review, see Ref. 3). CFTR contains two membrane-spanning domains, each with six membrane-spanning sequences, that form an anion-selective pore (4–7). Like other members of the ABC transporter family, CFTR possesses two cytosolic nucleotide binding domains (NBD1 and NBD2). ATP binding and hydrolysis by the two NBDs regulates the opening and closing of the channel pore (8–15). Several studies suggest that ATP hydrolysis at NBD1 may precede channel opening, and ATP hydrolysis at NBD2 may precede channel closing (10, 11, 13). Thus, despite shared amino acid sequences (~29% identity), NBD1 and NBD2 appear to have different roles in controlling CFTR channel activity.

However, our understanding of how nucleotides regulate CFTR is incomplete. Although several models have been proposed (13, 14, 16), the mechanism and consequences of the ATP hydrolytic cycle at the NBDs is still not known. Studies of CFTR and other ABC transporters indicate that there is a functional interdependence between the NBDs in mediating ATPase activity (15, 17–20); both NBDs must be functional for ATPase activity to occur. The physical basis of this interdependence has not been determined. Similarly, the timing and the role of nucleotide binding and hydrolysis at each NBD relative to channel opening and closing are not fully established.

At least three consensus sequences have been identified in the NBDs of CFTR and other ABC transporters: the Walker A and Walker B motifs and the LSGGQ motif (21, 22). Crystallographic and mutagenesis studies on other proteins have clearly shown the importance of the Walker A and B motifs in nucleotide binding and hydrolysis (for a review, see Ref. 23). However, the function of the LSGGQ motif, a motif unique to and well conserved across all ABC transport proteins, is not well understood (22, 24–26). Modeling studies have suggested that this motif may mediate an interaction between the NBDs and the membrane-spanning domains (24, 25, 27); sequence similarities with G proteins and mutagenesis studies, however, have suggested a role for this motif in mediating ATP hydrolysis (26, 28).

To learn more about ATP regulation of CFTR and its structural basis, we introduced cysteine residues at analogous sites in the two NBDs of CFTR and studied the consequences of their covalent modification with the sulfhydryl-reactive reagent N-ethylmaleimide (NEM). Earlier work showed that the ATPase activity of P-glycoprotein is inhibited in a nucleotide-dependent manner by NEM modification of an endogenous cysteine residue in the Walker A motif of either NBD1 or NBD2 (17, 18). We therefore used P-glycoprotein sequence to guide us in introducing a cysteine residue into the Walker A motif of NBD1 or NBD2 of CFTR. We also introduced a cysteine at the serine position of the LSGGQ motif in NBD1 or the LSGGQ-like motif (LSHGH) in NBD2. Following transient expression in HeLa cells, these mutants were studied using the excised, inside-out patch clamp technique. We examined their functional response to modification by NEM and the effect of nucleotides on the rate and the consequences of NEM modification.

EXPERIMENTAL PROCEDURES
Site-directed Mutagenesis and Transfection—CFTR mutants were prepared in the pTM1-CFTR4 plasmid using the method of Kunkel (29).
Mutations were verified by restriction digestion and by sequencing around the site of the mutation. *In vitro* transcription and translation of each mutant was performed to assess expression of full-length protein. Wild-type and mutant CFTRs were transiently expressed in HeLa cells using the vaccinia virus/T7 bacteriophage hybrid expression system as described previously (9). Cells were studied routinely 4–24 h after transfection, depending on the level of expression desired.

Patch Clamp Technique—Methods used for excised, inside-out patch clamp recordings were as described previously (13, 30). Voltages were referenced to the extracellular side of the membrane. Macropatch and clamp recordings were as described previously (13). Cells were studied routinely 4–24 h after transfection, depending on the level of expression desired.

To evaluate reaction of 5-thio-2-nitrobenzoic acid with NEM (32)—Covalent modification of CFTR, NBD2-Cys was performed to assess expression of full-length protein.

Results are presented as means ± S.E. for n observations. Statistical
Covalent Modification of CFTR

RESULTS

NEM Modification of Either NBD Inhibits CFTR Cl⁻ Channel Activity—Fig. 1 shows the amino acid sequence for the Walker A motif of NBD1 and NBD2 of both CFTR and P-glycoprotein. We introduced a cysteine residue by site-directed mutagenesis at residue 462 in NBD1 (termed the NBD1-Cys mutant) or residue 1248 in NBD2 (termed the NBD2-Cys mutant). These sites correspond to the location of cysteines in P-glycoprotein (Fig. 1). Both NBD1-Cys and NBD2-Cys mutants also contain the C832A mutation. This mutation largely removes the stimulatory effect of 100 μM NEM on wild-type CFTR but does not otherwise alter channel function (33).

To determine the functional consequences of NEM modification of the NBDs, we applied 100 μM NEM to the cytoplasmic side of excised membrane macropatches containing CFTR-C832A (control), NBD1-Cys, or NBD2-Cys mutant channels. As we have shown previously (33), 100 μM NEM had a slight stimulatory effect on CFTR-C832A channel activity (Fig. 2A). In contrast, NEM irreversibly inhibited the activity of both NBD1-Cys and NBD2-Cys (Fig. 2, B and C). NBD2-Cys was modified more rapidly than NBD1-Cys. The addition of vehicle alone (ethanol) had no effect on channel activity (33).

Because the NBDs of CFTR are thought to interact with ATP, we hypothesized that ATP may alter the rate of NEM modification. In addition, because NEM modification occurs at a potential ATP binding site, we further speculated that after channels were completely modified by NEM, the ATP dependence would be altered, perhaps due to altered ATP affinity. To test this, we examined the effect of NEM in the presence of increasing concentrations of ATP. Fig. 2, B and C, shows that as ATP concentration increased, inhibition by NEM occurred more slowly. In Fig. 3, we quantify this effect. For the NBD2-Cys mutant, the decline in current following NEM application was well fit by a single decay exponential plus a constant (see legend to Fig. 3A). This allowed us to quantify differences in the rate of modification and the effect of ATP concentration on fully modified channels. The reciprocal of the time constant, τ, derived from this analysis and shown in Fig. 3A represents the pseudo-first order rate constant for NEM modification. The constant, Iₙ, represents current through completely modified channels relative to that measured before NEM modification under the same conditions. When we studied the NBD1-Cys mutant at ATP concentrations > 0.1 mM, the time course of inhibition was not well fit by a single exponential; therefore, in Fig. 3B we report only the percentage of current remaining at 5 min (however, the data at 0.1 mM ATP were well fit and are reported below). The NEM response of the CFTR-C832A mutant is also quantified in Fig. 3B. For the NBD2-Cys mutant, the estimated τ increased with increasing ATP (Fig. 3A); for the NBD1-Cys mutant, the percentage of current remaining at 5 min also increased with increasing ATP (Fig. 3B). Because ATP decreased the rate of NEM modification, ATP binding must alter the accessibility or reactivity of the introduced Walker A cysteine.

After complete modification by NEM of all the channels in a patch, some channel activity remained in NBD2-Cys (Fig. 3A, Iₙ). This indicates that NEM-modified channels retain function, albeit diminished. Fig. 3A also shows the effect of ATP concentration on activity relative to that in unmodified channels. Increasing ATP concentrations at least partially overcame the inhibitory effects of NEM modification. This observation suggests that at least part of the effect of NEM modification is due to decreased ATP binding.

The effects observed in the presence of increasing ATP were not due simply to increasing Mg²⁺ or a decrease in NEM reactivity. The rate of modification and ATP dependence of the fully modified NBD2-Cys mutant were similar at high and low MgCl₂ concentrations (τ = 16 ± 3 versus 10 ± 1 s, and Iₙ = 2 ± 1 versus 8 ± 4% with 0.3 mM ATP and in the presence of either 13 mM MgCl₂ or 3 mM MgCl₂, respectively; n = 3 each). Similarly, the rate of NEM modification of 5-thio-2-nitrobenzoic acid was not affected by 5 mM ATP or 5 mM PP₁ (see “Experimental Procedures”).

To learn how NEM inhibited current, we studied single channels. Fig. 4 shows current records from excised, inside-out patches containing either a single wild-type CFTR channel (A) or a single NBD1-Cys (B) or NBD2-Cys (C) mutant channel before and after NEM modification. Fig. 4 and Table I show that neither mutant behaved markedly different from the wild-type molecule. NBD2-Cys had a slightly increased open state probability (P₀) relative to wild-type CFTR due primarily to a slightly increased duration of burst activity. Neither single channel conductance nor any of the other time constants calculated for either mutant were significantly different from wild-type CFTR. Our earlier work showed that NEM did not alter the single channel properties of CFTR-C832A (33). However, NEM inhibited NBD1-Cys and NBD2-Cys channels by decreasing the Pₐ (Fig. 4, Table I). The decreased Pₐ was due to a marked increase in the long closed time between bursts of activity (τₐ). Consistent with the macroscopic currents, some activity remained in both mutants following NEM modification. After NEM modification, the mean burst duration decreased in the NBD2-Cys mutant and tended to increase in the NBD1-Cys mutant. However, these changes were small compared with the changes in τₐ.

Pyrophosphate Protects Both NBDs from NEM Modification.
brane voltage clamped at NBD1-Cys (A), NBD2-Cys (B), or NBD2-Cys (C). Tracings were obtained with membrane voltage clamped at −80 mV in the presence of 1 mM cytosolic ATP and, where indicated, following modification by 200 μM NEM. The dashed lines indicate closed state. PKA was removed prior to data collection.

**DISCUSSION**

**NEM Modification of the Walker A Motifs—** Our data show that cysteine residues introduced into the Walker A motifs of both NBD1 and NBD2 are accessible to NEM modification. ATP protected both NBDs from modification, and modification of either NBD decreased channel activity by markedly prolonging the interburst duration. These data provide direct evidence that both NBDs interact with ATP and support the previous conclusion, based on studies of CFTR containing site-directed mutations in the NBDs (9, 13–15), that the functions of both NBDs contribute to channel activity. Protection by ATP might occur if the bound ATP sterically interferes with access of NEM to the introduced cysteines. Alternatively, ATP binding might induce a conformational change in the domain that inhibits NEM access or alters the chemical reactivity of the introduced cysteine. For example, the Walker A motif (P-loop) in other proteins is known to undergo a large conformational change following nucleotide binding (36).

Although there were similarities in the effect of NEM on NBD1 and NBD2, there were also differences. At all ATP concentrations tested, the NBD2-Cys mutant was more readily modified than the NBD1-Cys mutant. This difference in reactivity could have several explanations: the two NBDs may be structurally different, independent of nucleotide binding; the two NBDs might have different ATP affinities; there could be differences in how ATP is bound or positioned; ATP binding might alter the conformation of the two NBDs in different ways; or NBD2 might spend a greater fraction of time in an unbound nucleotide-free state relative to NBD1. Another difference between the two NBDs is that ADP protected NBD2 from modification, but not NBD1. This suggests that compared with NBD1, NBD2 has a higher apparent affinity for ADP, however, since both mutants were studied under different nucleotide concentrations, caution is warranted in making this
interpretation. Nevertheless, this conclusion is consistent with studies showing that site-directed mutations in NBD2, but not NBD1, interfere with the effect of ADP (9). 

PPi, at a stimulatory concentration also inhibited the rate of NEM modification, but the effect was apparent with both NBDs. It is possible that PPi binds to both NBDs, thereby slowing NEM modification. However, earlier studies showed that the effect of PPi was attenuated by site-directed mutations in NBD2 but not NBD1 (35). In addition, PPi increased the burst duration, suggesting an effect at NBD2 (12, 34). If PPi bound to NBD1, it would be expected to prolong the interburst interval, but PPi decreased the interburst interval, making an interaction at NBD1 seem less likely (34). How then does PPi slow NEM modification of both NBDs if it is likely to interact primarily with NBD2? We speculate that PPi binding at NBD2 impairs NEM access to NBD2. PPi binding at NBD2 locks the channel open and may thus lock NBD1 in a nucleotide-bound, NEM-inaccessible state. Future studies will be required to assess this more directly. However, consistent with this hypothesis, we have previously demonstrated that PPi enhances 8-azido-ATP photolabeling of CFTR (34).

After channels were modified by NEM, we found that an increase in ATP concentration increased the amount of current remaining in the NEM-modified NBD2-Cys mutant (Fig. 3A, bottom). Although we were not able to make a similar quantitative analysis of the NEM-modified NBD1-Cys variant, the data are consistent with a similar effect in that mutant. In addition, single-channel studies showed that NEM modification markedly prolonged the long closed period between bursts for both mutants. Earlier studies showed that ATP concentration influences mainly the long closed time, suggesting that ATP binding occurs during the long closed time interval (12, 15, 37). Based on these observations, we speculate that NEM modification may impair access of ATP to its binding site at NBD2 and possibly NBD1 during the long closed interval of channel gating.

Accessibility of the LSGGGQ Motif—The function of the LSGGGQ motif in ABC transporters is not certain. It has been suggested that this motif might be a "linker" between domains of ABC transporters (24, 25). Sequence similarities between the LSGGGQ motif of ABC transporters and a motif in G proteins was the basis for a model in which the motif might be involved in ATP hydrolysis (26, 28). In G proteins, the homologous residues are located on a surface-exposed "switch II" loop. In that position, the glutamine may activate a water molecule for nucleophilic attack on the bound nucleotide. Consistent with this notion, we found that modification of the glutamine by site-directed mutations affected gating as predicted if the glutamine influences the rate of hydrolysis (28). What do our NEM modification data tell us about the positioning of the LSGGGQ motif within the NBDs? Since NEM is lipophilic and can access the interior of a protein as well as its surface, the rate of modification must be considered. We found that the rate of modification of the NBD1-LSGGQ and the NBD2-LSGGQ channels was very rapid. The estimated second order rate constant for NEM modification of a model thiol in free solution, 2-mercaptoethanol (pK<sub>a</sub> = 9.61), at pH 7.3 and 25 °C is 5 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (38). The second order rate constant for NEM modification of the model thiol in free solution, 2-mercaptoethanol (pK<sub>a</sub> = 9.61), at pH 7.3 and 25 °C is 5 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (38). The second order rate constant for NEM modification of the NBD1-LSGGQ and the NBD2-LSGGQ channels was very rapid. The estimated second order rate constant for NEM modification of a model thiol in free solution, 2-mercaptoethanol (pK<sub>a</sub> = 9.61), at pH 7.3 and 25 °C is 5 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (38). The second order rate constant for NEM modification of the model thiol in free solution, 2-mercaptoethanol (pK<sub>a</sub> = 9.61), at pH 7.3 and 25 °C is 5 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup> (38).

![Figure 5. PPi slows the rate of NEM modification of both NBD1-Cys and NBD2-Cys mutants.](image-url) Data were obtained as in Fig. 2. Bars indicate interventions made at the cytosolic surface of the patch.

### Table I

|                | Wild type | NBD1-Cys | NBD1-Cys | NBD2-Cys | NBD2-Cys |
|----------------|-----------|----------|----------|----------|----------|
| NEM            | -         | -        | +        | -        | +        |
| g (pS)<sup>a</sup> | 9.6 ± 0.4 (8) | 9.8 ± 0.2 (9) | 10.3 (1) | 9.3 ± 0.4 (9) | 8.3 ± 0.3 (5) |
| P<sub>n</sub>  | 0.35 ± 0.02 (11) | 0.40 ± 0.02 (9) | 0.01 ± 0.01<sup>b</sup> (5) | 0.52 ± 0.01<sup>c</sup> (7) | 0.05 ± 0.01<sup>d</sup> (6) |
| T<sub>h</sub> (ms) | 149 ± 8 (5) | 145 ± 9 (4) | 205 ± 20 (4) | 200 ± 13 (3) | 120 ± 7<sup>e</sup> (6) |
| τ<sub>c</sub> (ms)<sup>f</sup> | 2.4 ± 0.1 (5) | 2.7 ± 0.3 (5) | ND<sup>j</sup> | 2.7 ± 0.3 (4) | ND<sup>j</sup> |
| τ<sub>e</sub> (ms)<sup>f</sup> | 152 ± 12 (5) | 186 ± 20 (4) | 3 ± 2 × 10<sup>16</sup><sup>b</sup> (4) | 172 ± 57 (3) | 3 ± 1 × 10<sup>16</sup><sup>b</sup> (6) |

<sup>a</sup> Single channel conductance (in picoSiemens).
<sup>b</sup> <i>p</i> < 0.05 relative to channel in absence of NEM.
<sup>c</sup> Fast, interburst closed time.
<sup>d</sup> ND, not done.
<sup>e</sup> Slow, interburst closed time.
<sup>f</sup> Values estimated from P<sub>n</sub> and T<sub>h</sub>.
<sup>g</sup> ND, not done.

### Table II

| Condition | ATP (mM) | PP<sub>i</sub> (mM) | ADP (mM) |
|-----------|---------|-----------------|----------|
| Wild type | 0.3     | 5               | 0        |
| NBD1-Cys | 0.3     | 5               | 0        |
| NBD2-Cys | 0.3     | 5               | 0        |

*ND, not done.*

![Graph showing the effect of PPi on channel activity of wild-type CFTR with no mutations and NBD1-Cys and NBD2-Cys mutants.](image-url) The effect of PPi and ADP on channel activity of wild-type CFTR with no mutations and NBD1-Cys and NBD2-Cys mutants.

Data are percentage of macroscopic current compared with that in the presence of ATP alone (100%). All data were derived from excised, inside-out membrane patches with the membrane potential clamped at −40 mV following the removal of PKA. All interventions were made to the cytosolic side of the patch. Statistical comparisons were made using an analysis of variance followed by a post hoc Bonferroni test. Asterisks indicate <i>p</i> < 0.05 relative to wild-type CFTR. Numbers in parentheses indicate <i>n</i>.

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**Covalent Modification of CFTR**

All data were collected from excised, inside-out membrane patches with the membrane potential clamped at −80 mV in the presence of 1 mM cytosolic ATP and, where indicated, 200 μM cytosolic NEM. Statistical significance was determined using Student’s t test. Numbers in parentheses indicate <i>n</i>.
the reasonable assumption that the reaction proceeds first order with respect to NEM concentration. Note that our data were collected at 34–36 °C instead of 25 °C; however, the reaction rate would probably only double with a 10 °C increase in temperature (39). The NBD1-LCGGQ mutant is therefore modified at a rate similar to that predicted for a thiol in free solution. This suggests that the motif is in a sterically accessible, solvent-exposed position within the NBD. Since it is overwhelmingly the thiolate species that reacts with NEM, our interpretation is based on the assumption that the ionization state of the NBD-LCGGQ cysteine is not grossly distorted by its environment (38).

Work with other proteins suggests that the glycine-rich Walker A motif also forms a surface-exposed loop (frequently referred to as the P-loop) adjacent to the nucleotide binding cleft (40). Interestingly, the rate of modification of a cysteine introduced into the Walker A motif in NBD2 was predicted to be $1.8 \times 10^5$ M$^{-1}$ s$^{-1}$ (based on extrapolation to zero ATP). This value is also comparable with that predicted for a thiol in free solution. Therefore, our data suggest that, like the Walker A motif, the LSGGQ motif is probably not buried within the membrane or within a domain; rather, it is positioned at the protein surface.

**Relationship to Data on P-glycoprotein**—Our data show that there are features of NBD structure and function conserved across at least two ABC transporters, CFTR and P-glycoprotein. Through introduction of a cysteine residue into either NBD of CFTR, we were able to confer upon CFTR NEM sensitivity similar to that of P-glycoprotein. As in P-glycoprotein, NEM sensitivity was competitively modulated by ATP (17, 18), and the NBD2 cysteine (in the context of a cysteineless P-glycoprotein molecule) was more readily modified than the NBD1 cysteine (18). There are some differences between the CFTR data and the P-glycoprotein data. For example, NEM did not modify either P-glycoprotein NBD at ATP concentrations greater than 2 mM. With the CFTR NBD1-Cys mutant, some inhibition was observed at 2 mM ATP, and with the NBD2-Cys mutant, significant inhibition occurred even with ATP concentrations of 10 mM. This observation may reflect structural differences between CFTR and P-glycoprotein, which may dictate differences in ATP-mediated protection or in ATP affinity. Our comparison is complicated by our choice of assay; we are comparing CFTR channel activity, which may or may not be a direct measure of CFTR ATPase activity, with P-glycoprotein ATPase activity. Overall, however, our data provide evidence...
that CFTR ATPase activity is similar to that observed in P-glycoprotein and that CFTR ATPase activity is intimately associated with channel gating.

Finally, our current data as well as earlier studies of CFTR and P-glycoprotein indicate a functional interdependence between NBD1 and NBD2. This suggests that there is a cycle of ATP hydrolysis involving an interaction between the two NBDs. For example, Li et al. showed that the G551D mutation in NBD1 eliminates most of CFTR ATPase activity (15). Valladares trapping experiments by Senior and co-workers (41) suggest that both NBDs hydrolyze ATP; however, when only one becomes “trapped,” neither can hydrolyze ATP. In P-glycoprotein, Loo and Clarke showed that NEM modification of either NBD completely inhibits its ATPase activity (18). Muller et al. (42) demonstrated that mutation of either NBD1 or NBD2 Walker A lysine virtually eliminated P-glycoprotein ATPase activity. Based on these data, Senior and co-workers (19) have proposed that P-glycoprotein ATPase activity involves a cycle of ATP hydrolysis in which NBD1 and NBD2 alternately hydrolyze ATP. In CFTR, models of channel gating also propose a sequential process, with ATP hydrolysis at NBD1 mediating channel opening and ATP hydrolysis at NBD2 mediating channel closing (10, 13, 14, 16). In this regard, it is interesting to speculate that differences in the rate of NEM modification at the two NBDs may reflect asymmetries in the hydrolytic cycle. For example, during a gating cycle, more rapid modification of NBD2 by NEM might occur if NBD2 spends a longer time in an unbound, nucleotide-free state than NBD1.

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