Cysteine Substitutions Reveal Dual Functions of the Amino-terminal Tail in Cystic Fibrosis Transmembrane Conductance Regulator Channel Gating*

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Previously, we observed that the cystic fibrosis transmembrane conductance regulator (CFTR) channel openings are destabilized by replacing several acidic residues in the amino-terminal tail with alanines (Naren, A. P., Cormet-Boyaka, E., Fu, J., Villain, M., Blalock, J. E., Quick, M. W., and Kirk, K. L. (1999) Science 286, 544–548). Here we determined whether this effect is due to the loss of negative charge at these sites and whether the amino-terminal tail also modulates other aspects of channel gating. We introduced cysteines at two of these positions (E54C/D58C) and tested a series of methanethiosulfonate (MTS) reagents for their effects on the gating properties of these cysteine mutants in intact Xenopus oocytes and excised membrane patches. Covalent modification of these sites with either neutral (MMTS) or charged (2-carboxyethylmethanethiosulfonate (MTSCE) and 2-(trimethylammonium)ethylmethanethiosulfonate (MTSET)) reagents markedly inhibited channel open probability primarily by reducing the rate of channel opening. The MTS reagents had negligible effects on the gating of the wild type channel or a corresponding double alanine mutant (E54A/D58A) under the same conditions. The inhibition of the opening rate of the E54C/D58C mutant channel by MMTS could be reversed by the reducing agent dithiothreitol (200 μM) or by elevating the bath ATP concentration above that required to activate maximally the wild type channel (>1 mM). Interestingly, the three MTS reagents had qualitatively different effects on the duration of channel openings (i.e. channel closing rate), namely the duration of openings was negligibly changed by the neutral MMTS, decreased by the positively charged MTSET, and increased by the negatively charged MTSCE. Our results indicate that the CFTR amino tail modulates both the rates of channel opening and channel closing and that the negative charges at residues 54 and 58 are important for controlling the duration of channel openings.

The cystic fibrosis transmembrane conductance regulator (CFTR)\textsuperscript{1} is a cAMP-regulated chloride channel that is expressed in a variety of epithelial tissues (1). Altered CFTR channel function contributes to several human diseases including cystic fibrosis (2) and secretory diarrhea (3). The CFTR channel possesses two transmembrane domains, each of which contains six transmembrane segments, two nucleotide binding domains (NBDs), and a large regulatory domain (R domain) with multiple phosphorylation sites (4). PKA phosphorylation of the R domain and ATP binding at the nucleotide binding domains are required for CFTR channel activation (5–8). CFTR channel gating probably involves physical and functional interactions among the R domain and the NBDs (7, 9, 10), although the precise roles of these domains in controlling channel opening and closing are still unclear. In addition, there have been reports that other regions of CFTR such as the cytoplasmic loops may also modulate CFTR channel function (11, 12).

We observed previously (13, 14) that a cluster of negatively charged residues in a putative helical region of the amino-terminal tail (N-tail) participates in CFTR channel gating. Alanine substitutions at these positions (i.e. Asp-47, Glu-54, Glu-58, and Asp-58) resulted in accelerated deactivation kinetics in intact oocytes and shortened open channel bursts in excised membrane patches (13). A disease-associated mutant that maps to one of these sites (D58N CFTR) exhibited similar alterations in macroscopic current kinetics and open channel burst duration (14). These mutations had no apparent effect on the ATP dependence of channel gating or on bulk phosphorylation of the CFTR protein by PKA but did prevent the very long channel openings that can be induced by the poorly hydrolyzable nucleotide analog AMP-PNP (14). Thus, these mutations in the N-tail destabilize channel openings (i.e. increase channel closing rate) apparently by affecting a gating transition that is downstream of channel activation by phosphorylation and ATP binding (14).

Although the results of our previous mutational analyses indicated that the N-tail regulates CFTR channel closing, these studies provided only limited insight into the specific role of this region in modulating CFTR gating. For example, most of our previous data were obtained for one kind of mutation (Glu/Asp to Ala), which limits our ability to make conclusions about the importance of the charge or bulk of the side groups of the relevant amino acids. In addition, the comparison between the gating properties of wild type and mutant channels is an unpaired comparison that could be confounded by effects of the mutations on the biosynthesis or general structure of the channel protein. Finally, the N-tail could play additional roles in

\textsuperscript{1} The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; MMTS, methylmethanethiosulfonate; MTSCE, 2-carboxyethylmethanethiosulfonate; MTSET, 2-(trimethylammonium)ethylmethanethiosulfonate; AMP-PNP, 5′-adenylylimidodiphosphate; NBDs, nucleotide binding domains; PKA, cAMP-dependent protein kinase; MTS, methanethiosulfonate; DTT, dithiothreitol; N-tail, amino-terminal tail; PCR, polymerase chain reaction; WT, wild type.
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CFTR channel gating that are not particularly sensitive to point mutations in this region. Although we failed to observe a dramatic effect of mutations in this region on channel opening rate, we did observe a modest increase in interburst duration (i.e. closed time) for D58N CFTR (14) as if this mutation also affected the ability of the channel to open. In addition, Chan et al. (15) have recently reported that CFTR channels that have been epitope-tagged (FLAG-tagged) at the extreme amino terminus exhibit fewer channel openings in excised membrane patches (i.e. reduced opening rate) relative to the untagged channel. These observations raise the possibility that the N-tail could participate in multiple aspects of CFTR gating (i.e. both channel opening and closing).

To address these issues we substituted cysteines for two negatively charged amino acids in the N-tail (Glu-54 and Asp-58) and covalently modified these residues in intact Xenopus oocytes and excised membrane patches. The advantages of studying cysteine mutants is that one can acutely and reversibly modify these residues with thiol-specific reagents (i.e. do paired comparisons within the same oocyte or membrane patch). In addition, since cysteines can be modified with methanethiosulfonate (MTS) derivatives with different chemistries (16), such experiments have the potential to provide initial insights into the structural requirements for the involvement of the N-tail in channel gating. We were encouraged to attempt this approach because of reports that the CFTR channel is relatively insensitive to low concentrations of thiol-specific reagents (17–19). Our results indicate that modification of these cysteines with either uncharged or charged MTS reagents markedly and reversibly reduces channel opening rate in excised membrane patches. This inhibition of opening rate was observed at concentrations of MTS reagents that had no effect on the gating of the wild type channel or of a corresponding alanine mutant (E54A/D58A). Interestingly, uncharged and charged MTS reagents had different effects on open channel burst duration (i.e. channel closing rate); specifically, burst duration was increased by the negatively charged MTSCE, decreased by the positively charged MTSET, and virtually unaffected by the neutral MMTS. Our results indicate that the amino-terminal tail can modulate both the opening and closing of the CFTR channel and that the duration of channel openings is dependent on the negative charges at positions Glu-54 and Asp-58.

EXPERIMENTAL PROCEDURES

Mutagenesis—The E54C and E54C/D58C CFTR mutants were generated by PCR mutagenesis. A 1-kilobase pair fragment of wild type CFTR in pCDNA3 (Invitrogen Corp.) was amplified by PCR in the presence of an appropriate mutagenic oligonucleotide. The upstream primer contained a Kpn21 site. After digestion the PCR product was ligated back into pCDNA3-wild type CFTR pre-digested with Asp-718 and Kpn21. Mutations were confirmed by sequencing the entire 1-kilobase pair fragment. For oocyte injections wild type and mutant CFTR cRNAs were prepared using the T7 Megascript transcription kit from Ambion Inc.

Electrophysiology—Female Xenopus laevis were purchased from X. laevis One (Ann Arbor, MI). Frogs were anesthetized in water containing 0.2% MS-222 (Tricaine, 3-aminobenzoic acid ethyl ester) for 30 min before surgery. Oocytes were removed from a 1-cm incision in the abdomen. After suturing the incision, the frog was allowed to recover in water. The frogs were humanely killed after the final collection. Clumps of oocytes were defolliculated in a 10-ml plastic tube of OR-2 solution (92 mM NaCl, 5 mM MgCl2, 2 mM KCl, 5 mM HEPES, pH 7.5) containing 2 mg/ml collagenase A. Defolliculated oocytes were transferred to 50 mM NaCl, 2 mM KCl, 1 mM MgCl2, 0.2 mM CaCl2, 5 mM HEPES, pH 7.5) by the addition of a mixture containing 1 mM isobutylmethylxanthine, 10 μM forskolin, and 200 μM dibutyryl-cAMP. Oocytes were clamped at ~50 mV throughout the experiments. All recordings were performed at 21–23 °C.

For patch clamp experiments the oocytes were shrunk briefly in a bath solution containing 140 mM N-methyl-D-glucamine, 0.5 mM MgCl2, 1 mM EGTA, 10 mM HEPES (pH 7.4 with HCl). The vitelline membrane was then removed with fine tip forceps. Single channel recordings were performed at 21–23 °C in an inside-out excised configuration with a holding potential of ~80 mV (pipette side ground). The pipette solution contained 140 mM N-methyl-D-glucamine, 0.5 mM MgCl2, 0.2 mM CaCl2, 10 mM HEPES (pH 7.4 with HCl). PFK catalytic subunit and Mg-ATP were added to the bath right after the formation of an excised inside-out patch and were present throughout the experiment. The final concentrations of Mg-ATP and PFK catalytic subunit were 1 mM and 80 units/ml, respectively, unless otherwise noted. Bath solutions containing MMTS, MTSET, MTSCE, or DTT were added in a low calcium ND-96 bath solution (two electrode voltage clamp experiments) or in the bath solution used for patch clamping (see above) before each experiment.

Data Analysis—Patch clamp records were filtered at 100 Hz. Only records with stable activity for at least 10 min were analyzed. Single channel analysis was performed using pClamp6 software (Axon Instruments). Closings shorter than 20 ms were considered to be intraburst flickerings (due presumably to block by the HEPES in the bath (20, 21)) and were ignored in our analysis. The kinetic differences between wild type CFTR and the cysteine mutants, and the effects of thiol modification on the cysteine mutants, were also obvious when longer cut-off times (50 or 80 ms) were used (data not shown). Since most patches contained multiple channels, mean open channel burst duration was calculated by the cycle time method described by Mathews et al. (22) and used by us previously (13, 14). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22). Channel open probability (Popen) was calculated as described (13, 14, 22).

RESULTS

The Functional Properties of the Cysteine Mutants Are Similar to Those of the Previously Studied Alanine Mutants—The functional properties of the cysteine mutants were first analyzed in intact oocytes to determine how the cysteine substitutions themselves affected CFTR activity. The cRNAs encoding wild type CFTR, a single cysteine mutant (E54C), or a double cysteine mutant (E54C/D58C) were injected into oocytes and assayed by two-electrode voltage clamp analysis. Both cysteine mutants exhibited lower cAMP-activated macroscopic currents than wild type CFTR when equivalent amounts of cRNA were injected (Fig. 1B), as we had observed previously (13, 14) for the alanine mutants at these positions. In addition, like the corresponding double alanine mutant (E54A/D58A) (13), the double cysteine mutant deactivated faster than wild type CFTR fol-
mixture. More cRNA was injected for the cysteine mutant than for WT CFTR (2 half-time of deactivation was defined as the time required for the current to decline to 50% of the peak level observed in the presence of the cAMP deactivation kinetics for the wild type (WT) channel and the double cysteine mutant observed following washout of the cAMP mixture. The half-time of deactivation was defined as the time required for the current to decline to 50% of the peak level observed in the presence of the cAMP mixture. More cRNA was injected for the cysteine mutant than for WT CFTR (2 versus 0.5 ng) to achieve approximately the same absolute current levels following activation with the cAMP mixture.

allowing removal of the cAMP-activating mixture (Fig. 1C). As will be shown below, E54C/D58C also exhibits reduced channel \( P_o \) and briefer channel openings in excised membrane patches relative to the wild type channel. Thus, the cysteine mutations in the N-tail affect CFTR channel activity much like the corresponding alanine substitutions that we had characterized previously (13, 14).

The Cysteine Mutants Are Inhibited by Covalent Modification with a Membrane-permeant MTS Reagent in Intact Oocytes—We next tested the responses of the cysteine mutants to MMTS, which covalently attaches a thio-methyl group to cysteine through disulfide bonding (see Fig. 1A for formula). MMTS is neutral, small, and lipid-soluble and, therefore, can cross to the cytoplasmic side of the membrane when applied extracellularly (23). The currents mediated by the double cysteine mutant and, to a lesser extent, the single cysteine mutant were inhibited when MMTS (10 \( \mu M \)) was applied to the bath after currents had been first activated with the cAMP-containing mixture (Fig. 2, A and B). The representative current-voltage relationship shown in Fig. 2C indicates that the MMTS-induced inhibition of the currents mediated by E54C/D58C was due to lowered macroscopic conductance (i.e. reduced slope) rather than to a change in concentration driving force (i.e. altered reversal potential).

We also tested the effects of MMTS on the wild type channel (WT CFTR) and the double alanine mutant (E54A/D58A) to determine if the inhibition was specifically due to modification of the engineered cysteines at residues 54 and 58. At this concentration MMTS had small effects (5–10% inhibition) on the macroscopic currents mediated by WT CFTR and E54A/D58A in intact oocytes (Fig. 2, A and B) and had no effects on the channel activities of these constructs in excised patches (see below). Greater inhibition of the currents mediated by E54C/D58C was observed at higher MMTS concentration (1–5 mM), but nonspecific effects on wild type CFTR became significant at these considerably higher doses (results not shown). Consequently, 10 \( \mu M \) MMTS was used for the patch clamp studies described below. We also tested two charged, hydrophilic MMTS analogs (MTSET and MTSCE; see Fig. 1A for formula) for their effects on the currents mediated by the double cysteine mutant in intact oocytes (Fig. 2D). These membrane-impermeant reagents had no effect when applied extracellularly at a 10-fold greater concentration (100 \( \mu M \)) than that used for our MMTS experiments, which is consistent with the presumed cytoplasmic orientation of the CFTR amino-terminal tail (4).

Covalent Modification of E54C/D58C in Excised Membrane Patches, Differential Responses to MMTS, MTSET, and MTSCE—Since the activity of the double cysteine mutant was substantially affected by thiol modification in intact oocytes, we performed a series of patch clamp studies of the E54C/D58C channel in excised inside-out patches. The goals of these experiments were as follows: (i) to compare the gating properties of this N-tail mutant to those of wild type CFTR, and (ii) to test the effects of thiol modification of the engineered cysteines on CFTR channel gating in excised inside-out patches. The unmodified E54C/D58C mutant channel exhibited an ~50% lower \( P_o \) than WT CFTR (Fig. 3) under conditions that maximally activate the wild type channel (80 units/ml PKA; 1.0 mM Mg-ATP). This reduction in \( P_o \) was due in part to shorter open channel bursts (Fig. 3D), as we had observed previously (13, 14) for the corresponding alanine mutants. No obvious effect of these mutations on channel opening rate was detected (Fig. 3D). We next tested the effects of the neutral MMTS on the gating properties of E54C/D58C in excised patches, since this compound inhibited the macroscopic currents mediated by the cysteine mutants in intact oocytes. When added to the cytoplasmic face of excised patches, MMTS had little effect on the channel activity in excised patches occurred within 1–5 min of adding MMTS to the patch (results not shown). Interestingly, the MMTS-induced reduction in the \( P_o \) of the double cysteine mutant was due almost entirely to a marked inhibition of opening rate (Fig. 3D). We could detect no significant effect of MMTS on open channel burst duration (i.e. the duration of channel openings).

If the effect of MMTS on the gating of the E54C/D58C channel is due to the formation of a mixed disulfide at these positions, then this effect should be reversed by a reducing agent such as DTT. Fig. 4 shows that the inhibitory effects of MMTS on the gating of E54C/D58C were completely reversed by the subsequent addition of 200 \( \mu M \) DTT. The \( P_o \) and channel opening rate of the double cysteine mutant completely recovered from MMTS inhibition within 1–5 min of adding DTT to the bath. At this low concentration DTT had negligible effects on the gating of the wild type channel (results not shown) or on the gating of E54C/D58C in the absence of MMTS (Fig. 4, A and B). The fact that DTT alone had no effect on the gating of E54C/
D58C indicates that the lower channel activity of this mutant is unlikely due to formation of an intramolecular disulfide bond between the two engineered cysteines in the N-tail.

We also tested the effects of the two charged MTS reagents on the gating properties of the double cysteine mutant in excised patches, i.e. the positively charged MTSET and the negatively charged MTSCE. Although these charged reagents had no effect on the activity of the double cysteine mutant when applied extracellularly to intact oocytes (Fig. 2D), each inhibited E54C/D58C channel activity when applied to the cytoplasmic face of an inside-out patch. Like MMTS, the positively charged MTSET inhibited $P_o$ and channel opening rate of the double cysteine mutant (Figs. 5, B and C) but had no effect on the channel activity of wild type CFTR (Fig. 5A) or the double alanine mutant (data not shown). However, unlike the case for the neutral MMTS, MTSET also significantly inhibited the open channel burst duration of the double cysteine mutant (Fig. 5C). Thus, modification of the E54C/D58C channel with the positively charged MTS reagent not only inhibited channel opening rate but also further shortened the channel openings in excised membrane patches.

The negatively charged MTSCE had a qualitatively different effect on the gating properties of the double cysteine mutant. Although modification with MTSCE also inhibited channel opening rate ($P_o$, burst duration, and channel opening rates for WT CFTR and the double cysteine mutant. *Asterisks indicate values that are significantly different ($p < 0.05$) from pre-MMTS values.

**Fig. 2.** A cell-permeant MTS reagent (MMTS) inhibits the N-tail cysteine mutants in intact oocytes. A, representative traces showing the effects of adding MMTS to the bath (10 μM) on the currents mediated by the indicated CFTR constructs in intact oocytes. B, mean data (±S.E.) showing effects of 10 μM MMTS on wild type and mutant CFTR currents. More cRNA was injected for the mutants than for WT CFTR to achieve approximately the same absolute current levels following activation with the cAMP mixture (2 ng for E54C/D58C; 1 ng for E54C and E54A/D58A, and 0.5 ng for WT CFTR). C, representative I-V curves before and after MMTS (10 μM) modification of the E54C/D58C mutant channel. D, lack of effect of extracellular MTSSET and MTSCE (100 μM each) on E54C/D58C CFTR currents.

**Fig. 3.** MMTS inhibits E54C/D58C channel activity in excised inside-out membrane patches primarily by decreasing channel opening rate. A and B, representative channel records showing negligible effects of 10 μM MMTS on the gating of WT CFTR and E54A/D58A CFTR. C, representative channel records showing the marked inhibition of E54C/D58C channel activity in excised patches by 10 μM MMTS. The records in A–C were obtained before and 3–5 min after MMTS addition to the bath. D, mean data (±S.E.) showing the effects of 10 μM MMTS on $P_o$, burst duration, and channel opening rates for WT CFTR and the double cysteine mutant. *Asterisks indicate values that are significantly different ($p < 0.05$) from pre-MMTS values.
E54C/D58C mutant channel. In fact, the mean open channel burst duration for the double cysteine mutant increased to wild type levels following modification with the negatively charged MTSCE (Fig. 6B; where mean burst duration was calculated for multichannel patches using the cycle time method (see “Experimental Procedures” and Refs. 14 and 22)). Similar results were obtained when we generated histograms of open channel bursts for patches containing only one detectable channel each (Fig. 7). Although the latter analysis is made more difficult by the fact that MTS-modified channels have low channel opening rates (and, hence, fewer openings to analyze), it is clear that modification with the positively charged MTSET and the negatively charged MTSCE caused shifts toward briefer and longer openings, respectively. Thus, the duration of CFTR channel openings can be increased by modification of the engineered cysteines in the N-tail with a negatively charged MTS reagent but not with a neutral or positively charged reagent. These results, taken together with our initial observation that open channel burst duration was reduced by replacing the original acidic residues at these positions (Glu-54 and Asp-58) with alanines (13, 14) or cysteines (this study), indicate that the negative charge in this region of the N-tail plays a significant role in stabilizing CFTR channel openings.

Covalent Modification of the Double Cysteine Mutant Alters AMP-PNP Activation and ATP Sensitivity—The channel openings exhibited by the wild type CFTR channel can be stabilized by AMP-PNP (a poorly hydrolyzable ATP analog) when added in combination with ATP at room temperature (14, 22). Fig. 8 shows that the activity ($P_\text{o}$) of the E54C/D58C mutant channel can also be stimulated by the addition of 3 mM AMP-PNP (in the presence of 1 mM ATP) and that this effect is due to a large increase in open channel burst duration. This is qualitatively similar to the effect of AMP-PNP on wild type CFTR, although the degree of activation for the cysteine mutant was somewhat smaller than that previously observed for the wild type channel (wild type $P_\text{o}$ increases to nearly 0.9 under these conditions (14)). Given these initial results we performed “order of addition” experiments on excised patches containing the double cysteine mutant.
cysteine mutant channel: (i) to determine if activating this channel with AMP-PNP protects against the inhibitory effects of MMTS modification on E54C/D58C channel activity and (ii) to determine if thiol modification of the N-tail cysteines influences subsequent activation by AMP-PNP. Fig. 8 shows that prior addition of AMP-PNP failed to protect the double cysteine mutant from MMTS inhibition. MMTS still markedly reduced the $P_o$ of the E54C/D58C channel, although in this case the inhibition by MMTS was due to reductions in both channel opening rate and burst duration. No differences in the time course of MMTS inhibition in the presence or absence of AMP-PNP were observed (results not shown).

When AMP-PNP was added after MMTS treatment, this nucleotide analog did induce small increases in $P_o$ and burst duration (Fig. 9). However, these increases were much smaller than those induced by treatment of the unmodified channel with AMP-PNP (compare with Fig. 8), and AMP-PNP failed to rescue the much reduced channel opening rate of the MMTS-modified channel. Conversely, AMP-PNP had a dramatic effect on the gating of the E54C/D58C channel when this cysteine mutant was first modified with the negatively charged MTSCE (Fig. 9). In 4 out of 4 patches we observed that AMP-PNP addition to the MTSCE-modified channel induced the appearance of very long open channel bursts that could last for several minutes. Such exceptionally long bursts are characteristic of the wild type channel when exposed to AMP-PNP under these conditions (14, 22) but are never observed for the unmodified E54C/D58C channel or the corresponding alanine mutants (14). Thus, covalent modification of the engineered cysteines in the N-tail with the negatively charged MTS reagent not only stabilized channel openings in the absence of AMP-PNP (Figs. 6 and 7) but also recovered the long open channel bursts that are normally induced by this poorly hydrolyzable nucleotide analog.

Finally, we determined if we could reverse the inhibitory effects of MTS modification on channel opening rate by elevating the bath ATP beyond that required to maximally activate the wild type channel (i.e., $>1$ mM). The rationale for these experiments were 2-fold: (i) CFTR channel opening is activated by ATP binding to one or both NBDS (5–8), and (ii) each MTS reagent inhibited the channel opening rate of the double cysteine mutant. Fig. 10 shows that increasing the ATP concentration to 10 mM following modification of the E54C/D58C channel with MMTS nearly completely reversed the inhibitory effects of thiol modification on $P_o$ and channel opening rate.

These data indicate that covalent modification of the double cysteine mutant reduces its ATP sensitivity, which probably explains the inhibitory effects of the thiol reagents on channel opening rate.

**DISCUSSION**

We reported previously (13, 14) that a cluster of negatively charged residues in the amino-terminal tail of CFTR (Asp-47, Glu-51, Glu-54, and Asp-58) participates in the gating of this chloride channel. Mutating these negatively charged residues to alanine destabilized CFTR channel openings in excised membrane patches (13, 14). We also observed that a disease-associated mutant (D58N) that maps to this region exhibited unstable openings (i.e. shortened open channel burst duration) in excised patches (14). Based on our previous results (14) we proposed that the N-tail controls channel gating by facilitating
the transition from a brief open state to a long open state and by stabilizing this long opening state. The biochemical mechanism by which the N-tail participates in channel gating is unknown, although it may involve an interdomain interaction with the R domain and/or NBD1 (13).

To explore further the role of the amino-terminal tail of CFTR in channel gating, we substituted cysteines for two of the acidic residues in the N-tail that appeared to play a significant role in channel gating (Glu-54 and Asp-58). These mutants allowed us to examine in more detail the involvement of the N-tail in CFTR channel gating by acutely modifying the engineered cysteines with different thiol-reactive reagents. The cysteine substitutions per se affected macroscopic currents in intact oocytes and single channel behavior in excised patches much like the alanine and asparagine substitutions at these positions. Subsequent modification of these cysteines with any of three MTS reagents reduced channel activity in excised membrane patches primarily by decreasing the rate of channel...
opening. This inhibitory effect was not observed for the wild type channel or for a corresponding double alanine mutant (E54A/D58A), which argues against a nonspecific effect of these reagents on channel activity. This inhibition could also be reversed by DTT at a concentration (200 μM) below that which affects the activity of the unmodified E54C/D58C channel (Fig. 4) or the wild type channel. (Gating of wild type CFTR can be affected by DTT at millimolar concentrations of this reducing agent.) Thus, the simplest explanation of our data is that the MTS reagents affected CFTR gating by forming mixed disulfides with the engineered cysteines at positions 54 and 58 in the CFTR amino-terminal tail.

The marked effect of thiol modification on channel opening rate differs from what we observed to be the most obvious effect of the N-tail mutations themselves, which was to decrease open channel burst duration (i.e. to increase channel closing rate). A role for the N-tail in modulating channel opening rate is also supported by the recent findings of Chan et al. (15), who reported that CFTR channels that had been epitope-tagged at the amino terminus exhibited reduced opening rates in excised patches. Our prior failure to detect an obvious effect of the alanine and cysteine substitutions on channel opening rate may be due to the fact that absolute rates of channel opening can be overestimated in multichannel patches that contain mutant channels with low activity (i.e. in patches where channel number can be underestimated). It is possible that these mutations do indeed decrease opening rate, but this effect is difficult to detect when performing unpaired comparisons between multichannel patches containing wild type and N-tail mutant channels. This uncertainty illustrates the value of acutely modifying channels in individual patches, as performed in this study. On the other hand, it is also possible that the regulation of channel opening rate by the N-tail is less sensitive to point mutations at these positions than the modulation of burst duration. This latter point is supported by our observation that the regulation of opening rate and of burst duration appear to have different charge dependencies. In particular, the regulation of opening rate appears to be independent of the net charge at these positions, since opening rate was inhibited by modification with uncharged, positively charged, or negatively charged MTS reagents. This is unlike the case for burst duration, which is dependent on the negative charges at these residues in the N-tail (as discussed below).

The inhibition of opening rate by thiol modification of the E54C/D58C mutant channel could be reversed by elevating the bath ATP concentration beyond that normally required to maximally activate the wild type channel. Channel opening is presumably driven by ATP binding to one or both NBDs (5–8). It would appear, then, that modification of the N-tail cysteines inhibited channel opening rate primarily by reducing the ATP sensitivity of channel activation. One possible mechanism by which thiol modification of the N-tail cysteines could inhibit the ATP sensitivity of channel opening would be to inhibit phosphorylation of the R domain (22). We observed previously that the isolated N-tail could bind in vitro to a peptide fragment of CFTR (residues 595–813) that included the distal portion of NBD1 followed by the R domain (13). (At the time of that study residues 595–813 were thought to constitute the R domain alone (4); however, the more recent functional data of Chan et al. (15) indicate that NBD1 probably extends to between residues 622 and 634.) N-tail mutations that disrupt binding to this NBD1/R domain fragment have no effect on channel phosphorylation (14), but it is possible that R domain phosphorylation could be affected by chemical modification of these sites. An alternative point of view would be that the N-tail influences more directly the interactions of ATP with the NBDs or the coupling of channel gating to nucleotide binding. This would be consistent with our recent evidence that the N-tail physically associates with the distal portion of NBD1 between residues 595 and 623. Thus, it is conceivable that the amino tail interacts with a docking site(s) at or near the NBDs (in particular, NBD1) and thereby influences the abilities of these domains to modulate channel gating.

Although the major effect of MMTS on E54C/D58C gating was to decrease channel opening rate, this reagent also inhibited the response of the double cysteine mutant to the poorly hydrolyzable AMP-PNP. AMP-PNP stimulates the activity of wild type CFTR and, to a lesser extent, the E54C/D58C mutant by stabilizing channel openings (i.e. by increasing burst duration). Treatment of the cysteine mutant with MMTS either before or after AMP-PNP addition attenuated the effect of this ATP analog on burst duration. This result is consistent with the notion that the N-tail can modulate both channel opening rate and the stability of channel openings (i.e. closing rate), as discussed below.

In contrast to their similar effects on channel opening rate, the three MTS reagents had qualitatively different effects on the duration of channel openings exhibited by the E54C/D58C mutant. Open channel burst duration was negligibly changed, decreased, and increased by the neutral MMTS, the positively charged MTSET, and the negatively charged MTSCE, respectively. Covalent modification with the negatively charged MTSCS recovered the very long open channel bursts (>1 min) that are normally exhibited by the wild type channel (but not unmodified E54C/D58C) following exposure to AMP-PNP. These data are consistent with the shorter channel openings that were previously observed for the disease-associated D58N mutant (14), which harbors a structurally subtle substitution with the exception of the loss of negative charge at this position (asparagine for aspartate). Thus, the negative charges at these positions in the N-tail appear to play a significant role in stabilizing CFTR channel openings.

In summary, our results indicate that the CFTR amino-terminal tail modulates both the rate of channel opening and the rate of channel closing (i.e. the duration of channel openings). The modulation of opening rate by the N-tail is ATP-dependent but relatively insensitive to the net charge at positions 54 and 58 in the N-tail. Conversely, the regulation of burst duration is highly sensitive to mutations or modifications that affect the net negative charge at these residues. The N-tail presumably exerts its effects on channel gating by means of intramolecular interactions with components of the gating machinery (e.g. the R domain and NBDs (13)) and/or with the pore itself. Perhaps the involvement of the N-tail in regulating both the opening and closing of the channel reflects two different sites of interaction for the N-tail (i.e. one interaction that modulates opening rate and another that modulates closing rate). However, other mechanisms are also possible. Further biochemical studies will be required to map precisely the docking site or sites within the CFTR polypeptide with which the N-tail interacts to modulate the opening and closing of the channel. Since the N-tail can regulate multiple aspects of CFTR channel gating, this region is a potentially interesting target for physiologic modulators of CFTR activity that can bind to this tail (24, 25) or for the development of drugs to treat diseases that are caused by defective regulation of this ion channel (2, 3).

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