Variable Reactivity of an Engineered Cysteine at Position 338 in Cystic Fibrosis Transmembrane Conductance Regulator Reflects Different Chemical States of the Thiol*

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In a previous study of T338C CFTR (cystic fibrosis transmembrane conductance regulator) we found that protons and thiol-directed reagents modified channel properties in a manner consistent with the hypothesis that this residue lies within the conduction path, but the observed reactivity was not consistent with the presence of a single thiolate species in the pore. Here we report results consistent with the notion that the thiol moiety may exist in at least three chemical states, the simple thiol, and two altered states. One of the altered states displays reactivity toward thiols like dithiothreitol and 2-mercaptopropanethanol as well as mixed disulfides (methanethiosulfonate reagents: MTSET, MTSES) and an alkylating agent (iodoacetamide). The other altered state is unreactive. The phenotype associated with the reactive, altered state could be replicated by exposing oocytes expressing T338C CFTR to CuCl₂, but not by glutathionylation or nitrosylation of the thiol or by oxidation with hydrogen peroxide. The results are consistent with the hypothesis that substituting a cysteine at 338 can create an adventitious metal binding site. Metal liganding alters thiol reactivity and may, in some cases, catalyze oxidation of the thiol to an unreactive form such as a sulfenic or sulfonic acid.

The technique of cysteine scanning mutagenesis has been widely used to probe the structure and functional properties of ion channels (1–8), including the cystic fibrosis transmembrane conductance regulator (CFTR) (9–11). The strategy is based on the notion that the reactive thiolate anion can interact with a variety of reagents including mixed disulfides (8, 12–14), alkylating reagents (12, 15, 16), and metals (17–20). In such experiments a typical first step is to screen various mixed disulfides (methanethiosulfonate reagents: MTSET, MTSES) and an alkylating agent (iodoacetamide). The other altered state is unreactive. The phenotype associated with the reactive, altered state could be replicated by exposing oocytes expressing T338C CFTR to CuCl₂, but not by glutathionylation or nitrosylation of the thiol or by oxidation with hydrogen peroxide. The results are consistent with the hypothesis that substituting a cysteine at 338 can create an adventitious metal binding site. Metal liganding alters thiol reactivity and may, in some cases, catalyze oxidation of the thiol to an unreactive form such as a sulfenic or sulfonic acid.

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4 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; WT, wild type; MTSET, 2-trimethylammoniummethyl methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; 2-ME, 2-mercaptopropanethiol; DTT, dithiothreitol; IAM, iodoacetamide; SNAP, S-nitroso-N-acetylpenicillamine; TAPS, 3-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-1-propanesulfonic acid.

MATERIALS AND METHODS

Mutagenesis and in Vitro Transcription—The methods used for mutagenesis and in vitro transcription were similar to those reported previously (11, 22). Briefly, CFTR mutants were generated using the QuikChange™ site-directed mutagenesis kit from Stratagene. The sequences in the region of the mutation and in the entire PCR-generated region were confirmed by direct DNA sequencing.

The T338C CFTR mutants used in this study were generated on two different CFTR backgrounds, either the conventional WT background or a Cys-less background in which all the 18 endogenous cysteines were substituted with alternative amino acids (serine or leucine). The Cys-less CFTR construct was a gift from Drs. Martin Mense and David Gadsby and was used in the pGEMHE vector as previously described by Chan et al. (23). Mense et al. (24) showed that the single-channel gating of Cys-less CFTR depends on protein kinase A and ATP in a manner similar to that of WT CFTR channels expressed in oocytes. Our preliminary experiments indicated that Cys-less CFTR behaved similarly to WT CFTR in terms of its anion selectivity, the I–V shape, and its nonresponsiveness to thiol-directed reagents. To distinguish the two T338C mutants, the construct generated on the Cys-less background is labeled as T338C/Cys-less CFTR and that generated on the WT background is labeled T338C or T338C/WT CFTR.

The CFTR cRNAs for Xenopus oocyte injection were synthesized using the in vitro transcription kit, mMessage mMachine or Ambion mMessage mMachine T7 Ultra transcription kit for the Cys-less CFTRs (Ambion, Inc., Austin, TX). The transcription products were purified and the quality and quantity of the transcripts were assessed on an agarose gel (11, 22).

Oocyte Preparation and Electrophysiological Recordings—Protocols for preparing oocytes are described in Liu et al. (25) and the methods for whole cell recordings were identical to those described in Liu et al. (25).
Briefly, individual oocytes were placed in the recording chamber and continuously perfused with frog Ringer solution containing (in mM): 98 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES-HemiNa, pH 7.4. HEPES was replaced with TAPS to buffer the bathing solution to pH 9.0. All experiments were conducted at pH 7.4 unless specified. The volume of the perfusion chamber was about 100 μl and the flow rate was about 4 ml/min. Room temperature was between 19 and 22 °C. A two-electrode voltage clamp system (TEVC-200, Dagan Corp., Minneapolis, MN) was used for data acquisition. Oocytes were maintained under open circuit conditions, and periodically, the membrane potential was ramped from -110 to +60 mV over a period of 1.8 s to construct the whole cell I–V plots. The conductance was calculated from the slope of the I–V plot at the reversal potential (g_{Cl} at V_m = E_{rev}) using a voltage range from V_m = E_{rev} - 10 mV and V_m = E_{rev} + 10 mV. Data are reported as mean ± S.E.

**RESULTS**

T338C/WT CFTR Conductance Was Markedly Altered by 2-ME or DTT Prior to Exposure to Exogenous Thiol-directed Reagents—Exposing oocytes expressing T338C/WT CFTR to 2-ME or DTT during steady state activation led to increases in conductance (without any discernable change in reversal potential) that were rapid (t_{1/2} = 20 s), and of variable amplitude and were not seen in oocytes expressing CFTR constructs lacking the cysteine at 338, such as WT, T338A, T338H, T338S CFTR, or Cys-less CFTR. Shown in Fig. 1A is a representative experiment in which exposing an oocyte expressing T338C/WT CFTR

Reagents—Highly polar derivatives of methanethiol sulfonate, MTSET⁺ (2-(trimethylammonium)ethyl methanethiol sulfonate bromide) and MTSES⁻ (sodium (2-sulfonatoethyl)methanethiol sulfonate) were obtained from Toronto Research Chemicals (Toronto, Canada). 2-Mercaptoethanol (2-ME), dithiothreitol (DTT), iodoacetamide (IAM), S-nitroso-N-acetylpenicillamine (SNAP), and CuCl₂ were obtained from Sigma.

Different Chemical States of an Engineered Cysteine (T338C)

![Figure 1: A, effect of 2-ME on T338C/WT CFTR conductance. Following activation by 10 μM Isop and 1 mM isobutylmethylxanthine (hatched bar), an oocyte expressing T338C CFTR was exposed to 1 mM 2-ME (open circles). Inset A1, changes in average conductance (Δg_{R}, n = 109) induced by exposure to 2-ME or DTT binned every 20 μS versus the initial conductance. Number of experiments included in each bin is shown (from left to right): 38, 30, 29, 24, 20, 16, 11, 7, 5, 3, 1, and 1. Inset A2, I–V plots prior to (dashed line) and after exposure to 2-ME (solid line). B, loss of effect of 2-ME (open circles) on Cys-less CFTR conductance. Inset, I–V plots as above. C, effect of 2-ME (open circles) on T338C/Cys-less CFTR conductance. Inset C1, fractional change in conductance (n = 34) induced by 2-ME or DTT versus initial conductance. Inset C2, I–V plots as above. Crosses in all of the graphs indicate time points where no reagents were applied to oocytes.

FIGURE 1. A, effect of 2-ME on T338C/WT CFTR conductance. Following activation by 10 μM Isop and 1 mM isobutylmethylxanthine (hatched bar), an oocyte expressing T338C CFTR was exposed to 1 mM 2-ME (open circles). Inset A1, changes in average conductance (Δg_{R}, n = 109) induced by exposure to 2-ME or DTT binned every 20 μS versus the initial conductance. Number of experiments included in each bin is shown (from left to right): 38, 30, 29, 24, 20, 16, 11, 7, 5, 3, 1, and 1. Inset A2, I–V plots prior to (dashed line) and after exposure to 2-ME (solid line). B, loss of effect of 2-ME (open circles) on Cys-less CFTR conductance. Inset, I–V plots as above. C, effect of 2-ME (open circles) on T338C/Cys-less CFTR conductance. Inset C1, fractional change in conductance (n = 34) induced by 2-ME or DTT versus initial conductance. Inset C2, I–V plots as above. Crosses in all of the graphs indicate time points where no reagents were applied to oocytes.
to 1 mM 2-ME at steady state activation induced a rapid, over 2-fold increase in conductance \( g_{\text{Cl}} \) at \( V_m = E_{\text{rev}} \). The conductance typically reached a maximum within the mixing time of the chamber, about 20–40 s following exposure. Similar results were obtained using DTT (see also Fig. A1, inset, for summary). In principle, an increase in conductance induced by 2-ME or DTT could reflect several possible mechanisms that are not mutually exclusive, including: 1) breaking an intra-protein disulfide bond, 2) reducing a protein sulfenic acid (Protein-SOH), 3) reducing a glutathionylated cysteine (Protein-SSG), 4) reducing protein nitrosothiol (Protein-SNO), or 5) ligation by thiol-based reducing agents of trace metals reacting with the cysteine thiol to form Protein-S-ML\(_n\), where \( L_n \) represents other metal ligands that could be derived from the peptide backbone, other side chains, or exogenous mobile species such as \( \text{Cl}^- \) or \( \text{OH}^- \) (26, 27).

To test the hypothesis that the reducing agent effect was the result of breaking an intramolecular disulfide bond, we used a Cys-less CFTR construct in which all 18 endogenous cysteines were replaced with alternative amino acids. Reducing agents had no effect on the conductance of oocytes expressing Cys-less CFTR (Fig. 1B). However, in oocytes expressing T338C/Cys-less CFTR, in which a cysteine was substituted at position 338 in the Cys-less background, 1 mM 2-ME induced a variable increase in conductance similar to that seen in oocytes expressing T338C/WT CFTR (Fig. 1C). These observations indicated that the cysteine substituted at position 338 was necessary and sufficient to account for the effects of reducing agents seen in oocytes expressing T338C/WT CFTR and ruled out the breaking of an intramolecular disulfide bond within a CFTR monomer as a mechanism for the increase in conductance.

We also considered the possibility that the reducing agent-induced increase in CFTR conductance might reflect the breaking of a disulfide bond linking two CFTR peptides that form a dimeric channel (28–30). Recent covalent labeling studies, however, provided strong evidence that a single CFTR polypeptide forms a single anion-conducting pore (31). Furthermore, detailed studies of T338C/WT CFTR suggest that residue 338 lies within the pore (25) where it exhibits reactivity toward mixed disulfides and alkylating agents that is not consistent with a disulfide bond (see also below). We concluded therefore, that the response depicted in Fig. 1, A and C, implies that the chemical state of the cysteine substituted at position 338 can be altered due to chemical reactions of the cysteine thiol at position 338 that do not involve formation of a disulfide bond with another peptide.

The response of individual oocytes expressing T338C/WT CFTR to 2-ME or DTT varied widely, as indicated by the results compiled in Fig. 1, inset A1. Oocytes injected with comparable amounts of mRNA and maintained under identical conditions exhibited initial conductances ranging from 4 to 240 \( \mu \text{S} \), and the response of these oocytes to 2-ME or DTT was proportionately greater in oocytes exhibiting a lower initial conductance. A similar pattern of reactivity was observed in oocytes expressing T338C/Cys-less CFTR (Fig. 1C, inset). The variable response to 2-ME or DTT was observed in oocytes from the same frog that were maintained under identical conditions and tested on the same day. The inverse correlation between the effect of a reducing agent and the initial conductance implies that the low, initial CFTR conductance exhibited by some of the oocytes was due to a reversible modification of the cysteine at position 338. The variability of the response to 2-ME or DTT suggested that, in any particular oocyte, the initial CFTR conductance reflected a mixed population of channels. In some of these channels, the cysteine was in the simple thiol form, \( \text{S}^- \) or \( \text{SH} \), whereas in others the thiol was modified to one or more of the thiol reaction products enumerated in the opening paragraph of "Results." Exposing oocytes to a reducing agent apparently regenerated the simple thiol with a concomitant net increase in conductance.

Evidence for a Terminal Oxidation State—In a previous study of T338C CFTR (25) it became apparent that there was a high degree of variability in the response to thiol-directed reagents even if oocytes had been previously exposed to 2-ME or DTT. This observation led us to suggest that a variable portion of the channels was in a terminal or unreactive oxidation state such as a sulfenic acid or sulfonic acid (32–35). This phenomenon is explored in more detail in the current study, and the results obtained using a variety of thiol-directed reagents uniformly suggest that whereas a variable subpopulation of T338C channels display altered reactivity that can be rescued by exposure to 2-ME or DTT, there is a second subpopulation of channels that is simply unreactive.

Oocytes Expressing T338C/WT CFTR Displayed Highly Variable Reactivity Toward MTSET\(^{\text{+}}\) and MTSES\(^{\text{−}}\)—The disparate response of oocytes expressing T338C CFTRs to 2-ME or DTT is compatible with the notion that, in any individual oocyte, a variable percentage of channels is in the simple thiol form (\( \text{S}^- \), \( \text{SH} \)). This hypothesis predicts that a similar variability should be apparent in the response of oocytes to mixed disulfide reagents like MTSET\(^{\text{+}}\) and MTSES\(^{\text{−}}\), which react only with the thiolate anion (36). The results summarized in Fig. 2 support this hypothesis. Shown in Fig. 2, A and B, are representative experiments in which, following activation of T338C/WT CFTR, oocytes not previously exposed to 2-ME and DTT were exposed to 1 mM MTSET\(^{\text{+}}\). In one case (Fig. 2A) the initial exposure to MTSET\(^{\text{+}}\) produced a 40% decrease in conductance that persisted following washout of the reagent as expected for a covalent interaction. Subsequent exposure to 2-ME (5 mM) increased the conductance as expected, but to a level greatly exceeding the initial conductance indicating that 2-ME not only reversed the reaction with MTSET\(^{\text{+}}\), but also increased the number of cysteines in the simple thiol form. A second exposure to MTSET\(^{\text{+}}\) then produced a decrease in conductance greater than that induced by the first exposure. In the second example (Fig. 2B), the initial response to 1 mM MTSET\(^{\text{+}}\) was a net increase in conductance. As indicated by the data plotted in Fig. 2B, inset, and C, this increase could be substantial. After a wash, exposure to 5 mM 2-ME increased the conductance further and a second exposure to 1 mM MTSET\(^{\text{+}}\) then decreased the conductance to a level that was comparable with that achieved by the first exposure to MTSET\(^{\text{+}}\). If the responses to MTSET\(^{\text{+}}\) after exposure to 2-ME or DTT, seen here and reported previously, represent the consequence of the reactions of the mixed disulfides with the thiolate anion, then the increase in conductance seen prior to exposure to 2-ME or DTT suggests that this reaction could represent the displacement of some other thiol adduct by the positively charged ethyl(trimethylammonium) thiol moiety. For example, if the altered state of the thiol bears some other thiol adduct by the positively charged ethyl(trimethylammonium) thiol moiety. For example, if the altered state of the thiol bears

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6 Similarly, exposure to reducing agents was without effect on oocytes expressing either T338A or T338S CFTR, constructs that retain the 18 endogenous cysteines (see below).
could arise because the population of channels that are sensitive to DTT and 2-ME is heterogeneous in some way not discerned in these studies.

The data summarized by the gray triangles in Fig. 2C demonstrates that exposure of different oocytes expressing T338C/WT CFTR to MTSET+/H11001, under identical conditions, prior to any exposure to 2-ME and DTT, could result in an increase, a decrease, or no change in conductance! As suggested by the examples shown in Fig. 2B the functional effect of MTSET+ exhibited an inverse correlation with the initial conductance of the oocyte; oocytes with a low initial conductance reacted with an increase in conductance, whereas those with a higher initial conductance reacted with a decrease in conductance. Responses of naive oocytes to MTSES-/H11002 were uniformly inhibitory and the magnitude of the inhibition was not as variable as that seen with MTSET+/H11001.

As indicated in Fig. 2D, pretreating oocytes with 2-ME or DTT altered the subsequent response to MTSET+ (open triangles) and MTSES- (open circles), but did not totally eliminate the variability. In pre-treated oocytes the effect of MTSET+ was uniformly inhibitory, as reported previously (25), but the extent of the inhibition varied widely, ranging from less than 20% to nearly 60% for MTSET+ and from 67 to nearly 93% for MTSES-. The greater average inhibition by MTSES- is consistent with the expected effect of depositing a negative charge in the anion conduction pathway (25) and suggests that, although both reactions may lead to partial obstruction of the pore, the effect of charge is, nevertheless, discernable.

The observation that reactivity remains highly variable even after exposure to 10–20 mM 2-ME or DTT suggests that a variable population of the T338C channels is simply unreactive, due perhaps to some higher order oxidation of the thiol (32–35). It should be noted that the cysteine at position 338 is essential for the effects of MTS reagents as well as 2-ME and DTT shown above, because neither the conductance due to T338A or T338S CFTR was sensitive to reducing agents or thiol-directed reagents.7

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Trapping Thiols with an Alkylation Agent, IAM—The results presented so far are compatible with a scheme in which the total conductance of an oocyte expressing T338C/WT CFTR or T338C/Cys-less CFTR comprises at least three components that we will label as $g_{SH}$, $g_{SX1}$, and $g_{SX2}$, where the total conductance, $g_{Cl}$, is given by Equation 1.

$$g_{Cl} = g_{SH} + g_{SX1} + g_{SX2} \quad \text{(Eq. 1)}$$

7 X. Liu and D. C. Dawson, unpublished observation.
Here $g_{SH}$ represents channels in which the cysteine at 338 is in the simple thiol form ($S^{−},SH$), and $g_{SX1}$ and $g_{SX2}$ represent channels containing one of two altered forms of the cysteine, one that reverts to the simple thiol in the presence of 2-ME and DTT ($g_{SX1}$) and another ($g_{SX2}$) that does not.

We sought to isolate populations of altered channels by first trapping those in the thiolate form using an alkylating agent. Such reagents (e.g. IAM, iodoacetic acid, N-ethylmaleimide) form an irreversible, thioether bond with protein thiols and have been widely used to trap thiols in other proteins (12, 15, 16, 37, 38). We reasoned that trapping those channels in the thiolate form would enable us to separate the two remaining populations of channels ($g_{SX1}/g_{SH}$ and $g_{SX2}$) using 2-ME or DTT and then a thiol-directed reagent. On the basis of preliminary experiments we chose IAM as the trapping agent because the predicted reaction results in the deposition of a relatively small substituent that does not carry a net charge (Equation 2) (12, 38).

$$\text{P–S}^- + \text{ICH}_2–\text{NH}_2 \rightarrow \text{P–S–CH}_2–\text{NH}_2 + \text{I}^- \quad \text{(Eq. 2)}$$

Fig. 3A shows a representative example ($n = 5$) of the somewhat surprising result of an attempt to trap thiols using an oocyte exhibiting a very low initial conductance (indicative of a high percentage of reversibly altered channels) that would be predicted to give rise to a large response to 2-ME or DTT. Exposure to millimolar concentrations of IAM, pH 7.4, induced a slow, monotonic increase in conductance. After the conductance reached a steady state, exposing the oocyte to 2-ME was without effect, nor was the conductance altered by exposure to MTSES$, as expected if all reactive thiols were blocked by reaction with IAM. Fig. 3A, inset, contains a plot of the response of several oocytes to IAM, representing a range of initial conductance. The change in conductance induced by IAM exhibited an inverse relationship with the initial conductance reminiscent of that seen with 2-ME or DTT (Fig. 1). This result suggested that the alkylation reaction trapped not only the simple thiolate form, but also those represented by $g_{SX1}$, channels that were spontaneously altered but could be converted to $g_{SH}$ by 2-ME or DTT.

The rate of the alkylation reaction, as judged from the time course of experiments such as that shown in Fig. 3A, was relatively slow suggesting that the rate of alkylation in a naive oocyte might be limited by the reactivity of the thiol. To examine the effect of thiol reactivity on the rate of reaction of IAM at pH 7.4, we compared the apparent rate of alkylation using oocytes pretreated with 2-ME or DTT. Reaction with IAM
had little or no effect on the macroscopic conductance of pretreated oocytes (Fig. 3B, n = 6). For this reason we assessed the rate of the alkylation reaction in such oocytes crudely by treating with IAM, washing, and then exposing the oocyte to MTSES\(^{-}\), a reagent that deposits a negative charge that is expected to profoundly decrease conductance due to reactive channels (25). The results depicted in Fig. 3B suggest that 10 min exposure to only 100 μM IAM blocked most of the free thiols such that MTSES\(^{-}\) was without further effect on the conductance. In the absence of pre-exposure to IAM, MTSES\(^{-}\) profoundly decreased the conductance under similar conditions (Fig. 3C, n = 5). Experiments of this sort, although lacking in precision, demonstrated that the rate of reaction of IAM was much more rapid after oocytes were exposed to a reducing agent.

We also compared the rate of alkylation by IAM under alkaline conditions that favor the highly reactive thiolate form of the SH group (12, 15, 39). Fig. 4A contains the results of an experiment (n = 3) in which after exposure to DTT, the solution perfusing a pre-reduced oocyte was changed from pH 7.4 to 9.0, resulting in a rapid decrease of the macroscopic conductance. We reported previously that this effect appears to be largely attributable to an increase in the partial negative charge on the thiolate anion and the resulting electrostatic retardation of anion flow (25). Subsequent exposure of the same oocyte to IAM (1 mM, pH 9) resulted in a rapid increase in conductance (t\(_{1/2}\) = 24 s) likely due, at least in part, to the elimination of the negative charge associated with the thiolate anion, although a concomitant change in gating (increase in P\(_{o}\)) cannot be excluded (see Liu et al. (25) and "Discussion"). Returning the perfusate to pH 7.4 had a minimal effect on the conductance and the channel was unreactive toward MTSES\(^{-}\) or 2-ME (Fig. 4B), confirming the alkylation reaction.

Fig. 4B contains the result of an experiment in which alkylation was monitored at pH 9 using a naïve oocyte expressing T338C/WT CFTR (n = 5). In this case the response to IAM at pH 9 comprised two distinct phases. The conductance initially increased toward a level comparable with the initial steady state conductance at pH 7.4 and then slowly increased further to a level greater than the initial conductance. The change in conductance was fitted to a function containing two exponentials with half-times (t\(_{1/2}\)) of 50 s and 12 min, respectively. The initial fast component is comparable with the accelerated rate of reaction between the thiolate and IAM seen at pH 9 in oocytes previously exposed to 2-ME or DTT (Fig. 4A), whereas the slower rate was comparable with that seen at pH 7.4 in naïve oocytes in which most of the channels were in the altered state (see Fig. 3A). This result is consistent
with the hypothesis that channels reacting with IAM originated from two populations. One population is in the thiolate form and reacted rapidly with IAM at pH 9. Channels in the other population exist in an altered state that reacts more slowly with IAM. Fig. 4C contains the result of an experiment showing that IAM did not affect the function of T338A CFTR \((n = 3)\). Here we compared the blocking effect of Au(CN)\(_2^–\), a permeant, pseudohalide anion, that blocks WT CFTR at millimolar concentrations (40–42). Block of T338A CFTR was unaffected by exposure to IAM.

Can Thiol-reactive Agents Mimic Aspects of the Spontaneous Alteration of T338C CFTR Reactivity?—The signature behavior of spontaneously occurring, reversibly altered T338C CFTR channels \((\xi_{\text{ox,1}})\) is a rapid increase in conductance upon exposure to 1 mM 2-ME or DTT \((t_{1/2} < 20–40 \text{ s})\), a net increase in macroscopic conductance when reacted with MTSET\(^+\), and relatively slow trapping by IAM. We sought, therefore, to compare the behavior of experimentally altered channels with this signature. Possible states of a cysteine thiol and their interconversion are depicted in Fig. 5. The dashed line delineates those states that are most likely to be encountered in cells (35, 43–48). Shown in blue are states that could, in principle, be visited reversibly, depending on the local environment. Shown in red are irreversible or terminal states. It is immediately apparent that reactivity with the monothiol, 2-ME, or the dithiol, DTT (indicated as green RSH in the diagram), is common to many of the possible altered states of the cysteine at 338. These agents can reduce mixed disulfides formed with glutathione (P-SSG) or MTS reagents (P-SSR\(^+\)) and regenerate the simple thiol from the nitrosothiol (P-SNO) or the sulfenic acid (P-SOH). In addition, both 2-ME and DTT are well known to be metal ligands that can coordinate metals in solution and act as “exogenous” ligands to protein-bound metals (49–53). Thus, on the basis of reactivity alone, neither 2-ME nor DTT can discriminate among these alternatives. The alkylation reactions, however, are predicted to be more limited, involving only the free thiol or the metal-bound thiol (54–60). This more limited reactivity is likely to be shared by MTS reagents. The thiol-disulfide exchange reaction has been observed with free and metal-bound thiolites (leading to displacement of the metal) but MTS reagents are not expected to react with P-SNO, P-SOH, or P-SSG (16, 61–63).

**FIGURE 5. Possible chemical states of the cysteine at position 338.** States shown in blue represent those that are reactive, including glutathionylated (P-SSG), sulfenic acid (P-SOH), nitrosothiol (P-SNO), mixed disulfides formed with MTS reagents (P-SSR\(^+\)), and a metal-bound state (P-S-ML\(^n\)). States shown in red represent states that are unreactive, including sulfonic acid (P-SO\(_2\)H) and sulfonic acid (P-SO\(_3\)H), and alkylation (P-SC\(_2\)H\(_4\)ON). Dashed lines encircle states that could, in principle, occur in oocytes without any external perturbation.

**T338C CFTR Channels Reacted with Either Glutathione or MTSET\(^+\) Could Not Be Trapped by IAM—**Fig. 6A illustrates a typical result of glutathionylation of T338C CFTR channels \((n = 3)\). Exposure to a mixture of 50 \(\mu\text{M}\) GSH and 100 \(\mu\text{M}\) diamide (64) produced a profound decrease in conductance that was not reversed by washing. Subsequent exposure to 2 mM IAM produced only a minimal effect on conductance, unlike the typical slow increase in conductance seen in naive oocytes (Fig. 3A). Furthermore, after washing out IAM, exposure of the oocyte to 1 mM 2-ME produced a modest reversal of the inhibition. Higher concentrations of 2-ME (10 and 20 mM) largely reversed the inhibition, indicating that IAM did not trap glutathionylated thiols. The rate of reversal of the glutathionylation by 2-ME was, however, much slower than the response to 2-ME seen in naive oocytes (Fig. 1). Similarly, following reaction with MTSET\(^+\), exposure of oocytes to IAM produced only a minimal effect on conductance. Exposure of the oocyte to 2 mM 2-ME reversed the MTSET\(^+\)-induced inhibition but at a rate much slower than that seen in naive oocytes (Fig. 6B, \(n = 3\)). This result is consistent with the expectation that neither mixed disulfide would react with the alkylation agents and strongly suggests that the \(\xi_{\text{ox,1}}\) state of the T338C channel is not due to the formation of a mixed disulfide. Diamide-GSH had no discernable effect on conductance of oocytes expressing T338A CFTR.

Oxidation by NO or \(\text{H}_2\text{O}_2\) Did Not Reproduce the Signature Behavior of Spontaneously Oxidized T338C CFTR Channels—Fig. 7A depicts a typical experiment \((n = 4)\) in which an oocyte expressing T338C CFTR was first exposed to 1 mM DTT to increase the number of cysteines in the simple thiol form. Exposure to 1 mM SNAP, a commonly used NO donor (46, 65), produced a minimal effect on conductance, but largely blocked the subsequent reaction with MTSES\(^–\), indicating oxidation of the cysteine to the nitrosothiol. This apparent oxidation was without effect on the macroscopic conductance but was readily reversed by exposing oocytes to 1 mM DTT, as indicated by an 80% decrease in conductance following the second exposure to MTSES\(^–\). SNAP had no discernable effect on conductance of oocytes expressing T338A CFTR.

Fig. 7B depicts a typical experiment \((n = 2)\) in which an oocyte expressing T338C CFTR was first exposed to 1 mM DTT to increase the number of cysteines in the simple thiol form. Exposure to 5 mM \(\text{H}_2\text{O}_2\)
produced a decrease in conductance that was not spontaneously reversible. Exposure to 1 mM IAM had no additional effect on conductance. However, exposure to 1 mM 2-ME led to a small increase in conductance, suggesting that some channels were oxidized to sulfenic acid, which is susceptible to reduction, whereas other channels were oxidized to sulfinic or sulfonic acids that are not susceptible to reduction. Up to 14 mM H$_2$O$_2$ had no discernable effect on conductance of oocytes expressing T338S or WT CFTR.7

Low Concentrations of Copper Mimicked the g$^{\text{SSX}}$ Phenotype—Metals like iron, zinc, and copper that react with free thiols are abundant in Xeno-
pus oocytes (66), so we explored the consequences of exposing oocytes expressing T338C CFTR to metals added to the perfusion solution. Iron, the metal most abundant in oocytes was without effect whether added to the perfusion solution as FeSO₄ plus ascorbate (Fe²⁺/H₂O₂) (67) or FeCl₃ plus nitrilotriacetic acid (to stabilize Fe³⁺/H₂O₂) (68–70) in concentrations ranging from 1 to 5 μM. Zinc, added as ZnCl₂, produced a significant inhibition (50% at 200 μM) of conductance in oocytes previously exposed to 2-ME or DTT, but the effect was reversed by washing. We have found that many constructs with a single added cysteine exhibit reversible block by Zn²⁺ with Kᵢ ranging from 15 μM to 1 mM (71, 72).

In contrast, exposure to low concentrations of copper in the perfusate induced a substantial decrease in T338C CFTR conductance that demonstrated a variable but substantial component that was not reversed by washing, indicative of a high affinity interaction. At 1 μM, copper induced an 80% (±5%, n = 5) decrease in T338C CFTR conductance, but was without effect on T338A or WT CFTR conductance. Washing often produced a slow recovery from inhibition that could vary from near zero to about 32% of the inhibited conductance. Following this transient the inhibition was not further alleviated by washing. The conductance was increased by exposing the oocyte to 2-ME or DTT, and the rate was similar to that seen in naïve oocytes (Fig. 8A).

Exposure of copper-inhibited oocytes expressing T338C/WT to MTSET⁺ mimicked the phenotype seen in naïve oocytes. As depicted in Fig. 8B (n = 3), after achieving an irreversible (by washing) inhibition by 1 μM copper, exposure to 1 mM MTSET⁺ increased conductance. Subsequent exposure to 1 mM 2-ME further increased the conductance after which a second exposure to MTSET⁺ with a concentration 10 times less than the first produced a decrease in conductance, a pattern identical to that seen in naïve oocytes. This result suggests that the bound copper can be displaced by reaction of the thiol with MTSET⁺. Similarly, exposure of a copper-inhibited oocyte to 1 mM IAM produced a slow increase in conductance that was strikingly reminiscent of that seen with naïve oocytes (Fig. 8C). The tᵢ is not significantly different from that seen with naïve oocytes (p value >0.3). The alkylation was confirmed by the lack of reactivity toward 2-ME and MTSES⁻. These observations are compatible with the hypothesis that the cysteine thiol at 338 forms part of an adventitious metal binding site. Copper binding

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**FIGURE 8.** Copper mimicked the phenotype seen in naïve oocytes expressing T338C CFTR. A an oocyte was exposed to: 1 mM 2-ME (open circles), 1 μM copper (crossed squares), 1 mM 2-ME, B an oocyte was exposed to: 1 mM 2-ME, 1 μM copper, 1 mM MTSET⁺ (gray triangles), 1 mM 2-ME, 100 μM MTSET⁺, 1 mM 2-ME, C an oocyte was exposed to: 1 mM DTT (open circles), 10 nM copper, 1 μM copper, 1 mM IAM (gray circles), 1 mM 2-ME, 100 μM MTSES⁻ (black circles). Crosses in all of the graphs indicate time points where no reagents were applied to oocytes.

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*The lowest copper concentration used was 1 nM (Liu, X., and Dawson, D. C., unpublished observation).*

*5 μM copper produced a modest inhibition of WT CFTR conductance (5%).*
reduces macroscopic conductance and alters, but does not eliminate, the reactivity of the thiol toward mixed disulfides and alkylating agents.

DISCUSSION

The Chemical State of a Cysteine Substituted at 338 Can Vary—The results presented here support the hypothesis that when CFTR channels containing T338C are expressed in Xenopus oocytes the chemical state of the engineered cysteine can vary. The effect of the altered thiol states on conductance and reactivity can be dramatic. Exposure of an oocyte expressing either T338C/WT or T338C/Cys-less CFTR to 2-ME or DTT can result in large and highly variable changes in conductance; and the reactivity of the cysteine-substituted constructs toward MTS reagents can differ qualitatively from one oocyte to another. The variability in thiol reactivity at 338 appears to be further complicated by the fact that the simplest interpretation of the results presented here and in a previous report (25) requires at least two altered states of the thiol, one that can revert to the simple thiolate in the presence of 2-ME or DTT (gSX1) and another that cannot (gSX2). For this reason the altered reactivity of a cysteine at 338 is only partially mitigated by pretreating oocytes with 2-ME or DTT.

Regardless of the origin of these altered states the variable reactivity that results is an important consideration in the interpretation of the screening assays that are typically the initial phase of a cysteine scanning protocol. The dramatic difference in reactivity toward MTS reagents may begin to explain the disparate results obtained by different laboratories with T338C CFTR (compare Refs. 9 and 25). At present it is not possible to identify unequivocally the chemical states that are represented by the conductance components labeled gSX1 and gSX2, but inhibition of conductance by copper and its reversal by reducing agents and a thiol-trapping agent suggests this trace metal as a possible candidate.

Effects of exogenous copper have been reported for several types of ion channels (73–75), but the binding sites are unknown. Recently, Li et al. (76) reported effects of reducing agents on the Torpedo CIC-0 chloride channel, but exogenous copper (10 μM) was without effect.

Altered States of T338C CFTR Are Defined by Reactivity—T338C CFTR channels in which the cysteine is in the simple thiol (or thiolate) form can be recognized by their reactivity toward mixed disulfides like MTSET+ and diamide-GSH as well as alklylation by IAM. We demonstrated previously that the thiol could be titrated by varying the pH of the bathing solution (25).

T338C CFTR channels in the gSX1 state were recognized initially by their reactivity toward 2-ME or DTT, a result incompatible with the simple thiol (or thiolate) form of the cysteine. The widespread use of these compounds as reducing agents suggested that the gSX1 state might reflect the formation of a disulfide bond, but reactivity with MTSET+ and IAM argue strongly against this possibility. Rather, it seems that the pattern of reactivity exhibited by the gSX1 state is most consistent with the behavior of a metal-bound thiolate.

The reactivity of metal-bound thiobates has been studied extensively in metal-containing proteins as well as model systems. Both exhibit reduced rates of alkylation similar to that reported here, consistent with the notion that the nucleophilicity of the thiolate is reduced by its interaction with the bound metal (57, 58, 60). The alkylation of the thiol is expected to lead to displacement of the metal and the formation of a thioether bond. Similarly, thiol-disulfide exchange reactions can release protein-bound metal (77).

It seems appropriate to view the action of 2-ME and DTT on T338C CFTR as a reflection of their ability to ligand metals rather than their activity as “reducing agents.” Liganding of metals by mono- and diethiol is well established in the literature of metallobiochemistry (53, 49), and the efficacy of these compounds in converting gSX1 to gSX2 is much greater than that seen when they are breaking a mixed disulfide bond (Fig. 1). We propose, therefore, that 2-ME, DTT, IAM, and MTSET+ all act on channels in the gSX1 state by displacing a bound copper ion, albeit by different mechanisms.

The copper binding hypothesis also provides a possible explanation for the variable conductance component labeled here as gSX2 that was distinguished by its lack of reactivity. This variable, unreactive fraction of the conductance was seen with all classes of thiol-directed reagents, thiol (2-ME and DTT), mixed disulfide (MTS reagents), and alkylating agents (IAM, iodoacetic acid, and N-ethylmaleimide) as well as a per- meanate coordination compound, Au(CN)2–, which reacts with the cysteine thiol via ligand exchange (78). The lack of reactivity of the gSX2 species suggests that a variable portion of the thiols can be oxidized to a “terminal” state, i.e. a sulfonic or sulfonic acid species, which is not susceptible to reducing agents (32–35). This sort of oxidation is known to be catalyzed by copper in solution (79) and at metal-bound thiobates in proteins (80). Although the results presented here do not provide definitive evidence for either a sulfonic or sulfonic acid species, the reactions summarized in Fig. 5 suggest that one of these is a strong candidate. Alternatively, the liganding of the copper could vary from one channel to another in such a way as to render a variable population of channels less susceptible to rescue by 2-ME or DTT or alkylation by IAM.

The copper-binding hypothesis may also explain the observation that exposure of “naïve” oocytes (not previously exposed to 2-ME or DTT) to MTSET+ can result in an increase in conductance. Metal binding reduces macroscopic conductance either by reducing single-channel conductance, open probability, or both. In the case of MTSET+ reaction with the metal-bound thiol would be predicted to displace the metal (along with its bound ligands) and also add a positive charge. This could result, for example, in a net increase in single-channel conductance even though the final value was less than that of the unmodified channels as reported previously (25). We reported previously that single T338C CFTR channels exhibited variability in conductance consistent with different chemical states of the cysteine thiol (25).

Potential Origin of Bound Copper—Copper is abundant in Xenopus oocytes, second only to iron and zinc. Nomizu et al. (66) estimated the total concentration to be of the order of 10 μM. Studies of mammalian cells suggest that the concentration of free copper is vanishingly small due to the presence of a variety of copper-binding proteins and chaperones in the cytoplasm (81, 82). It is also common to find copper in tap water, however, so we considered the possibility that the copper that binds to T338C CFTR could originate in one or more of the solutions that bathe the oocytes after they are removed from the frog. The Milli-Q® Plus (Millipore, Billerica, MA) purified water that is used for all of the solutions employed in these experiments was assayed by means of atomic absorption spectrophotometry and inductively coupled plasma mass spectrometry (Severn Trent Laboratories Inc., STL, Seattle, WA). Both methods, however, reported copper levels below the limit of sensitivity of the instrument, about 10 nM. As described above, concentrations of copper below 10 nM were sufficient to produce significant inhibition of T338C CFTR. Unfortunately, assays of the bathing solutions themselves were compromised by interference from chloride ions. Additional studies will be required to identify definitively the source of bound copper.

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