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CFTR: Temperature-dependent cysteine reactivity suggests different stable conformers of the conduction pathway

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Abstract

Cysteine scanning has been widely used to identify pore-lining residues in mammalian ion channels, including the cystic fibrosis transmembrane conductance regulator (CFTR). These studies, however, have been typically conducted at room temperature rather than human body temperature. Reports of substantial effects of temperature on gating and anion conduction in CFTR channels as well as an unexpected pattern of cysteine reactivity in the sixth transmembrane segment (TM6), prompted us to investigate the effect of temperature on the reactivity of cysteines engineered into TM6 of CFTR. We compared reaction rates at temperatures ranging from 22°C to 37°C for cysteines placed on either side of an apparent size-selective, accessibility barrier previously defined by comparing reactivity toward channel-permeant and channel-impermeant, thiol-directed reagents. The results indicate that reactivity of cysteines at three positions extracellular to the position of the accessibility barrier, 334, 336 and 337, is highly temperature dependent, such that at 37°C cysteines at these positions were highly reactive toward MTSES[−], whereas at 22°C the reaction rates ranged from two to six-fold slower to undetectable. An activation energy of 157 kJ/mole for the reaction at 337 is consistent with the hypothesis that, at physiological temperature, the extracellular portion of the CFTR pore can adopt conformations that differ significantly from those accessible at room temperature. However, the position of the accessibility barrier defined empirically by applying channel-permeant and channel-impermeant reagents to the extracellular aspect of the pore is not altered. The results illuminate previous scanning results and indicate that assay temperature is a critical variable in studies designed to use chemical modification to test structural models for the CFTR anion conduction pathway.

Cysteine scanning, using both channel-permeant and channel-impermeant, thiol-directed reagents has been employed by several labs to investigate the structure of the anion conduction pathway of the CFTR chloride channel (1,2,3,4,5,6,7,8,9). A comparison of the reactivity of cysteines engineered into TM6 toward channel-permeant probes ([Au(CN)₂][−], [Ag(CN)₂][−]) and channel impermeant probes (MTSET⁺, MTSES[−]), assayed at room temperature, suggested that pore geometry imposed a size-selective, accessibility cut-off that prevented reaction of channel-impermeant reagents with engineered cysteines that lay cytoplasmic to position 338 (10). One exception to the pattern, however, was position 337. When reagents were applied from the *extracellular* side, a cysteine substituted at 337 reacted readily with channel-*permeant* probes, but, in our hands, reaction with channel-*impermeant* probes, MTSET⁺ or MTSES[−], was not detectable. A second concern was that previous studies of cysteine reactions at position 337, also assayed at room temperature, had produced contradictory results. Cheung and Akabas (1,2) reported reactivity of F337C

CFTR towards MTSEA⁺, MTSET⁺ and MTSES⁻. Fatehi et al. (5) reported reactivity of F337C CFTR towards MTSES⁻ but not MTSET⁺, while Beck et al. (6) reported no reactivity at this locus towards MTSEA⁺.

Cysteine scanning studies are routinely carried out at room temperature, rather than at 37°C, the temperature at which human CFTR would normally function. To probe for possible temperature-induced changes in the accessibility of different protein conformations we investigated the temperature-dependence of cysteine reactivity in TM6. The results indicate that reactivity toward a channel-impermeant reagent, MTSES⁻, for at least three positions, 334, 336 and 337, is highly temperature dependent. In particular, reactivity of a cysteine substituted at position 337 was readily detectable at temperatures above 27°C, whereas modification by extracellular MTSES⁻ could not be detected at 22°C. The apparent activation energy for the reaction exceeded that expected for diffusion or thiol-disulfide exchange in solution as well as CFTR gating, suggesting that the change from low to physiological temperature is associated with a change in conformation of the outer vestibule of the pore. The position of the size-selective, accessibility cut-off for externally applied thiol-reactive probes, however, was unaffected by temperature.

MATERIALS AND METHODS

Mutagenesis and In Vitro Transcription

The methods used for mutagenesis and *in vitro* transcription were similar to those reported previously (3,11,12). Briefly, CFTR mutants were generated using site-directed mutagenesis PCR. Ambion mMessage mMachine T7 Ultra transcription kit (Ambion) was used to generate the CFTR cRNAs for *Xenopus* oocyte injection. The sequences in the region of the mutation were confirmed by direct DNA sequencing.

Preparation and Microinjection of Oocytes

Preparation and microinjection of *Xenopus laevis* oocytes was as previously described in detail (3,11). The follicular membranes were removed by mechanical agitation (1–2 hours) in a Ca²⁺-free solution containing (mM): 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES, pH 7.5, with 0.2 Wünsch units/mL Liberase Blendzyme 3 (Roche Molecular Biochemicals, Indianapolis, IN). Defolliculated oocytes were washed and maintained in a modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 2.4 NaHCO₃, 10 HEPES (hemi-Na), and 250 mg/L Amikacin plus 150 mg/L Gentamicin at pH 7.5. Stage V to VI oocytes were injected with 50 nL CFTR cRNA plus cRNA encoding the human β_2 -adrenergic receptor per oocyte. CFTR RNA concentration was adjusted so that the maximum steady state stimulated conductance is less than 200 μ S (~12.5 ng/oocyte).

Whole-cell Recordings

Individual oocytes were placed in a 200 μ l recording chamber (RC-1Z, Warner) and continuously perfused with Frog Ringer's solution (4 ml/min). The Ringer's solution contained (in mM): 98 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES-Hemi Na, at pH 7.4. CFTR channels were activated using 10 μ M isoproterenol (a β -adrenergic agonist) and 1 mM IBMX (a phosphodiesterase inhibitor) as the stimulating cocktail (Isop+IBMX). The Oocyte 725 amplifier (Warner) and the pClamp 8 data acquisition program (Axon Instruments, Inc. CA) were used for whole-cell recordings. Oocytes were maintained in the open circuit condition and the membrane potential was periodically ramped from -120 to +60 mV over 1.8 sec to construct the whole cell I-V plots.

Temperature control

A Dual Automatic Temperature Controller (CL-200) and an in-line solution heater/Cooler (SC-20) (Warner Instruments, Hamden, CT) were used to apply acute temperature change under constant flow. Bath temperature was monitored. The temperature signal was digitized and recorded using a USB Data Acquisition Device (DI-158, DATAQ Instruments, Inc. Akron, OH).

Determination of apparent activation energy

The activation energy was determined using the Arrhenius equation. The equation was rearranged so that the *log* of the rate constant (*k*) is a function of the inverse of the temperature (*T*), Eq 1.,

$$\ln(k) = \frac{-E_a}{R} \frac{1}{T} + \ln(A) \quad (1)$$

Where *A* is the pre-exponential factor, *R* is the gas constant and *E_a* is the activation energy determined from the slope of the *ln(k)* vs. *1/T* plot. We refer to *E_a* as apparent activation energy which may be determined by the nature of the thiol-probe reaction, diffusional access of thiol-directed reagents to the cysteine thiolate and protein conformational change.

Reagents

Isobutylmethyl xanthine (IBMX), isoproterenol (Isop), 2-mercaptoethanol (2-ME), Diethyldithiocarbamate (DDTC), Dithioerythritol (DTE), Diethyldithiocarbamate (DDTC) and KAUCN₂ were purchased from Sigma (St Louis, MO). Sodium (2-Sulfonatoethyl)methanethiosulfonate (MTSES[−]) were purchased from Toronto Research Chemicals (Toronto, Canada). Potassium cyanide (KCN) was obtained from Fisher Chemicals (Fairlawn, NJ). Dr. Robert Bridges (Rosalind Franklin University, Chicago, IL) and Cystic Fibrosis Foundation (CFF) kindly provided CFTR_{inh}-172 (4-[4-Oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl]-benzoic acid, labeled as CF172 here).

RESULTS

The representative experiments compiled in Figure 1 illustrate the dramatic effect of increased temperature on the rate of reaction of MTSES[−] with F337C CFTR. MTSES[−] was chosen for this comparison for two reasons. First, the thiol-disulfide exchange reaction deposits a negatively charged substituent in the pore that is expected to profoundly inhibit anion conduction (4,10) rendering the progress of the reaction readily detectable. Second, the anionic character of MTSES[−] makes it most comparable to the channel permeant probe, [Au(CN)₂][−]. In a previous study we reported that, whereas the channel-permeant probe, [Au(CN)₂][−] reacted with a cysteine substituted at position 337, the channel impermeant probe, MTSES[−], did not. In many such experiments conducted at room temperature, no change in conductance was detectable upon exposure of an oocyte expressing F337C CFTR to 1 mM MTSES[−], even for periods exceeding 10 minutes. Repeated attempts to detect a reaction at room temperature sometimes gave rise to results like that depicted in Figure 1A, however. In this instance exposure of the oocyte expressing F337C CFTR to MTSES[−] at room temperature evoked what at first appeared to be a very slow rate of reaction, but the decline spontaneously reversed upon removal of the reagent from the superfusate. Subsequent exposure of the oocyte to the channel-permeant probe, [Au(CN)₂][−], produced a profound inhibition as earlier reported (10), confirming that the cysteine thiolate remained

un-reacted. The inhibition following the reaction of $[\text{Au}(\text{CN})_2]^-$ was not reversed by exposure to a competing thiol, 2-ME, but was readily reversed by exposure to the high-affinity metal ligand, CN^- , as expected from previous studies (10,12). Figure 1B depicts an experiment in which an oocyte expressing F337C CFTR was exposed to 1 mM MTSES $^-$ at 22°C, and superfusate temperature was then raised to 32°C in the presence of the mixed disulfide. Raising the superfusate temperature in the presence of 1 mM extracellular MTSES $^-$ resulted in a 64% (± 1 , $n=3$) decrease in conductance. Addition of 1 mM $[\text{Au}(\text{CN})_2]^-$ afterwards produced a small irreversible decrease in conductance that was reversed by exposure to 1 mM KCN, suggesting that even after a 20 minute exposure, a small fraction of the cysteine thiols were not modified by MTSES $^-$ at 32°C. Similar results were seen at 37°C (Figure 1C), except that raising bath temperature to 37°C in the presence of the mixed disulfide resulted in a more rapid reduction in conductance, indicative of a robust reaction of the mixed disulfide probe with the substituted cysteine at the elevated temperature. Figure 1D depicts an experiment designed to test the reversibility of the temperature induced changes in the reactivity of a cysteine at 337. An oocyte expressing F337C CFTR was exposed to 37°C for 10 min and then returned to 22°C. The temperature excursion evoked an increase in conductance that was sustained during the period of elevated temperature, but reversed rapidly when the temperature of the superfusate was returned to 22°C. This behavior is identical to that reported previously for wt CFTR channels (13,14) and reflects increases in open probability and single-channel conductance evoked by the elevated temperature. Subsequent exposure to MTSES $^-$ at 22°C produced no evidence of a reaction, but block by 1 mM $[\text{Au}(\text{CN})_2]^-$ confirmed the presence of the reactive thiolate. This result indicates that, although reaction of MTSES $^-$ with F337C CFTR was readily detectable at 37°C, exposure to the elevated temperature did not result in any irreversible change in the channel.

Figures 2, 3 and 4 contain the results of similar experiments conducted with oocytes expressing R334C, I336C and T338C CFTR channels. In the case of R334 and I336, exposure to MTSES $^-$ (3 μM and 1mM, respectively) produced reactions at 22°C which were slow, but unlike that of F337C CFTR, readily discernable at room temperature. Both reaction rates were enhanced at 30°C and 37°C. The reaction of MTSES $^-$ with T338C CFTR was the most rapid at 22°C and was nevertheless increased at 37°C.

The second order rate coefficients for the reaction of MTSES $^-$ with CFTR constructs bearing cysteines positioned at 334, 336, 337 and 338 are collected in Table 1. Shown also is the apparent activation energy calculated based on Eq. 1 using the slope obtained from fitting the Arrhenius plots shown in Figure 5. At room temperature the measured rates of reaction varied over four orders of magnitude, despite the relative proximity of these four positions in the protein, suggesting that the orientation or electrostatic environment of the engineered cysteine side chains may differ significantly in different locations. Reactions at all four cysteines were enhanced by raising the temperature to 37°C, but the effect varied from about two-fold for a cysteine at 338 to greater than seven-fold for a cysteine at 337. As expected, the apparent activation energies varied widely, ranging from being that expected for disulfide exchange (15,16) for T338C CFTR to values in the range of those generally associated with protein conformational change (17,18,19,20).

Figure 6 summarizes the inhibition by MTSES $^-$ of CFTR conductance at 22°C (27°C for F337C CFTR) and 37°C in oocytes expressing CFTR constructs bearing substituted cysteines at positions extracellular to (334, 336, 337, 338) and cytoplasmic to (339, 340, 341, 342 and 344) the apparent accessibility cut-off defined by Alexander et al. (10). It is apparent that, despite the dramatic increases in the reaction rates of cysteines extracellular to the cut-off, the position of the cut-off was unchanged at 37°C. That is, whatever conformational restriction prevents the reaction of extracellularly-applied MTSES $^-$ with

cysteines cytoplasmic to position 338 remained intact at 37°C. The ability of cysteines at more cytoplasmic positions to react with channel-permeant, thiol-directed probes is documented in Alexander et al. (10). Recently, Bai et al. (7) and El Hiani and Linsdell (8) used detached membrane patches to demonstrate reactivity of deeper-lining cysteines to MTSES⁻ applied from the *cytoplasmic* side. However, the two studies differ substantially in the position of the apparent size cut-off defined by the reactivity toward cytoplasmically applied reagents.

DISCUSSION

The results presented here provide evidence for a striking temperature-dependence of the reactivity of cysteines engineered into the sixth transmembrane domain (TM6) of the CFTR chloride channel toward a channel-impermeant, thiol-directed reagent, MTSES⁻. To our knowledge this is the first report comparing substituted cysteine reactivity at different temperatures, in particular, “physiological temperature,” 37°C. The findings have implications for assays routinely used to test molecular models of the CFTR channel as well as for our understanding of what structure of the pore domain is most relevant to the function of the channel *in vivo*.

Previous room temperature cysteine scanning studies from several labs implicated three residues in TM6, R334, I336 and T338, as being likely to lie within the outer vestibule of the CFTR pore (3,4,5,6,10). Cysteines substituted at these positions reacted with both channel-permeant reagents like [Au(CN)₂]⁻ and [Ag(CN)₂]⁻ as well as larger, channel-impermeant reagents like MTSES⁻ and MTSET⁺, as expected if these residues formed part of a wide, extracellular end of the channel that coupled the rate-limiting portion of the pore to the extracellular solution. Raising the temperature increased the reaction rate for the extracellularly applied, channel-impermeant reagent, MTSES⁻, at all three positions. At room temperature, the most rapid modification was seen at position 338. The rate at 22°C, about 4000 M⁻¹s⁻¹, is about an order of magnitude less than that reported for the reaction of MTSES⁻ with 2-mercaptoethanol in free solution (21). The activation energy, 35 kJ/mol, is in the range reported for thiol-disulfide exchange, 30–40 kJ/mol (15,16) and exceeds that reported for the diffusion of alanine in water, 19 kJ/mole (22). The room temperature rate of reaction at 334 was comparable to that at 338, but the rate of reaction was more temperature-dependent as evident from the higher energy of activation. The activation energy, 98 kJ/mol, lies within the range reported for CFTR channel gating, 70–150 kJ/mol (14). In addition, the rate at 37°C actually exceeded that seen at 338 despite the fact that 338 is thought to lie somewhat deeper in the pore. These findings point to increased accessibility of channel conformations that result in de-occlusion of the substituted cysteine at 334 with increasing temperature.

Modification rates at 336 and 337 were dramatically lower than those at 334 and 338, by three orders of magnitude at 22°C. Given the proximity of these positions in the protein the low rates suggest some sort of occlusion of the substituted cysteine or some other environmental feature such as a nearby negative charge that would attenuate the rate of the thiol-disulfide exchange reaction at room temperature (21,23,24). The activation energy seen for the reaction at 336 was well above that reported for thiol-disulfide exchange, as if an apparent occlusion of the substituted cysteine is reduced at elevated temperature due to a conformational change.

Cysteine modification at position 337 appears to represent a limiting case in that the reaction rate was undetectable at 22°C (Figure 1A) and robust at 37°C, exceeding that seen at position 336 at 37°C. The estimated energy of activation, 157 kJ/mol, was much larger than

at other positions and exceeded that associated with the opening of CFTR channels (14), suggestive of a conformational change that accompanied the increased temperature.

The observed accessibility of a cysteine at position 337 to the channel-impermeant reagent, MTSES⁻, at 37°C was important for two reasons. First, it is clear that at elevated temperature, the channel can access conformations that permit a cysteine at position 337 to react with both channel-permeant and larger, channel-impermeant reagents; thereby bringing the reactivity of a cysteine at 337 in line with that seen at 334, 336 and 338. The observed nonselective reactivity at 337 places this position in the outer-vestibule of the pore as defined by probe accessibility. This finding does not, in and of itself, resolve the discrepancy between our data and that of Cheung and Akabas (1,2) and Fatehi et al. (5) who reported reactivity of F337C CFTR towards externally applied MTSES⁻ at room temperature, but it does at least raise the possibility of some, as yet unidentified, condition of their experiments that enables the CFTR channel to access conformations at room temperature in which the thiol-disulfide exchange reaction can occur at position 337.

The temperature-induced changes in cysteine reactivity reported here, in particular that of a cysteine at position 337, are similar to the non-denaturing conformational changes reported for enzymes like adenosine deaminase (19) and carbonic anhydrase (20). These enzymes can apparently access different functional states, some dominant at physiological temperature and others dominant at lower temperatures. Massey et al. (17) had previously reported similar, thermally-induced, reversible changes in the structure of D-amino acid oxidase. These thermally-induced transitions between relatively stable, functional states have been likened to a partial unfolding from more compact low-temperature forms to more open, high-temperature forms characterized by increased exposure of non-polar amino acid side chains to the aqueous environment (18,19). Similarly, the large changes in cysteine reactivity reported here suggest that the outer portion of the CFTR pore can access more open conformations at 37°C that render a cysteine substituted at 337 reactive toward larger, externally applied reagents. Importantly, however, the position of the previously defined cut-off in reactivity toward channel-impermeant reagents appears to be unchanged at physiological temperature, indicating that the barrier to the permeation of MTSES⁻ remains intact and located cytoplasmic to T338.

The reactivity of cysteines substituted at either of two adjacent locations 337 and 338 differed dramatically. The results suggest that the cysteine side chain at 337 is occluded in the substates of the channel that predominate at room temperature, and it is less reactive even at 37°C for reasons that could involve steric as well as electrostatic effects (23,24). A goal of future modeling and MD simulations will be to understand the striking difference in cysteine reactivity at these two positions in the protein.

There are over 1800 mutations known in the CFTR gene. The most common, $\Delta F508$, results in a protein characterized by a temperature-sensitive folding defect (25). The $\Delta F508$ CFTR channel is expressed in the plasma membrane of mammalian cells at 28°C, but elevating the temperature leads to the disappearance of the surface protein with a half-time of 1–2 hours (26,27,28,29,30,31), suggesting that exposure to physiologic temperature produces unfolding that leads to internalization of surface protein and ultimately degradation within the cell. The results presented herein suggest that there may be a general propensity of the CFTR protein toward low-to-physiological-temperature conformational change that is apparent even in the outer vestibule of the pore, well away from site of the most common mutation in the first nucleotide binding domain, NBD1. Temperature-induced, partial unfolding can produce, as in the present instance, stable conformers, whereas in disease-related mutants like $\Delta F508$, the natural tendency to unfold at physiological temperature may be exacerbated by a defect that drives the conformational change farther along the unfolding

pathway toward a state of denaturation which triggers degradative, quality control processes in the cell. Understanding this continuum may offer insights into the unfolding process seen in mutant CFTRs.

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ABBREVIATIONS

CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
IBMX	Isobutylmethyl xanthine
ISOP	isoproterenol
2-ME	2-mercaptoethanol
DTE	Dithioerythritol
KCN	Potassium cyanide
DDTC	Diethyldithiocarbamate
MTSES⁻	Sodium (2-Sulfonatoethyl)methanethiosulfonate
CF172 (CFTR_{inh}-172)	4-[4-Oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl]-benzoic acid

References

1. Cheung M, Akabas MH. Identification of cystic fibrosis transmembrane conductance regulator channel-lining residues in and flanking the M6 membrane-spanning segment. *Biophysical Journal*. 1996; 70:2688–2695. [PubMed: 8744306]
2. Cheung M, Akabas MH. Locating the anion-selectivity filter of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. *J Gen Physiol*. 1997; 109:289–299. [PubMed: 9089437]
3. Smith SS, Liu X, Zhang ZR, Sun F, Kriewall TE, McCarty NA, Dawson DC. CFTR. Covalent and noncovalent modification suggests a role for fixed charges in anion conduction. *J Gen Physiol*. 2001; 118:407–432. [PubMed: 11585852]
4. Liu X, Zhang ZR, Fuller MD, Billingsley J, McCarty NA, Dawson DC. CFTR: A Cysteine at Position 338 in TM6 Senses a Positive Electrostatic Potential in the Pore. *Biophys J*. 2004; 87:3826–3841. [PubMed: 15361410]
5. Fatehi M, Linsdell P. State-dependent Access of Anions to the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore. *J Biol Chem*. 2008; 283:6102–6109. [PubMed: 18167343]
6. Beck EJ, Yang Y, Yaemsiri S, Raghuram V. Conformational changes in a pore-lining helix coupled to cystic fibrosis transmembrane conductance regulator channel gating. *J Biol Chem*. 2008; 283:4957–4966. [PubMed: 18056267]
7. Bai Y, Li M, Hwang TC. Dual roles of the sixth transmembrane segment of the CFTR chloride channel in gating and permeation. *J Gen Physiol*. 2010; 136:293–309. [PubMed: 20805575]
8. El Hiani Y, Linsdell P. Changes in accessibility of cytoplasmic substances to the pore associated with activation of the cystic fibrosis transmembrane conductance regulator chloride channel. *J Biol Chem*. 2010; 285:32126–32140. [PubMed: 20675380]

9. Wang W, El Hiani Y, Linsdell P. Alignment of transmembrane regions in the cystic fibrosis transmembrane conductance regulator chloride channel pore. *J Gen Physiol.* 2011; 138:165–178. [PubMed: 21746847]
10. Alexander C, Ivetac A, Liu X, Norimatsu Y, Serrano JR, Landstrom A, Sansom M, Dawson DC. Cystic fibrosis transmembrane conductance regulator: using differential reactivity toward channel-permeant and channel-impermeant thiol-reactive probes to test a molecular model for the pore. *Biochemistry.* 2009; 48:10078–10088. [PubMed: 19754156]
11. Liu X, Alexander C, Serrano J, Borg E, Dawson DC. Variable reactivity of an engineered cysteine at position 338 in cystic fibrosis transmembrane conductance regulator reflects different chemical states of the thiol. *J Biol Chem.* 2006; 281:8275–8285. [PubMed: 16436375]
12. Serrano JR, Liu X, Borg ER, Alexander CS, Shaw CF 3rd, Dawson DC. CFTR: Ligand Exchange between a Permeant Anion ([Au(CN)₂][−]) and an Engineered Cysteine (T338C) Blocks the Pore. *Biophys J.* 2006; 91:1737–1748. [PubMed: 16766608]
13. Mathews CJ, Tabcharani JA, Hanrahan JW. The CFTR chloride channel: nucleotide interactions and temperature-dependent gating. *Journal of Membrane Biology.* 1998; 163:55–66. [PubMed: 9569250]
14. Csanady L, Nairn AC, Gadsby DC. Thermodynamics of CFTR channel gating: a spreading conformational change initiates an irreversible gating cycle. *J Gen Physiol.* 2006; 128:523–533. [PubMed: 17043148]
15. Iversen R, Andersen PA, Jensen KS, Winther JR, Sigurskjold BW. Thiol-disulfide exchange between glutaredoxin and glutathione. *Biochemistry.* 2010; 49:810–820. [PubMed: 19968277]
16. Liang J, Fernandez JM. Kinetic measurements on single-molecule disulfide bond cleavage. *J Am Chem Soc.* 2011; 133:3528–3534. [PubMed: 21341766]
17. Massey V, Curti B, Ganther H. A temperature-dependent conformational change in D-amino acid oxidase and its effect on catalysis. *J Biol Chem.* 1966; 241:2347–2357. [PubMed: 5911617]
18. Bischof JC, He X. Thermal stability of proteins. *Ann N Y Acad Sci.* 2005; 1066:12–33. [PubMed: 16533916]
19. Bodnar MA, Britt BM. Transition state characterization of the low- to physiological-temperature nondenaturational conformational change in bovine adenosine deaminase by slow scan rate differential scanning calorimetry. *J Biochem Mol Biol.* 2006; 39:167–170. [PubMed: 16584631]
20. Hollowell HN, Younvanich SS, McNevin SL, Britt BM. Thermodynamic analysis of the low- to physiological-temperature nondenaturational conformational change of bovine carbonic anhydrase. *J Biochem Mol Biol.* 2007; 40:205–211. [PubMed: 17394770]
21. Karlin A, Akabas MH. Substituted-cysteine accessibility method. *Methods Enzymol.* 1998; 293:123–145. [PubMed: 9711606]
22. Longworth L. Temperature dependence of diffusion in aqueous solutions. *J Phys Chem.* 1954; 58:770–773.
23. Snyder GH, Cennerazzo MJ, Karalis AJ, Field D. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. *Biochemistry.* 1981; 20:6509–6519. [PubMed: 6796114]
24. Britto PJ, Knipling L, Wolff J. The local electrostatic environment determines cysteine reactivity of tubulin. *J Biol Chem.* 2002; 277:29018–29027. [PubMed: 12023292]
25. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, CROR, Smith AE. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell.* 1990; 63:827–834. [PubMed: 1699669]
26. Sharma M, Benharouga M, Hu W, Lukacs GL. Conformational and temperature-sensitive stability defects of the delta F508 cystic fibrosis transmembrane conductance regulator in post-endoplasmic reticulum compartments. *J Biol Chem.* 2001; 276:8942–8950. [PubMed: 11124952]
27. Heda GD, Tanwani M, Marino CR. The Delta F508 mutation shortens the biochemical half-life of plasma membrane CFTR in polarized epithelial cells. *Am J Physiol Cell Physiol.* 2001; 280:C166–C174. [PubMed: 11121388]
28. Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR. Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol Biol Cell.* 2004; 15:2684–2696. [PubMed: 15075371]

29. Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H, Lukacs GL. Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J Cell Biol.* 2004; 164:923–933. [PubMed: 15007060]
30. Varga K, Goldstein RF, Jurkuvenaite A, Chen L, Matalon S, Sorscher EJ, Bebek Z, Collawn JF. Enhanced cell-surface stability of rescued DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) by pharmacological chaperones. *Biochem J.* 2008; 410:555–564. [PubMed: 18052931]
31. Okiyoneda T, Barriere H, Bagdany M, Rabeh WM, Du K, Hohfeld J, Young JC, Lukacs GL. Peripheral protein quality control removes unfolded CFTR from the plasma membrane. *Science.* 2010; 329:805–810. [PubMed: 20595578]
32. Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galletta LJ, Verkman AS. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest.* 2002; 110:1651–1658. [PubMed: 12464670]

Figure 1.

Increased temperature dramatically increased the reactivity of F337C CFTR toward MTSES⁻. Oocytes expressing F337C CFTR were activated using stimulatory cocktail (10 μM Isop + 1 mM IBMX, hatched bar and cross hairs) and then exposed to 1 mM 2-ME to reverse any spurious reactions of the substituted cysteine (11). (A) Exposure to 1 mM MTSES⁻ (dark gray bar and circles) produced no reaction, but 1 mM [Au(CN)₂]⁻ (black bar and circles) produced profound inhibition that was not reversed by 1 mM 2-ME, but was reversed by 1 mM KCN (white bar and triangles); (B) Raising the temperature to 32°C in the presence of MTSES⁻ produced a modest reaction rate; (C) Raising the temperature to 37°C in the presence of MTSES⁻ produced a rapid reaction; (D) After exposure to 37°C and returning to 22°C, exposure to 1 mM MTSES⁻ produced no evidence of a reaction.

Figure 2.

Increased temperature altered the rate of modification of I336C CFTR by MTSES⁻. Oocytes expressing I336C CFTR were activated using stimulatory cocktail (10 μM Isop + 1 mM IBMX, hatched bar and cross hairs). **(A)** Increased temperature reversibly increased CFTR conductance (gray bar and circles). After returning the temperature to 22°C, the oocyte was exposed to 10 μM CF172, a CFTR inhibitor (32); **(B)** Raising superfusate temperature to 30°C in the presence of MTSES⁻ resulted in an increased rate of reaction. After returning the temperature to 22°C, the oocyte was exposed to 10 μM CF172. **(C)** Raising superfusate temperature to 37°C in the presence of MTSES⁻ further increased the rate of reaction with MTSES⁻. After returning the temperature to 22°C, the oocyte was exposed to 10 μM CF172.

Figure 3.

Increased temperature altered the rate of modification of R334C CFTR by MTSES⁻. An oocyte expressing R334C CFTR was activated using stimulatory cocktail and then (A) exposed to 1 mM 2-ME (white bar and circles) and 3 μM MTSES⁻ (dark gray bar and circles) at room temperature; (B) Exposure to 3 μM MTSES⁻ at 30 $^{\circ}C$ (gray bar and circles) resulted in an increased rate of reaction. (C) Exposure to 3 μM MTSES⁻ at 37 $^{\circ}C$ (gray bar and circles) further increased the rate of reaction.

Figure 4.

Increased temperature increased the rate of reaction of T338C CFTR with MTSES⁻. An oocyte expressing T338C CFTR was activated using stimulatory cocktail (10 μM Isop + 1 mM IBMX, hatched bar and cross hairs) and then (A) exposed to 100 μM DDTC to reverse spurious thiol reactions (white bar and circles). Exposure to 5 μM MTSES⁻ (dark gray bar and circles) profoundly inhibited the T338C CFTR conductance; (B) The bath temperature was increased to 30 $^{\circ}C$ (gray bar and circles) and oocyte exposed to 5 μM MTSES⁻ at the elevated temperature. (C) The bath temperature was increased to 37 $^{\circ}C$ (gray bar and circles) and oocyte exposed to 5 μM MTSES⁻ at the elevated temperature. Oocytes were pre-treated with 1 mM DTE (>24 hours) in order to reverse any reaction with contaminating trace metals (11).

Figure 5. Arrhenius plots for **(A)** R334C, **(B)** I336C, **(C)** F337C and **(D)** T338C CFTR. E_a Value from Alexander et al. (10). Values, based on Table 1, represent mean $\pm$ SEM.

Figure 6.

Summary of temperature effects on the reactivity of CFTR constructs toward MTSES⁻. Percent block of CFTR conductance by MTSES⁻ was defined as the change in conductance induced by MTSES⁻, Δg_{ES} , divided by the conductance at time zero of exposure, $g_{t=0}$, at 22°C (27°C for F337C, white bars) and 37°C (dark gray bars). The small hatched bars at the base of the Y-axis indicate cases where the value was indeterminate or near zero. The values at 37°C (gray bars) from T339C to V345C reflect small changes in conductance in response to the temperature pulse, rather than reactivity towards MTSES⁻. §Data taken from Alexander et al. (10). Values represent mean $\pm$ SEM.

Table 1

Temperature-dependence of MTSES⁻ modification

Mutant	k _{MTSES} (M ⁻¹ s ⁻¹)	k _{MTSES} (M ⁻¹ s ⁻¹)	k _{MTSES} (M ⁻¹ s ⁻¹)	k _{MTSES} (M ⁻¹ s ⁻¹)	E _a (kJ/M)
	22°C	30°C	32°C	37°C	
R334C	2648 ± 259 (n=3)	9411 ± 1210 (n=5)		18407 ± 3240 (n=3)	98
I336C	1.2 [§]	2.3 ± 0.1 (n=3)		6.9 ± 0.4 (n=4)	88
F337C	2.6 ± 0.7 (27°C) [*] (n=3)		5.1 ± 1.2 (n=3)	19.4 ± 4.4 (n=4)	157
T338C	4067 ± 573 (n=5)	7192 ± 370 (n=4)		7972 ± 1019 (n=6)	35

[§] Value from Alexander et al. (10).^{*} Reaction rate was undetectable at 22°C so value determined at 27°C was used. Values are mean ± SEM.