The magnitudes and distributions of subconductance states were studied in chloride channels formed by the wild-type cystic fibrosis transmembrane conductance regulator (CFTR) and in CFTRs bearing amino acid substitutions in transmembrane segment 6. Within an open burst, it was possible to distinguish three distinct conductance states referred to as the full conductance, subconductance 1, and subconductance 2 states. Amino acid substitutions in transmembrane segment 6 altered the duration and probability of occurrence of these subconductance states but did not greatly alter their relative amplitudes. Results from real time measurements indicated that covalent modification of single R334C-CFTR channels by [2-(trimethylammonium)ethyl]methanethiosulfonate resulted in the simultaneous modification of all three conductance levels in what appeared to be a single step, without changing the proportion of time spent in each state. This behavior suggests that at least a portion of the conduction path is common to all three conducting states. The time course for the modification of R334C-CFTR, measured in outside-out macropatches using a rapid perfusion system, was also consistent with a single modification step as if each pore contained only a single copy of the cysteine at position 334. These results are consistent with a model for the CFTR conduction pathway in which a single anion-conducting pore is formed by a single CFTR polypeptide.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel and a member of the large, ATP-binding cassette transporter superfamily. The predicted domain architecture includes two membrane-spanning domains (MSDs), each with six transmembrane (TM) domains, two nucleotide-binding domains, and a unique regulatory (R) domain (1). Although it is well known that CFTR functions as a chloride channel, the composition of the minimal functional unit remains unclear; neither the number of CFTR polypeptides required nor the number of pores per functional channel is known. Three alternative scenarios have been proposed: (i) one polypeptide/one pore (e.g. see Ref. 2), (ii) two polypeptides/one pore (3), and (iii) one polypeptide/two pores (4). Evidence for each of these possibilities has been derived from biochemical, structural, and/or electrophysiological studies of wild-type CFTR (WT-CFTR) and selected mutants (for a review, see Ref. 5).

Single WT-CFTR channels exhibit multiple conductance levels. Although the channel spends the majority of its time shuttling between the main (full) conductance level and the closed level, careful inspection of the fine structure of open-channel bursts led to the observation of one or two other levels of intermediate conductance (4, 6–13). These subconductance levels could represent permeation through completely separate pores (which, when summed, comprise the full conductance level) or permeation through a single pathway that may reside in multiple configurations differing in conductance. In this study, we made use of mutants containing cysteines engineered at putative pore-lining positions in TM6 to determine the minimal functional unit of the CFTR channel. These mutants were readily covalently modified by the sulphydryl modifying reagent MTSET from the extracellular side (14, 15). First, we used the inside-out single-channel recording configuration to study the amplitude and distribution of subconductance and full conductance states of channels before and after modification in single- and double-site mutants. Second, we used the outside-out macropatch configuration to study the kinetics of modification in real time. The results of this study are consistent with a model for the CFTR protein in which a single pore is formed from a single CFTR polypeptide.

**EXPERIMENTAL PROCEDURES**

**Preparation of Oocytes and cRNA**—For mutant R334C, site-directed mutagenesis used a nested PCR strategy in which the mutation was designed into antiparallel oligomers (14). R334C was prepared from a construct carrying the full coding region of CFTR in the pBluescript vector. The rest of the mutants used in this study were prepared with the QuikChange protocol (Stratagene; La Jolla, CA) using oligonucleotide-mediated mutagenesis. All mutant constructs were verified by sequencing across the entire open reading frame before use. WT-CFTR cRNA was prepared from a construct carrying the full coding region of CFTR in the pAlter vector (Promega, Madison, WI). For macropatch recordings, cRNAs were prepared from a construct encoding CFTR in the pGEMHE vector, which was kindly provided by Dr. D. Gadsby.
CFTR Is a One-pore Channel

(rockefeller University). Oocytes were injected in a range of 5–100 ng of CFTR cRNAs; for experiments using two-electrode voltage clamp, CFTR cRNAs were injected along with 0.4 ng of cRNA for the β2-adrenergic receptor, allowing activation of CFTR by exposure to isoproterenol in the bathing solution. Oocytes were incubated at 18 °C in modified Liebowitz’s L-15 medium with the addition of HEPES (pH 7.5), gentamicin, and penicillin/streptomycin. Recordings were made 24–72 h after the injection of cRNAs.

Electrophysiology—For single-channel recording, CFTR channels were studied in excised, inside-out patches at room temperature (22–23 °C). Since preliminary experiments showed that the full single channel conductance of unmodified R334C-CFTR was very low (1.5 pS) compared with that of WT-CFTR (14, 15), most single-channel experiments in this study used asymmetrical [Cl] in order to increase the single channel amplitude at VM = −100 mV. Oocytes were prepared for study by shrinking in hypertonic solution (200 mM monopotassium aspartate, 20 mM KCl, 1 mM MgCl2, 10 mM HEPES, and 10 mM HEPES-KOH, pH 7.9) followed by manual removal of the vitelline membrane. Pipettes were pulled in four stages from borosilicate glass (Sutter Instrument Co., Novato, CA) and had resistances averaging −10 megohms when filled with low chloride pipette solution: 30 mM NMDG-Cl, 270 mM NMDG-aspartate, 5 mM MgCl2, 10 mM TES (pH 7.4). MTSET+ (100–200 μM) was back-filled into the pipettes before seal formation to diffuse into the tip during and after seal formation; solution lacking MTSET+ was used to fill the very tip of the pipette. Typical seal resistances were 200 gigohms or greater. Channels were activated by excision into intracellular solution containing 300 mM NMDG-Cl, 1.1 mM MgCl2, 2 mM Tris-EGTA, 1 mM MgATP, 10 mM TES (pH 7.4), and 50 units/ml PKA. CFTR currents were measured with an Axopatch 200B amplifier (Axon Instruments; Union City, CA) and were recorded at 1 kHz to DAT tape. For subsequent analysis, records were filtered at a corner frequency of 100 Hz and acquired using a Digidata 1322A interface (Axon) and computer at 2.5 ms/poin with pClamp 8.0.

For outside-out macropatch recordings, electrode tips were filled with a modified intracellular solution (150 mM NMDG-Cl, 1.1 mM MgCl2, 2 mM Tris-EGTA, 10 mM TES, pH 7.4) and then back-filled with the bath solution containing 1 mM MgCl2 and 100 mM NMDG-Cl. Through time, MgATP and PKA diffused to the intracellular face of the outside-out patch; CFTR channels were fully activated in a 50% cut-off between open and closed levels was employed during dephosphorylation. Prior to analysis, the single channel traces were filtered twice (see Fig. 5), we determined the relationship between the magnitude of the fractional increase in current upon first exposure (fractional ΔI) and the rate coefficient for MTSET+ modification during the second exposure (k2). Fractional ΔI upon first exposure was calculated as A/(A + B), where A represents the macroscopic current increment resulting from the first MTSET+ modification, and B is the macroscopic current increment from the second MTSET+ modification.

Source of Reagents—Unless otherwise noted, all reagents were obtained from Sigma. MTSET+ was purchased from Toronto Research Chemicals Inc. MTSET+ was first suspended in deionized water at a concentration of 100 mM, frozen in aliquots at −20 °C, and thawed and diluted into recording solution immediately before use. L-15 medium was from Invitrogen. PKA was from Promega.

Statistics—Unless otherwise noted, values given are mean ± S.E. Statistical analysis was performed using the t test for unpaired or paired measurements by Sigma Stat 2.03 (Jandel Scientific, San Rafael, CA), with p < 0.05 considered indicative of significance.

RESULTS

Wild-type and Mutant CFTRs Exhibit Comparable Subconductance States with Differing Stability and Probability of Occurrence—CFTR channels have been reported to exhibit subconductance states (4, 6–13). In our recordings of WT-CFTR single-channel currents from detached inside-out patches, subconductance states, although clearly discernible, were rare events, occurring in only 18% of bursts. For the purposes of this study, we defined a subconductance state as a conductance level that was visited during open-channel bursts and that was sufficiently stable to be recognized in an all-points histogram (16, 17). By careful, manual evaluation of the fine structure of open-channel bursts, multiple conductance levels of wild-type and mutant CFTRs were easily identified in patches containing 1–2 channels. Conduction levels that were identified were then confirmed using all-points amplitude histogram analysis; sojourns at these current levels are readily detectable by pClamp so that this operational definition permits an unambiguous separation of subconductance events from other small, non-CFTR single-channel events that contaminate some records.

Fig. 1 contains an example of the subconductance behavior of WT-CFTR recorded in a detached patch bathed by asymmetrical [Cl−]. Three states that differ in conductance are discernible: 7.6 pS, referred to here as the full conductance state (f), and two subconductance states of 5.4 and 3.1 pS, referred to as s2 and s1.
CFTR Is a One-pore Channel

Fig. 1. Sample traces of CFTR channel openings. Each current level is indicated by a dashed line. A–C, records for WT-, R334C-, and T338A-CFTR, respectively, were generated in excised, inside-out mode with asymmetrical [Cl−], where the pipette was filled with 40 mM [Cl−] and bath (cytoplasmic) solution contained 302 mM [Cl−]. The overall conductance of the single channel amplitude. All traces were recorded at $V_m = -100$ mV and were filtered at 100 Hz.

and s1, respectively. The dominant single-channel current in WT-CFTR is the full conductance state (i.e. transitions between c and f levels in Fig. 1A). The majority of WT-CFTR openings exhibit transitions from c directly to f and back again without sojourns in subconductance states.

Fig. 1 also contains records illustrating the subconductance behavior of two mutant CFTRs: R334C and T338A. The former exhibits a full conductance that is less than that of WT-CFTR under comparable conditions (14), and the latter exhibits an increased full conductance (9.8 pS in T338A-CFTR). Despite these differences in full conductance, however, the relative amplitudes of the three conductance states for WT-CFTR and the two mutants are quite similar (i.e. s1 is ~40% of f, and s2 is ~70% of f). The s2 subconductance of WT-CFTR was previously reported by Gunderson and Kopito (10) to reflect ~84% of the full conductance level, measured under conditions different from ours; they did not identify other subconductance levels in their data, perhaps because their data were filtered even more heavily (10 Hz). This result suggests that neither the R334C nor T338A mutation, although they involve residues reputed to lie within the CFTR pore (14, 18), greatly altered the relative magnitude of the subconductance states. The subconductance states seen in the mutant channels, however, differed markedly in their stability and probability of occurrence from that seen in WT-CFTR. In a longer record of single-channel currents from oocytes expressing R334C-CFTR (Fig. 2, A and B), the most prominent state is discernible as a conductance of ~1.2 pS that was reported previously (14), although upon closer examination of the fine structure of the open bursts, it is found that within nearly every burst there are transitions to three conductance states: one lower in conductance than that of the most frequent state and one higher in conductance. These results suggest that amino acid substitutions in TM6 alter the relative occurrence of subconductance states but not their relative amplitude in relation to the full conductance state, as if at least some portion of the conduction path for the three states (s1, s2, and f) is shared.

Deposition of a Positive Charge at 334 Amplifies All Conductance States Proportionately—We reasoned that if the three conductance states reflect different behaviors of a shared portion of the conduction path, which includes the amino acid at position 334 in TM6, it would be possible to use the properties of the R334C mutant to investigate the architecture of the functional CFTR pore. We showed previously that covalent modification of R334C-CFTR channels with MTSET+ increased the amplitude of the most prominent single-channel conductance (referred to here as s2) without altering gating (14). In our previous experiments, we assayed the impact of MTSET+-induced chemical modification in two ways, by comparing single-channel amplitudes in patches detached from different oocytes, either untreated or exposed to MTSET+ prior to recording, and by monitoring the modification of single channels in real time using recording pipettes back-filled with MTSET+ (see “Experimental Procedures”). In the present experiments, we monitored modification in real time to increase the likelihood that we would be able to observe the consequences of the reaction while in progress (19). The record in Fig. 2A is representative of such experiments. Electrode tips for these inside-out recordings were back-filled with solution containing 200 $\mu$M MTSET+. R334C-CFTR chloride channels were modified in ~15 min by MTSET+ diffusing down the electrode tip, as reflected by an increment of s1, s2, and f conductance levels ~2.1–2.3-fold (Fig. 2, B–F). We analyzed eight paired, inside-out single channel records (both pre- and postmodified channels included in the same patch) that contained only one R334C-CFTR channel per patch, as shown in Fig. 2A. In every case, only a single modification event was ever observed. Furthermore, all of the subconductance states appeared to be modified simultaneously. We maintained the patches that contained modified R334C-CFTR channels for up to 45 min in some experiments and found that following modification by MTSET+, the amplitudes of the s1, s2, and f conductance states consistently stayed at the same level, with no further modification observed.

The all-points histograms in Fig. 2, D and E, compiled by analyzing periods of 4 min immediately before and after the single modification event, confirm that the amplitudes of all three conductance states were increased by chemical modification. Furthermore, the mean values indicate that the amplitude of each conductance state increased in approximately the same proportion, between 2- and 2.3-fold (see Fig. 2F). Covalent modification by MTSET+ did not change the apparent reversal potential for either subconductance state or the full conductance state but only increased the slope conductance of each state (Fig. 3, A–C). Hence, the shared impact of covalent modification by MTSET+ on the amplitude of all conductance states exhibited by R334C-CFTR channels was also consistent with the hypothesis that the three conductance states have at least a portion of the conduction path in common.

Covalent Modification of R334C-CFTR Did Not Alter Gating—We previously reported that modification of R334C-CFTR by MTSET+ did not alter gating as defined by comparing open probability in patches from treated and untreated oocytes as well as that of channels monitored during the process of modification (14). Analysis of the subconductance behavior of R334C-CFTR provided an opportunity to reexamine the question of possible gating effects by asking whether modification of R334C-CFTR channels by MTSET+ altered the prevalence or duration of the three conductance states. As described under “Experimental Procedures,” we calculated values for $P_o$ according to the distribution of the times spent in each conductance state derived from the amplitude histograms. The overall $P_o$ of R334C-CFTR channels before and after MTSET+ modification was 0.24 ± 0.04 and 0.23 ± 0.04, respectively (p = 0.91; see Fig. 3D). Furthermore, as shown in Fig. 2G, the fractional abundance of the s1, s2, and f conductance states did not change upon MTSET+-induced modification in R334C-CFTR channels.
These results confirm and amplify our previous findings that covalent modification at the 334 locus alters the conduction properties of CFTR but is without effect on channel gating or the number of channels at the plasma membrane (14, 20). In contrast, modification of engineered cysteines in ClC-0 channels affected both conduction and gating (21). Furthermore, the observation in R334C-CFTR that the amplitudes of the s1, s2, and f states increased by an equivalent proportion, and apparently simultaneously, upon modification by MTSET+ suggests that each of these states reflects the activity of a single pore, or a portion of a shared conduction pathway rather than the activity of two separate pores. Miller and co-workers (22), in contrast, showed that modification of cysteines substituted in ClC-0 channels occurred in two steps, as predicted for a di-
Fig. 3. Modification of R334C-CFTR does not affect reversal potential and open probability. A–C, current-voltage relations for the s1, s2, and f conductance levels before (labeled s1, s2, and f) and after MTSET\textsuperscript{-} induced modification (labeled s1\textsuperscript{1}, s2\textsuperscript{1}, and f\textsuperscript{1}), measured with symmetrical \(-200 \text{ mM} \, [\text{Cl}^{-}]\). D, \(P_o\), determined before (filled circles) and after (filled triangles) covalent modification by MTSET\textsuperscript{-} for eight paired patches containing single R334C-CFTR channels. Each value represents mean \(P_o\) over \(\sim 4\) min before and after modification, which was assumed to have occurred at the midpoint between the last unmodified and first modified openings. The two isolated symbols (filled circle and triangle with error bars) are the mean \(\pm\) S.E. \(P_o\) values for records pre- and postmodification by MTSET\textsuperscript{-}.

How Many Cysteines Are in One Pore?—If the portion of the conduction path occupied by Arg\textsuperscript{334}\textsuperscript{+} is common to all three conductance states, the question remains, how many of these arginine residues are present in the functional pore of WT-CFTR? In other words, is the single common pathway formed from a single CFTR polypeptide, or are perhaps two polypeptides required, each contributing a single Arg\textsuperscript{334}\textsuperscript{+}? If each CFTR pore contained two copies of Arg\textsuperscript{334}\textsuperscript{+}, then the process of covalent modification should, in principle, proceed in two steps coinciding with the serial modification of the two cysteines. As described above, however, we were never able to observe more than a single modification event in single-channel experiments. In addition, despite many hours of recording, we were never able to observe the process of modification occurring while a channel was in the open state. In all cases, the modification event appeared to have occurred during an interburst closed interval, so that we could not eliminate the hypothesis that the modification reaction occurred in two steps. A similar result was obtained using a double mutant, R334C/K1250A, that exhibits a prolonged open state duration (data not shown). This observation suggests that modification at this site may be favored by the closed state.

To investigate the number of cysteines per pore, we examined the time course of the modification of CFTR channels by MTSET\textsuperscript{-}, in outside-out macropatches using a rapid perfusion system. We reasoned that if retaining the full conductance state required the modification of more than one cysteine, the kinetics of modification might be expected to reflect this. For example, it seemed likely that the change in local electrostatic potential caused by the modification of one cysteine (14) would significantly alter the thiol-disulfide exchange reaction at the second cysteine by two mechanisms that might partially cancel. A positive local electrostatic potential would reduce the local concentration of the MTSET\textsuperscript{-}, but it would also shift the p\(K_a\) of the target cysteine to more acidic values, rendering it more reactive (23–25).

Following activation of channels by diffusion of PKA and ATP into the patch from the pipette, a rapid perfusion system was used to apply MTSET\textsuperscript{-} to the extracellular surface of the patch. In Fig. 4, activated R334C-CFTR channels were first exposed to the bath solution containing no MTSET\textsuperscript{-} for a time period of \(-20\) s and then perfused by bath solution containing 50 \(\mu\text{M}\) MTSET\textsuperscript{-}. R334C-CFTR macroscopic current increased rapidly, reflecting modification by MTSET\textsuperscript{-} (14). The final, steady-state macroscopic current amplitude of modified R334C-CFTR was increased by 2.3 \(\pm\) 0.22-fold after prolonged exposure to 50 \(\mu\text{M}\) MTSET\textsuperscript{-}. This is consistent with the results of single-channel recordings and is further evidence that MTSET\textsuperscript{-} induced modification does not change \(P_o\) or channel number because the increase in macropatch current can be fully explained by the increase in single-channel amplitudes (Fig. 2F). More importantly, the kinetics of the modification process were fit best with a single exponential function in all five experiments (e.g. red line in Fig. 4A). The final, steady-state macroscopic current amplitude of modified R334C-CFTR was increased by 2.3 \(\pm\) 0.22-fold after prolonged exposure to 50 \(\mu\text{M}\) MTSET\textsuperscript{-}. This is consistent with the results of single-channel recordings and is further evidence that MTSET\textsuperscript{-} induced modification does not change \(P_o\) or channel number because the increase in macropatch current can be fully explained by the increase in single-channel amplitudes (Fig. 2F). More importantly, the kinetics of the modification process were fit best with a single exponential function in all five experiments (e.g. red line in Fig. 4A). The mean value of the time constant describing this relaxation (\(\tau\)) was 2.37 \(\pm\) 0.24 s (\(n = 5\)). The half-time for solution change was on the order of 0.03 s (see “Experimental Procedures”), so the observed time course most likely reflects the kinetics of the reaction of MTSET\textsuperscript{-} with the target thiol.

Previous studies from our laboratories provided evidence that both Arg\textsuperscript{334}\textsuperscript{+} and Lys\textsuperscript{335}\textsuperscript{+} contribute to the development of a positive electrostatic potential in the outer vestibule of CFTR (14). This observation provided an opportunity to test directly the notion that a nearby positive charge would modify the rate of modification of the cysteine at position 334 by comparing the rate of modification of R334C-CFTR with the rate of modification of R334C/K335A-CFTR (Fig. 4B). The amplitude of macroscopic current was increased 2.97 \(\pm\) 0.24-fold by 50 \(\mu\text{M}\) MTSET\textsuperscript{-} in R334C/K335A-CFTR. The kinetics of current increase were fit best with a single exponential function in each patch (red line in Fig. 4B), and the mean value of \(\tau\) was decreased to 1.25 \(\pm\) 0.11 s (\(n = 4\)). These data are compatible with the notion that the rate of modification of a cysteine at position 334 is sensitive to the local electrostatic potential, partially determined by the amino acid at position 335, although we cannot discount the possibility that the K335A mutation altered the pore structure.
the brief exposure to MTSET to that seen in experiments using the single exposure protocol follows. Solutions used for perfusion contained 5–10 MMTSET for 2.5 s and then zero MMTSET until the current increased to a new steady-state level (Fig. 5A). Upon brief exposure to a relatively low concentration of MMTSET, which was terminated before modification ran to completion, the amplitude of the macroscopic current was increased by a small fraction; the macroscopic current amplitude increased rapidly upon the second prolonged application of 50 MMTSET. The magnitude of the total increase in conductance with the dual exposure protocol was the same as with the single-exposure protocol (2.35 ± 0.27-fold). Hence, the brief exposure to MMTSET resulted in modification of a subset of the available cysteines. Most importantly, the kinetics of the second modification were fit best by a single exponential function having a rate constant of 2.2 s−1 (n = 6), virtually identical to that seen in experiments using the single exposure protocol (τ = 2.37 ± 0.24 s, p = 0.891).

We reasoned that if two cysteines in each channel must be modified to attain the complete conductance change, there might be a relationship between the fraction of cysteines modified in the first exposure and the rate of modification of the remaining cysteines in the second exposure to MMTSET. If there were two cysteines in each one-pore CFTR, then following the first brief exposure to MMTSET at a low concentration, the pool of R334C-CFTR channels should comprise a mixed population of unmodified, singly modified, and doubly modified channels (Fig. 5C); longer exposure to MMTSET in the first treatment would lead to a greater increase in current, due to modification of more cysteines. The change in electrostatic potential due to modification of one cysteine would be expected in the vicinity of R334C. We have also recently found that the rate of modification of a cysteine engineered at Thr332, predicted to lie one helical turn cytoplasmic to position Arg334, is sensitive to the charge at position 334 (25).

As an additional test for the presence of multiple cysteines, we studied the kinetics of modification of R334C-CFTR channels in outside-out macropatches using a two-pulse protocol as follows. Solutions used for perfusion contained 5–10 MMTSET for 2.5 s and then zero MMTSET for 10 s and finally 50 MMTSET until the current increased to a new steady-state level (Fig. 5A). Upon brief exposure to a relatively low concentration of MMTSET, which was terminated before modification ran to completion, the amplitude of the macroscopic current was increased by a small fraction; the macroscopic current amplitude increased rapidly upon the second prolonged application of 50 MMTSET. The magnitude of the total increase in conductance with the dual exposure protocol was the same as with the single-exposure protocol (2.35 ± 0.27-fold). Hence, the brief exposure to MMTSET resulted in modification of a subset of the available cysteines. Most importantly, the kinetics of the second modification were fit best by a single exponential function having a rate constant of 2.3 s−1 (n = 6), virtually identical to that seen in experiments using the single exposure protocol (τ = 2.37 ± 0.24 s, p = 0.891).

We reasoned that if two cysteines in each channel must be modified to attain the complete conductance change, there might be a relationship between the fraction of cysteines modified in the first exposure and the rate of modification of the remaining cysteines in the second exposure to MMTSET. If there were two cysteines in each one-pore CFTR, then following the first brief exposure to MMTSET at a low concentration, the pool of R334C-CFTR channels should comprise a mixed population of unmodified, singly modified, and doubly modified channels (Fig. 5C); longer exposure to MMTSET in the first treatment would lead to a greater increase in current, due to modification of more cysteines. The change in electrostatic potential due to modification of one cysteine would be expected to alter the rate of modification of the remaining cysteine, as suggested by the difference in response in R334C- and R334C/K335A-CFTR. Fig. 5B contains a plot of the modification rate coefficient (k2) during the second exposure to MMTSET as a function of the fractional change in current resulting from the first brief exposure to MMTSET (see “Experimental Procedures”). It can be seen that there was no relationship between the magnitude of the increase in current upon first exposure, relative to the total increase in current, and the rate of modification during the second exposure. The modification rate co-
efficient, $k$, in experiments with the single exposure protocol, such as in Fig. 4A, and the modification rate coefficient, $k_2$, in experiments with the two-pulse exposure, such as in Fig. 5A, were $8,569 \pm 518$ s$^{-1}$ m$^{-1}$ $(n = 5)$ and $9,142 \pm 863$ s$^{-1}$ m$^{-1}$ $(n = 6)$, respectively $(p = 0.561)$. Hence, the data do not support the presence of a mixed population of channels with multiple cysteine targets but rather support a model in which each one-pore CFTR contains a single cysteine at 334.

**Does Modification of One Cysteine Absolutely Prohibit Modification of a Second Cysteine in the Same Pore?**—Our interpretation of the preceding set of experiments rests on the assumption that modification of one cysteine by MTSET$^+$ would not simply prevent modification of a second, nearby cysteine due to an absolute steric/electrostatic block of the access pathway. To determine whether any engineered cysteines remain unmodified in R334C-CFTR channels after prolonged exposure to MTSET$^+$, we took advantage of the sensitivity of unmodified cysteines to bath pH. R334C-CFTR channels were examined by two-electrode voltage clamp, and channels were activated via the $\beta_2$-adrenergic receptor by exposure to isoproterenol. As reported previously, the channel conductance of unmodified cysteines to bath pH. R334C-CFTR macroscopic conductance. (Fig. 6, A and C). The macroscopic conductance of R334C-CFTR was increased $-2.5$-fold $(n = 3$, Fig. 6B) upon MTSET$^+$-induced modification at bath pH 7.5, which is consistent with our previous report (14). However, after R334C-CFTR channels were covalently modified by 200 $\mu$M MTSET$^+$, the macroscopic conductance was no longer sensitive to pH titration (Fig. 6, B and D). If an unmodified cysteine remained within the pore of channels that had been previously exposed to MTSET$^+$, macroscopic conductance should remain sensitive to pH, although the $pK_a$ might be shifted in the acidic direction due to the effect of the nearby positive charge (23–25). These results indicate that all engineered cysteines in the CFTR pore were modified by MTSET$^+$ during a single exposure, consistent with formation of the channel pore by a single CFTR polypeptide.

The possibility remains, however, that two separate copies of R334C contribute to each functional pore and that MTSET$^+$-induced modification of these two targets occurs with identical rates as expected if the two sites are far enough apart in the folded channel polypeptide that the electrostatic charge change that accompanies modification of one site is not sensed at the other site. In this case, the $-2.5$-fold change in conductance between unmodified and modified channels would represent the summed effects of two modification events per channel. One strong argument against this model exists in the fact that we never saw two-step increases in single-channel current during real time modification experiments, despite many hours of observation.

Although our previous experiments showed that the number of R334C-CFTR channels resident at the oocyte plasma mem-

**FIG. 6.** R334C-CFTR was not accessible to protons after modification by MTSET$^+$. A and C, oocytes expressing R334C-CFTR and $\beta_2$-adrenergic receptor were first activated by ND96 plus 5 $\mu$M isoproterenol at pH 7.5 for 6 min. Following activation, bath pH was then changed to either pH 6.0 or pH 9.0 and then to pH 7.5. On average, the macroscopic conductance of R334C-CFTR increased 22 $\pm$ 3% $(p = 0.02$, $n = 3$) in pH 6.0 and decreased 50 $\pm$ 5% $(p = 0.01$, $n = 3$) in pH 9.0. B and D, oocytes expressing R334C-CFTR and $\beta_2$-adrenergic receptor were first activated by ND96 plus 5 $\mu$M isoproterenol at pH 7.5 for 6 min and then followed by the same solution containing 200 $\mu$M MTSET$^+$ for 4 min; the macroscopic conductance was increased by $-2.5$-fold upon application of MTSET$^+$. Modification by MTSET$^+$ prevented the pH-induced response seen in R334C-CFTR macroscopic conductance.
brane does not change during short term PKA-mediated activation (20), we considered the possibility that the number of active channels might change during the course of these prolonged experiments. To control for potential changes in channel number, we analyzed single-channel recordings containing multiple R334C-CFTR channels in excised mode, while MTSET/H11001 diffused down to the tip from a back-filled pipette; the example shown in Fig. 7 contained at least three active channels. The sample traces (Fig. 7A) represent the 30-s spans near the beginning of the experiment, near the middle, and near the end of the experiment, such that the first modified channel opening (indicated by a filled arrowhead) is shown in the first trace, and all openings in the third trace are already modified. One can see that the single channel amplitude of the last modified opening is almost identical to that of the first modified opening. We counted the number of modified and unmodified openings within successive 30-s windows and plotted the number of unmodified openings (open circles) and modified openings (closed circles) as a function of time (Fig. 7B). As MTSET$^-$ diffused down the pipette, more channel openings exhibited the modified conductance, and fewer exhibited the unmodified conductance. This confirms that the modified openings with higher conductance arose from the same channels as the unmodified openings with low conductance and were not due to the MTSET$^-$-induced appearance of other channels in the patch. To account for changes in channel number due to rundown during the long recording, we plotted the fraction of all openings per segment that exhibited the modified conductance as a function of time (Fig. 7C). The concentration of MTSET$^-$ at the membrane surface should increase through time in an exponential fashion by diffusion; Fig. 7C shows that the fraction of openings that exhibited the modified conductance in each 30-s segment also increased with time in an exponential fashion. This was true for the single experiment shown in Fig. 7C and for five other multichannel patches, where the time to reach complete modification was normalized in order to account for differences in tip diameter and volumes of MTSET$^-$-free and MTSET$^-$-containing pipette solution. The symbols are mean ± S.E. for each time point; the solid line represents fit to an exponential function.

DISCUSSION

**Anion Conduction by CFTR: One Pore per Polypeptide**—The results presented here are compatible with the hypothesis that
a single, 170-kDa CFTR polypeptide forms a single, anion-selective conduction pathway (Fig. 8). Previous studies of CFTR, including of its substate behavior, have been interpreted in terms of two other, rather disparate conduction models (for a review, see Ref. 5). In one, a single CFTR polypeptide can form two pores (one from MSD1 and another from MSD2) (4, 26, 27), whereas in the other, formation of a single pore requires the association of two CFTR monomers (11, 3, 28, 29). Evidence supporting the one-polypeptide, two-pore model was derived from studies of channel activity recorded from cells expressing fragments of the CFTR polypeptide. For example, Guggino and co-workers (4) suggested that full-length WT-CFTR exhibited two subconductance levels that summed to the full conductance level, and it was proposed that each subconductance represented the properties of pores derived from different parts of the polypeptide. These studies, while intriguing, suffer from several limitations. First, the observation of channel activity that could be attributed to a CFTR fragment would not necessarily imply that such a fragment would form a similar structure when it resided within the parent molecule; CFTR fragments do not produce activity in all cases (30). Second, it is difficult to unequivocally attach a particular observed channel activity to a particular fragment; two copies of the front half (31) or two copies of the back half (32) may dimerize to form channels that are not related to channels formed by the intact protein.

Evidence for the one-pore, two-polypeptide model has been inferred from studies of channels in cells expressing CFTR concatemers comprising two polypeptides expressed in tandem. Ma and co-workers (3, 11) reported that channels formed from such constructs opened to a single level with the same conductance as channels expressed individually. However, the possibility exists that the observed channel behavior can be attributed to the activity of only one of the two monomers, with the other monomer silenced by the method of tethering the two together. Low resolution structures of CFTR were interpreted as suggesting that two CFTR polypeptides can dimerize in plasma membranes (33, 34), but these images do not make any predictions as to whether these putative dimeric structures comprise one pore or two. In contrast, recent structural data from electron microscopy was interpreted as suggesting that CFTR resides in the membrane as a monomer (35). Several recent studies reported that two CFTR molecules indeed may be induced to interact through binding of scaffolding proteins containing bivalent or multivalent PDZ domains, such as CAP70 (28) and NHERF (29), which appeared to result in an increase in the activity of a single pore rather than an increase in the number of open levels. However, it is not clear that the MSDs from both of the interacting CFTR peptides were contributing to chloride permeation. Further, because channels were evident in the record before the addition of the PDZ peptides, it is apparent that the formation of the minimum functional unit does not require PDZ-mediated interactions between multiple CFTR peptides.

Several laboratories have attempted to identify the structure of the pore of CFTR by examining the behavior of CFTR protein isolated and detergent-solubilized using a variety of methods, but these studies have not produced entirely consistent results (5). Using chemical cross-linking and nondissociative polyacrylamide gel electrophoresis, Bear and co-workers (36) studied the quaternary structure of purified, reconstituted CFTR, and suggested that CFTR exists in monomeric form, which gated to a single open level in planar lipid bilayers, but that reconstitution of CFTR monomers often led to formation of dimers. Chen et al. (37) could find no evidence of hybrid channels when WT-CFTR was co-expressed with either of several pore domain mutants.

Rosenberg and co-workers (38) reported that lectin-gold labeling of the single glycosylation site in P-glycoprotein, another member of the ATP-binding cassette transporter superfamily, resulted in particle size that was consistent with the monomeric form. Loo and Clarke (39, 40) used site-directed mu-
tagenesis to study the substrate-binding pocket of P-glycoprotein; TM domains in both the front and back halves of the full-length polypeptide contributed to the substrate-binding pocket and may be cross-linked to each other by aqueous reagents. Finally, the solution structures of two Escherichia coli ATP-binding cassette transporters, MscA and BtuCD, clearly show that two copies of these half-transporters dimerize to form the functional complexes, with one substrate-binding pocket (not two) formed from TM helices from each of the two MSDs (not four) (41, 42). By analogy, these structures suggest that the single substrate-binding pocket (or pore) of full-length CFTR may be composed of TM domains from both MSDs in a single CFTR peptide.

In the present experiments, we compared the subconductance behavior of wild type and mutant CFTRs and investigated the effect on subconductance behavior of covalent charge deposition using R334C-CFTR. The amplitudes of all three conductance states were simultaneously increased in nearly identical proportion upon modification by MTSET\(^{+}\). This result demonstrates unequivocally that the subconductance states reported here are, in fact, properties of the CFTR channel and strongly suggests that all three conducting conformations share at least a portion of the same conduction path, which contains the arginine at position 334. This finding is not compatible with the notion that subconductance states represent the properties of two completely separate conduction pathways (Fig. 8B). Rather, the present results support a model in which the subconductance pathways utilize at least a common outer vestibule that contains Arg\(^{334}\) (although divergence at the cytoplasmic end of the pore cannot be ruled out). It is also difficult to reconcile the results presented here with any model requiring the anion-conducting pore to be formed at the interface between two CFTR monomers (Fig. 8C), in a manner analogous to the structures of K\(^{+}\)-selective channels (43). In the simplest conception of such a model (3), a dimeric pore would be expected to contain two copies of Arg\(^{334}\) (or Cys\(^{334}\)). Neither the impact of covalent labeling nor the kinetics of labeling provided by a simplified model incorporating a charged vestibule in the subconductance states reflect different conformations of a single pore. Taken together, the results in this study are consistent with the notion that a single functional CFTR channel is built from a single CFTR polypeptide. Whereas there remains a possibility that multiple CFTR polypeptides may dimerize in epithelial cells, perhaps due to interaction with PDZ-domain proteins, each of these CFTR polypeptides would be expected to comprise a separate pore.

Acknowledgments—We thank Drs. Chris Hartzell and Zhiqiang Qu for helpful discussions and Christopher Thompson for reading the manuscript.

REFERENCES

1. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Flavest, N., Chou, J. L., Drumm, M., Jannuzzi, M. C., and Collins, P. S. (1989) Science 245, 1066–1073
2. McCoy, N. A. (2000) J. Exp. Biol. 203, 1947–1962
3. Zerhusen, B., Zhao, J. Y., Xie, J. X., Davis, P. B., and Ma, J. J. (1999) J. Biol. Chem. 274, 7627–7630
4. Vue, Y., Devidas, S., and Guggino, W. B. (2000) J. Biol. Chem. 275, 10030–10034
5. Dawson, D. C., Liu, X., Zhang, Z.-R., and McCoy, N. A. (2003) in The CFTR Chloride Channel (Kirk, K., and Dawson, D. C., eds) pp. 1–34, Landes Biosciences, Georgetown, TX
6. Haws, C., Krouse, M. E., Xia, Y., Gruner, D., and Wainwright, B. J., and Sheppard, J. (1997) J. Mol. Biol. 263, L692–L707
7. Ma, J., and Davis, P. B. (1995) Nature 378, 293–298
8. Gallet, X., Festy, F., Ducarme, P., Brasseur, R., and Thomas-Soumaré, A. (1998) J. Biol. Chem. 273, 29373–29380
9. Antosiewicz, J., Immonen, J. A., and Vilcek, J. (2000) Nature 405, 500–503
10. Wang, S., Yue, H., Derin, R. B., and Guggino, W. B. (2002) Cell 109, 169–179
11. Raghuram, V., McLaughlin, J., and Foskett, J. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1300–1305
30. Chan, K. W., Csanady, L., Seto-Young, D., Nairn, A. C., and Gadsby, D. C. (2000) J. Gen. Physiol. 116, 163–180
31. Sheppard, D. N., Ostergaard, L. S., Rich, D. P., and Welsh, M. J. (1994) Cell 76, 1091–1098
32. Ramjeesingh, M., Ugwu, F., Li, C., Dhani, S., Huan, L. J., Wang, Y., and Bear, C. E. (2003) Biochemistry 375, 633–641
33. Eskandari, S., Wright, E. M., Kreman, M., Starace, D. M., and Zampighi, G. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11235–11240
34. Schillers, H., Shahin, V., Albermann, L., Schafer, C., and Oberleithner, H. (2004) Cell Physiol. Biochem. 14, 1–10
35. Rosenberg, M. F., Kamis, A. B., Alexsandrov, L. A., Ford, R. C., and Riordan, J. R. (2004) J. Biol. Chem. 279, 39051–39057
36. Ramjeesingh, M., Li, C., Kogan, I., Wang, Y., Huan, L. J., and Bear, C. E. (2001) Biochemistry 40, 10700–10706
37. Chen, J.-H., Chang, X.-B., Aleksandrov, A. A., and Riordan, J. R. (2002) J. Membr. Biol. 188, 55–57
38. Rosenberg, M. F., Callaghan, R., Ford, R. C., and Higgins, C. F. (1997) J. Biol. Chem. 272, 10685–10694
39. Loo, T. W., and Clarke, D. M. (1999) Biochemistry 38, 5124–5129
40. Loo, T. W., and Clarke, D. M. (1999) J. Biol. Chem. 274, 35388–35392
41. Chang, G., and Roth, C. B. (2001) Science 293, 1793–1800
42. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) Science 296, 1091–1098
43. Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
44. Gong, X., and Linsdell, P. (2003) J. Physiol. 549, 387–397
45. Miller, C. (1982) Phil. Trans. R. Soc. London 299, 401–411
46. Ludewig, U., Pusch, M., and Jentsch, T. J. (1996) Nature 383, 340–343
47. Weinreich, F., and Jentsch, T. J. (2001) J. Biol. Chem. 276, 2347–2353
48. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 415, 287–294
49. Schwalbe, R. A., Wingo, C. S., and Xia, S. L. (2002) Biochemistry 41, 12457–12466

CFTR Is a One-pore Channel
Determination of the Functional Unit of the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel: ONE POLYPEPTIDE FORMS ONE PORE

Zhi-Ren Zhang, Guiying Cui, Xuehong Liu, Binlin Song, David C. Dawson and Nael A. McCarty

J. Biol. Chem. 2005, 280:458-468.
doi: 10.1074/jbc.M409626200 originally published online October 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409626200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 21 of which can be accessed free at http://www.jbc.org/content/280/1/458.full.html#ref-list-1