State-dependent Access of Anions to the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore*

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The cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel is gated by intracellular factors; however, conformational changes in the channel pore associated with channel activation have not been identified. We have used patch clamp recording to investigate the state-dependent accessibility of substituted cysteine residues in the CFTR channel pore to a range of cysteine-reactive reagents applied to the extracellular side of the membrane. Using functional modulation of the channel current-voltage relationship as a marker of modification, we find that several positively charged reagents are able to penetrate deeply into the pore from the outside irrespective of whether or not the channels have been activated. In contrast, access of three positively charged cysteine-reactive methanesulfonate (MTS) reagents to several different sites in the pore is strictly limited prior to channel activation. This suggests that in nonactivated channels some ion selectivity mechanism exists to exclude anions yet permit cations into the channel pore from the extracellular solution. We suggest that activation of CFTR channels involves a conformational change in the pore that removes a strong selectivity against anion entry from the extracellular solution. We propose further that this conformational change occurs in advance of channel opening, suggesting that multiple distinct closed pore conformations exist.

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR)¹ (1). CFTR is a member of the ATP-binding cassette family of membrane transport proteins that functions as a Cl⁻ channel in the apical membrane of many different epithelial cell types (2, 3). The CFTR protein contains 12 transmembrane (TM) α-helices that are presumed to form the pore region through which Cl⁻ ions cross the membrane (1, 4, 5). Channel activity is regulated by cytoplasmic factors. Phosphorylation of the regulatory domain by cAMP-dependent protein kinase A (PKA) is a prerequisite for channel opening (2, 3). Following phosphorylation, channel opening and closing are controlled by ATP interactions with the two intracellular nucleotide binding domains (NBDs) (6, 7). How these cytoplasmic processes lead to a structural rearrangement of the TM regions that results in opening of the Cl⁻ permeation pathway is not known.

Once open, the CFTR channel pore allows rapid permeation of Cl⁻ and other small anions but selects strongly against small cations (8). The mechanism of anion selectivity is not known. One factor that likely contributes is the presence of positively charged arginine and lysine side chains in the permeation pathway. Indeed, several positively charged residues have been shown to be involved in attracting Cl⁻ ions into the pore by an electrostatic mechanism (9, 10). The importance of charge at these positions can be demonstrated not only by mutagenesis to residues of different charge but also by mutation to cysteine followed by covalent modification by charged cysteine-reactive methanesulfonate (MTS) reagents. Such modification mimics the effects of mutagenesis, leading to side chain charge-dependent effects on the shape of the channel current-voltage relationship that reflect changes in unitary Cl⁻ conductance (9, 10). In the present study, we take advantage of charge-dependent changes in current-voltage relationship shape that result from the modification of introduced cysteines by charged reagents to report structural rearrangements of the channel pore that occur during the channel activation process.

EXPERIMENTAL PROCEDURES

Experiments were carried out on baby hamster kidney cells transfected with wild type or mutant forms of human CFTR (11). Patch clamp recording from inside-out patches excised from these cells was performed as described previously (11, 12). Following patch excision and recording of background currents, CFTR channels were activated by exposure to PKA catalytic subunit (20 nM) plus MgATP (1 mM) in the cytoplasmic solution. Activated channels were then “locked” in the open state by addition of 2 mM sodium pyrophosphate (PPi) to ensure state-dependent effects on the shape of the channel current-voltage relationship that reflect changes in unitary Cl⁻ conductance (9, 10). In the present study, we take advantage of charge-dependent changes in current-voltage relationship shape that result from the modification of introduced cysteines by charged reagents to report structural rearrangements of the channel pore that occur during the channel activation process.

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² The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-1-methylxanthine; MBTA, 4-(N-maleimidobenzyl)-α-trimethylammonium iodide; MTS, methanesulfonate; MTSES, sodium (2-sulfonatoethyl)methanesulfonate; MTSET, 2-(trimethylammonium)ethyl methanesulfonate bromide; NBD, nucleotide binding domain; pCMBS, p-chloromercuriphenylsulfonic acid; TM, transmembrane; PKA, protein kinase A; TMAEM, N-(2-(trimethylammonium)ethyl)-maleimide chloride; I-V, current-voltage; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonate.
Channels were exposed to extracellular cysteine-reactive reagents in two different ways. First, these reagents were present in the pipette solution throughout the experiment. Second, cells were pretreated with reagents in normal bath solution for 5–10 min prior to the experiment. Pretreated cells were then washed with bath solution to remove these reagents prior to transfer to the recording chamber and immediate use. In some cases, cells were pretreated with cysteine-reactive reagents together with the cAMP-activating compounds forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX; 100 μM). Cysteine-reactive reagents used were the MTS reagents sodium (2-sulfonatoethyl) methane sulfonate (MTSET) and 2-(trimethylammonium)ethyl methanesulfonate (TMAEM), and the organic mercurial p-chloromercuriphenylsulfonic acid (pCMBS). These substances were initially prepared as high concentration stock solutions in distilled water and stored frozen as small volume aliquots until the time of use, when they were diluted in bath solution and used immediately.

For experiments using Au(CN)2− to covalently modify an introduced cysteine, cells were pretreated with KAu(CN)2 in normal bath solution for 1–5 min either with or without cotreatment with forskolin plus IBMX. Following pretreatment, cells were washed with normal bath solution, transferred to the recording chamber, and used for patch clamp experiments immediately. Inside-out patches excised from cells pretreated in this way were then exposed to PKA, ATP, and PPi, as usual, to activate CFTR channel currents. Following attainment of full current amplitude, patches were then exposed to KCN (50 μM) in the bath solution to reverse covalent modification of cysteines by Au(CN)2− (13). We found that application of higher concentrations of KCN led to immediate loss of the gigaseal. All chemicals were from Sigma except for PKA (Promega, Madison, WI) and MTSES, MTSET, MBTA, and TMAEM (Toronto Research Chemicals, North York, ON, Canada).

Current traces were filtered at 100 Hz using an 8-pole Bessel filter, digitized at 250 Hz, and analyzed using pCLAMP software (Molecular Devices, Sunnyvale, CA). Macroscopic current-voltage (I-V) relationships were constructed using depolarizing ramp protocols (8, 14). Background (leak) currents recorded before addition of PKA and ATP have been subtracted digitally, leaving uncontaminated CFTR currents (8, 12). Relative I-V shape under different conditions was compared by plotting current as a fraction of that recorded at −80 mV (Irel).

Rectification of the I-V relationship was quantified as the "rectification ratio": the slope conductance at −50 mV as a fraction of that at +50 mV (15, 16). Experiments were carried out at room temperature, 21–24 °C. Values are presented as mean ± S.E. Unless stated otherwise, tests of significance were carried out using an unpaired t test, with p < 0.05 being considered statistically significant.

RESULTS

Modification by MTS Reagents—Arg-334 contributes an important surface charge in the outer mouth of the CFTR channel pore (9, 17). The importance of side chain charge at this position has been demonstrated previously by reaction with charged MTS reagents following mutation to cysteine (9, 18). Thus side chain charge at this position, whether determined by mutation or by covalent modification of an introduced cysteine by charged reagents, plays an important role in controlling Cl− entry into the pore from the extracellular solution and hence the shape of the I-V relationship (9, 18).

Similar effects have been observed for other surface charges in the CFTR pore (10). Indeed, when MTS reagents were included in the pipette solution, we found similar effects on I-V relationship shape in inside-out patches (Fig. 1, A–D) to those described previously in Xenopus oocytes (9). As shown previously (9, 15), the R334C mutation itself induced inward rectification because of removal of the native positive charge and reduced electrostatic attraction of extracellular Cl− ions. Inclusion of the positively charged MTSET in the pipette solution then restored a near-linear I-V relationship, whereas the negatively charged MTSES significantly increased the degree of inward rectification. All of these results are consistent with changes in the electrostatic attraction of extracellular Cl− ions as a result of the deposition of charge near the outer mouth of the pore following modification of the introduced cysteine side chain, as proposed previously (9). As described previously (9, 18, 19), neither MTSET nor MTSES had any effect on wild type CFTR, which exhibited a near-linear I-V relationship under all conditions studied (Fig. 1, A and B). These changes in I-V relationship shape therefore serve as a convenient reporter of MTS modification of the introduced cysteine residue.

In contrast to these results obtained with inclusion of MTS reagents in the pipette solution, we found that MTSES was apparently unable to modify R334C using a pretreatment protocol. Pretreatment with MTSET for 5 min prior to cell transfer to the recording chamber and removal of the MTS had a similar effect to inclusion of MTSET in the pipette; however, using the same protocol MTSES failed to alter I-V relationship shape (Fig. 1, E and F). This lack of effect could reflect that MTSES does not covalently label R334C and so must be present during the experiment to exert its effect, that covalent modification does occur but is relatively transient, or that covalent modification takes place during pipette application but not during pretreatment.

We considered that the factor most likely to contribute to differential covalent modification under these two conditions was the state of the CFTR channels: during pretreatment the channels should be closed, whereas when it is present in the pipette solution during channel activation MTSES should have access to both closed and open channels. We therefore repeated the MTSES pretreatment in the presence of two cAMP-activating compounds, forskolin and IBMX, to activate the channels during the pretreatment period. Following cAMP stimulation, MTSES was able to modify R334C channels and induce increased inward rectification of the I-V relationship (Fig. 1, G and H), indicating that stable covalent modification of this introduced cysteine could take place but was somehow dependent on the activation state of the channels. This suggests that access of the negatively charged MTSES, but not the positively charged MTSET, to the CFTR pore was severely limited prior to channel activation.

Conformational Change in the Pore on Activation of CFTR
Next we asked if this apparent state-dependent access of MTSES was limited to R334C or if a similar effect could be seen with cysteines introduced at other sites in the pore. Previously it has been shown that cysteines introduced at a number of positions in the pore-lining TM6 region of CFTR were accessible to extracellular MTS reagents (9, 19, 20). An example is S341C; as shown in Fig. 2A, inclusion of MTS reagents in the pipette solution also gave charge-dependent changes in $I-V$ shape in this mutant, indicating that deposition of charge at this position also alters anion movement in the pore. In fact, similar charge-dependent effects were observed in R334C, K335C, T338C, and S341C (Fig. 3). Another mutant, F337C, became significantly more inwardly rectified in the presence of MTSES but was apparently not affected by inclusion of MTSET in the pipette solution (Fig. 3). Thus, changes in $I-V$ shape because of charge deposition by covalent modification of introduced cysteines seem to be a common feature of accessible side chains in TM6 that are presumably arranged along the axis of the CFTR pore.

The state dependence of modification of cysteines introduced at these sites was studied using the MTS pretreatment protocol described above for R334C. Again, results from the example mutant S341C are shown in Fig. 2. As with R334C (Fig. 1), MTSES induced inward rectification of the $I-V$ relationship (indicating covalent modification of the substituted cysteine) when included in the pipette solution but not when preincubated with the cells. Results from all five reactive cysteine mutants studied using charged MTS reagents are summarized in Fig. 4. For each of the five, MTSET had similar effects on $I-V$
shape when included in the pipette solution or when preincubated with cells (or, in the case of F337C, the same lack of effect) (Fig. 4A). In contrast, in each mutant MTSES was able to induce inward rectification of the I-V relationship when present in the pipette solution but had no significant effect following pretreatment (Fig. 4B). State-dependent modification by external

MTSES, but not MTSET, therefore appears to be common to TM6 residues along the axis of the pore.

Access of Other Charged Reagents to the Pore—The results with MTS reagents suggest that the positively charged MTSET can enter CFTR channels and covalently modify introduced cysteine residues prior to activation, whereas the negatively charged MTSES cannot enter the pore until activation takes place. To investigate if this reflected some unusual property of MTS reagents or if it was more generally applicable to charged reagents, we sought other non-MTS reagents that would covalently modify introduced cysteines to alter channel properties.

Two positively charged maleimide derivatives, MBTA and TMAEM, had similar effects on R334C as those observed with MTSET (Fig. 5), leading to a more linear I-V relationship. In contrast, these reagents had no effect on I-V shape in wild type (data not shown). The similar functional effects of MTSET, MBTA, and TMAEM confirm that side chain charge is the important factor controlling I-V shape. As with MTSET, these other positively charged reagents appeared equally effective when present in the pipette solution or when used to pretreat cells. This result suggests that different kinds of cysteine-reactive reagents can be used to deposit a positive charge in the pore and that different kinds of organic cations can access the pore prior to channel activation.

The effects of MTSES on R334C were mimicked by another negatively charged reagent, pCMBS (Fig. 6). As with MTSES, inclusion of pCMBS in the pipette solution led to increased inward rectification of the I-V relationship. In contrast, pCMBS had no effect on I-V shape when preincubated with cells, but modification could be restored under these conditions by cAMP stimulation. pCMBS had no effect on wild type under any of these conditions (data not shown). These results indicate that pCMBS exhibits the same state-dependent modification of R334C as MTSES, suggesting that it is the negative charge of the cysteine-reactive reagent that results in its exclusion from the pore prior to channel activation.

FIGURE 2. Modification of S341C-CFTR by external MTS reagents. A, example I REL-V relationships recorded with or without MTS reagents in the pipette solution. B, example I REL-V relationships recorded with or without pretreatment with MTS reagents. C and D, mean rectification ratios estimated for the experimental conditions shown in A and B, respectively (mean of data from 3–5 patches). Asterisks indicate a significant difference from control (p < 0.001).

FIGURE 3. Modification by charged MTS reagents induces rectification of the I-V relationship in different cysteine mutant forms of CFTR. Shown are mean rectification ratios estimated for different CFTR variants with or without MTS reagents in the pipette solution, plotted as a function of the predicted side chain charge caused by MTS modification (+1 with MTSET, 0 control, −1 with MTSES). It is presumed that in wild type no modification takes place, and here charge reflects only the charge of the modifying reagent applied. Side chain charge of the introduced cysteine may not in fact be zero, but slightly negative (9). ○, wild type (both panels); ○, R334C (left); □, K335C (left); △, F337C (right); ◊, T338C (right); ♦, S341C (right) (mean of data from 3–9 patches). All MTS treatments led to significant changes in rectification ratio (p < 0.05) except MTSET-wild type, MTSES-wild type, and MTSET-F337C.
Because MTSES and pCMBS are large organic anions, it is difficult to extrapolate their state-dependent access to the pore with smaller anions like Cl\(^{-}\)/H\(_2\)O\(_2\). We therefore used Au(CN)\(_2\)/H\(_2\)O\(_2\), a highly permeant anion that has been shown to covalently modify the introduced cysteine in T338C-CFTR to block the permeation pathway (13). This blocking effect of Au(CN)\(_2\)/H\(_2\)O\(_2\) is very stable but can be reversed using an excess of CN\(^{-}\)/H\(_2\)O\(_2\) ions (13). We therefore reasoned that if Au(CN)\(_2\)/H\(_2\)O\(_2\) entered the pore to modify the introduced cysteine of T338C covalently, only very small currents could be activated by PKA, ATP, and PPi but that currents would subsequently be activated on exposure to CN\(^{-}\)/H\(_2\)O\(_2\). Because Au(CN)\(_2\)/H\(_2\)O\(_2\) is a permeant blocker (11, 21) it could not be included in the pipette solution; however, we could investigate its state-dependent access to the pore by preincubation with and without cAMP stimulants.

As shown in Fig. 7, pretreatment with Au(CN)\(_2\) did not appear to modify wild type CFTR either with or without cAMP stimulation. Thus, following pretreatment with 10 \(\mu\)M Au(CN)\(_2\) for 5 min, large currents could be activated by PKA, ATP, and PPi, and these were not significantly enhanced by exposure to 50 \(\mu\)M KCN in the bath solution (Fig. 7, A and B). In T338C, however, pretreatment with the same concentration of Au(CN)\(_2\) for only 1 min led to the appearance of a KCN-sensitive component of current (Fig. 7A). We interpret this to reflect CN\(^{-}\) reversal of channel block caused by Au(CN)\(_2\) modification of the introduced cysteine in this mutant. Whereas this apparent modification did take place in the absence of cAMP stimulation, it can be seen that the degree of modification was greatly enhanced by cAMP stimulation (Fig. 7). Thus, without cAMP stimulation, KCN treatment increased CFTR Cl\(^{-}\)/H\(_2\)O\(_2\) conductance by 1.95 \(\pm\) 0.24-fold (\(n = 6\)), whereas after cAMP stimulation KCN treatment increased conductance by 13.7 \(\pm\) 1.2-fold (\(n = 4\)) (Fig. 7B). As shown in Fig. 7C, this dramatic increase in CFTR conductance induced by KCN in patches excised from cAMP-pretreated cells appears to reflect activation of channels that had been inhibited, presumably by Au(CN)\(_2\) covalent modification. Thus, although it appears that Au(CN)\(_2\) can modify T338C-CFTR with or without cAMP stimulation, the dramatic increase in the proportion of channels apparently modified by Au(CN)\(_2\) when cAMP stimulation is applied concurrently with Au(CN)\(_2\) exposure suggests that the rate of modification is far greater in activated channels than in nonactivated channels.

Similar state-dependent inhibition by Au(CN)\(_2\) was also observed for other introduced cysteines (Fig. 7, D and E). For
each of R334C, K335C, and S341C, like T338C, the apparent degree of Au(CN)$_2$/H$_{11002}$ modification as determined by the KCN-sensitive component of the current was significantly enhanced by cAMP stimulation (Fig. 7E). This confirms that Au(CN)$_2$/H$_{11002}$ access to different sites within the pore is greater in activated than in nonactivated channels. In contrast, F337C was only very weakly inhibited by Au(CN)$_2$/H$_{11002}$ either with or without cAMP prestimulation. Alteration of I-V shape in F337C by MTSES (Figs. 3 and 4B) confirms the previous report that the side chain at this position is in contact with the aqueous lumen of the pore (19). However, this mutant was not functionally modified either by MTSET (Fig. 4A) or Au(CN)$_2$ (Fig. 7E). Currently it is not clear if this reflects relatively weak reactivity of the substituted cysteine at this position or if modification by MTSET and/or Au(CN)$_2$ fails to alter the experimental parameters that we are measuring. Whatever the explanation, this lack of effect appears to be specific for F337C because the four other reactive cysteine mutants studied could be modified functionally by all reagents tested.

**DISCUSSION**

Covalent modification of introduced cysteines has been used previously to illustrate the important surface charge roles played by native arginine residues at both the outer mouth (Arg-334 (9)) and inner mouth (Arg-303 (10)) of the CFTR pore. Our results suggest that a similar approach can be used to introduce charge at other locations throughout the pore that do not normally have charged side chains and that these introduced charges appear to have similar electrostatic effects on Cl$^{-}$ movement in the pore (Fig. 3). In the present work we have used these functional effects as convenient markers of the covalent modification of introduced cysteine residues by different reagents and under different conditions.

The main finding of the current work is that access of external anions to the CFTR channel pore is severely limited prior to channel activation. This applies both to the large negatively charged reagents MTSES and pCMBS and to the small, permeant anion Au(CN)$_2$. In contrast, large cations including MTSET, MBTA, and TMAEM are apparently able to enter into the pore prior to channel activation. In the case of MTSET at least, this large organic molecule is able to enter far enough into the pore of nonactivated channels to react with a cysteine substituted for Ser-341, purportedly located in the pore inner vestibule (22). In contrast, MTSES cannot even modify R334C in the outer mouth of the pore under these conditions. This suggests that the activated and nonactivated channel pores are functionally distinct, presumably reflecting some change in pore conformation that accompanies channel activation.

Reactivity of external MTSET and MTSES with R334C-CFTR has been studied previously, and it was shown that following channel activation both of these reagents modified this introduced cysteine only when the channel was closed (18). However, in this case both MTSET and MTSES modified the same state of the channel. The crucial difference from our own study is that these channels were studied following activation by PKA, whereas we show that prior to channel activation, no modification by MTSES takes place. This result implies that a change in anion access to the pore occurs prior to channel opening during the activation process (Fig. 8). We propose that prior to cAMP stimulation the channel pore is in an “inactive”
state that is separate from the “closed” state that separates openings during normal channel gating. Thus, at least two functionally distinct, nonconducting states of the CFTR channel pore exist. The switch from inactive to closed may involve, for example, phosphorylation by PKA. This would be consistent with the idea that activation of CFTR is a two-step process; the first step is controlled by phosphorylation of the regulatory domain, and the second step is controlled by ATP interactions with the NBDs (2, 3, 6, 7). Once the channel has been “activated” it will cycle between the closed and open states because of ATP-dependent channel gating governed by the NBDs (6, 7). This gating cycle also appears to involve changes in accessibility to both MTSET and MTSES, at least as reported by a cysteine substituted for Arg-334 (18), although in this case there is no apparent charge dependence of accessibility.

When the channel pore is in the inactive state (Fig. 8), pore-lining residues can be modified by cationic reagents (MTSET, MBTA, TMAEM) but not anionic MTSES or pCMBS. This distinction appears to hold for several accessible cysteines within the TM6 region that are presumably arranged along the axis of the pore, suggesting a global difference in access to the pore. Access of permeant Au(CN)₂⁻ ions to the pore also appears to increase dramatically on channel activation (Fig. 7). This implies that prior to channel activation some mechanism of charge selectivity exists that allows external cations, but not anions, to enter the channel pore. In contrast, size selectivity does not appear to be highly stringent because large cations (such as MTSET) can penetrate far into the pore of inactive channels. We propose that channel activation (which, we note, is separate from channel opening) is associated with a structural rearrangement of the pore that removes a charge-selective barrier and allows anions to enter from the extracellular solution. This may

![Figure 7](http://www.jbc.org/)

**FIGURE 7. State-dependent modification of introduced cysteines by external Au(CN)₂⁻ ions.** A, the effect of KCN reveals covalent modification by Au(CN)₂⁻. Example I-V relationships recorded following pretreatment with 10 μM KAu(CN)₂ for 5 min (wild type) or 1 min (T338C) is shown. For those traces indicated +cAMP, the pretreatment also included forskolin (10 μM) and IBMX (100 μM). In each case, currents are shown following channel stimulation with PKA, ATP, and PP (control) and following subsequent treatment with 50 μM KCN. B, the mean change in CFTR macroscopic conductance following addition of KCN without (white bars) or with (black bars) cAMP pretreatment is shown (mean of data from 3–9 patches). The asterisk indicates a significant difference from control conditions for the same channel variant (p < 0.0001). C, KCN-induced changes in CFTR macroscopic conductance for individual patches expressing T338C-CFTR, following pretreatment with KAu(CN)₂ alone (○) or KAu(CN)₂ plus forskolin and IBMX (●) are shown. Note the very low conductance prior to KCN treatment for (●) but similar conductance following treatment, which suggests similar numbers of functional channels in each patch. In both cases, KCN treatment led to a significant increase in conductance (p < 0.005, paired t test). CFTR conductance was quantified as the chord conductance over the entire voltage range studied (±80 mV). nS, nanosiemens. D, an example I-V relationship for R334C pretreated with 10 μM KAu(CN)₂, plus forskolin (10 μM) and IBMX (100 μM) for 1 min recorded before (control) and after treatment with 50 μM KCN is shown. E, the mean change in CFTR macroscopic conductance for R334C, K335C, F337C, and S341C following addition of KCN without (white bars) or with (black bars) cAMP pretreatment is shown (mean of data from 4–5 patches). Asterisks indicate a significant difference from control conditions for the same channel variant (p < 0.05).
result from, for example, rearrangement of charged residues close to the outer mouth of the pore that influence entry of charged substances from the extracellular solution. Because modification of R334C, which is thought to be located at the outer mouth of the pore, is state-dependent, we suggest that the conformational change associated with channel activation must affect the very outermost part of the pore to influence access of external ions to Arg-334. Because this conformational change is presumably triggered by intracellular events at the regulatory domain and/or NBDs, it must be signaled across the entire membrane-spanning part of the CFTR protein. This structural rearrangement may represent a crucial early step in the formation of an anion-selective permeation pathway. A separate conformational change subsequently leads to opening of this pathway and permeation by Cl− ions. Currently nothing is known concerning the physical mechanism or structural basis of this second conformational change that opens the pore.

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