ORIGINAL PAPER

The role of protons in fast and slow gating of the *Torpedo* chloride channel ClC-0

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Abstract Transmembrane proton transport is of fundamental importance for life. The list of H^+ transporting proteins has been recently expanded with the discovery that some members of the CLC gene family are stoichiometrically coupled Cl^-/H^+ antiporters. Other CLC proteins are instead passive Cl^- selective anion channels. The gating of these CLC channels is, however, strongly regulated by pH, likely reflecting the evolutionary relationship with CLC Cl^-/H^+ antiporters. The role of protons in the gating of the model *Torpedo* channel ClC-0 is best understood. ClC-0 is a homodimer with separate pores in each subunit. Each protopore can be opened and closed independently from the other pore by a "fast gate". A common, slow gate acts on both pores simultaneously. The opening of the fast gate is controlled by a critical glutamate (E166), whose protonation state determines the fast gate's pH dependence. Extracellular protons likely can arrive directly at E166. In contrast, protonation of E166 from the inside has been proposed to be mediated by the dissociation of an intrapore water molecule. The OH^- anion resulting from the water dissociation is stabilized in one of the anion binding sites of the channel, competing with intracellular Cl^- ions. The pH dependence of the slow gate is less well understood. It has been shown that proton translocation drives irreversible gating transitions associated with the slow gate. However, the relationship of the fast gate's pH dependence on the proton translocation and the molecular basis of the slow gate remain to be discovered.

Introduction: CLC channels and transporters

Ion channels and active ion transporters are generally assumed to be constructed according to different design principles as they have to fulfill opposite functions. A passive ion channel must allow very high throughput, while a primary or secondary active transporter must strictly avoid the passive slippage of the substrate that is pumped against its gradient. In fact, the structure of classical cation channels reveals the presence of an uninterrupted vertical pore through the membrane (Doyle et al. 1998), whereas no such pore is seen in the crystal structure of ion pumps and transporters (see e.g., Toyoshima et al. 2000). The family of Cl^- transporting CLC proteins provides an interesting exception to this general rule: some family members, like the prototype *Torpedo* channel ClC-0, are ion channels with a sizable conductance of ~ 10 pS (Miller 1982); others, like the bacterial ClC-ec1 or the human ClC-5, are stoichiometrically coupled 2 Cl^-:1 H^+ antiporters (Accardi and Miller 2004; Picollo and Pusch 2005; Scheel et al. 2005).

Recent reviews have highlighted the important roles of CLC proteins in human physiology and pathophysiology (Jentsch 2008; Jentsch et al. 2005; Zifarelli and Pusch 2007), and the general biophysical properties of CLC proteins have been described in detail (Zifarelli and Pusch 2007). In the present short review, we will restrict our attention to the role of protons in the gating of the model *Torpedo* channel, ClC-0.

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The architecture of CLC proteins

All CLC proteins are homodimers with each subunit bearing a proper ion translocation pathway that is physically separate from that of the neighboring subunit (Ludewig et al. 1996; Middleton et al. 1996; Weinreich and Jentsch 2001). This structural architecture was confirmed by the X-ray structure of the bacterial ClC-ec1 (Dutzler et al. 2002) (Fig. 1). The location of the ion pathway was indicated by the presence of two Cl^- ions in each subunit, an inner Cl^- (defining the binding site S_{int}) and a central Cl^- (S_{cen}) (Dutzler et al. 2003) (Fig. 1). The most important insight from these structural studies was the identification of a glutamate residue (E148 in ClC-ec1) whose side chain blocks the exit of Cl_{cen} towards the extracellular space (Fig. 1). In the crystal structure of a mutant ClC-ec1, in which E148 was changed to an alanine (E148A), a third Cl^- ion (defining S_{ext}) was found at the position that is occupied by the E148 side-chain in the WT (Dutzler et al. 2003). This third Cl^- ion was also seen in the structure of the E148Q mutant; the side-chain of the glutamine, pointed towards the extracellular space (Dutzler et al. 2003). Thus, E148 appears to be a gate in ClC-ec1 that controls the occupation of S_{ext} by a Cl^- ion, blocking any anion transport if its side-chain occupies S_{ext}. The fundamental role of E148 has been confirmed using functional studies: E148A, and other mutants of E148, completely lose the Cl^-/H^+ antiport activity; they behave as passive Cl^- selective permeabilities (Accardi and Miller 2004). Thus, the mechanisms of transport coupling involve E148, which most likely oscillates through protonated and deprotonated states for each transport cycle.
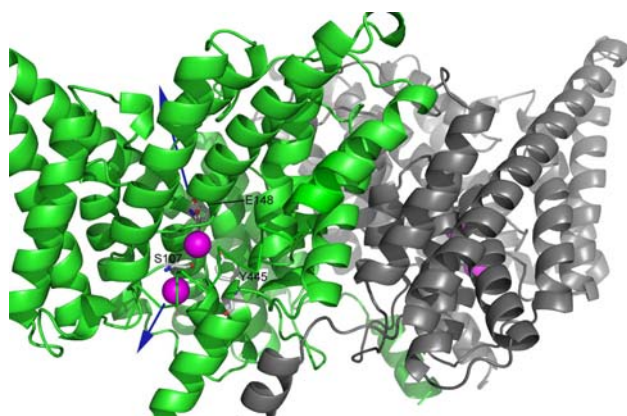

Fig. 1 The structure of the bacterial ClC-ec1. One subunit is shown in *green*, the other in *gray*. The view is approximately from within the membrane: the extracellular side is on *top*. Cl^- ions are shown in *pink*. Residues E148, Y445, and S107 are shown as *sticks*. The *arrows* indicate the approximate ion permeation pathway. Figure was prepared using Pymol based on pdb entry 1OTS

E148 is part of a "signature sequence" that is one of the most highly conserved regions among all CLC proteins (Mindell and Maduke 2001). The role of the equivalent residue, E166, for the gating of the *Torpedo* channel ClC-0 will be discussed after providing a brief overview of the properties of ClC-0.

Gating of the model channel ClC-0

At the single-channel level, ClC-0 has a "double-barreled" appearance. Openings occur in bursts during which the two protopores open and close independently from each other. The process governing the opening of the individual pores is called protopore gate or "fast" gate; openings/closings have dwell times in the millisecond time range. A common or "slow" gate is able to shut off both pores simultaneously on a timescale of seconds. The slow gate is a function of both subunits of the homodimeric protein, whereas the fast gate of each protopore works independently for each protopore (for details of slow gate function see later section). Both gates depend on voltage, $[Cl^-]$ and pH (Pusch 2004). In fact, it has previously been proposed that part of the voltage dependence of fast gating arises indirectly from a voltage-dependent binding and/or translocation of a Cl^- ion, entering the channel from the outside (Chen and Miller 1996; Pusch et al. 1995). This hypothesis is in agreement with the fact that the fast gate opens at positive voltages, with an apparent "gating valence" of ~ 1.

However, the voltage dependence of the fast gate does not follow a simple Boltzmann distribution. In particular, the open probability does not approach zero at negative values but reaches a finite value (Chen and Miller 1996; Ludewig et al. 1997). Phenomenologically, this has been ascribed to two possible pathways that lead to channel opening: one opening route with a rate constant that increases at positive voltages with a relatively large voltage dependence, and a less steeply voltage-dependent route whose rate increases at negative voltages (Chen and Miller 1996) (Fig. 2a). The open probability, p_{open}, is given by $p_{open} = 1/(1 + \beta/\alpha)$, and the parallel increase of α and β at negative voltages explains the finding that p_{open} saturates at a non-zero value.

In order to discuss the role of protons for the fast gate, it is convenient to call these two routes "route 1" and "route 2", respectively. The closing rate constant shows a monotonic exponential voltage dependence (Chen and Miller 1996; Zifarelli et al. 2008).

The role of the above-mentioned conserved glutamate residue for the gating of ClC-0 has been quite extensively studied. Neutralizing E166 by mutating it to alanine, glutamine, or serine, completely abolishes the normal
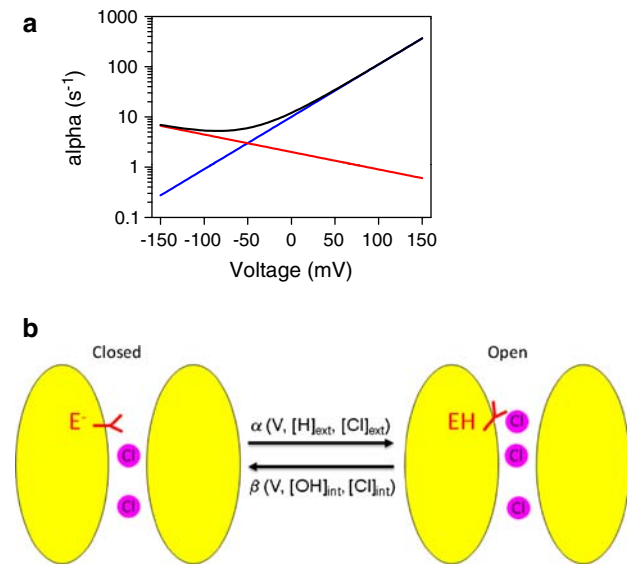
Fig. 2 Overview of fast gating of ClC-0. **a** Voltage dependence of the two gating routes (*blue* route 1, *red* route 2, *black* sum) (see text for details). **b** Schematic view of the elements and parameters involved in fast gating

voltage-dependent gating relaxations and locks the channel in an almost permanently open state (Dutzler et al. 2003; Traverso et al. 2003). Thus, E166 appears to be the major determinant of the fast protopore gate of the channel, even though additional conformational changes may accompany channel opening (Accardi and Pusch 2003).

Extracellular pH dependence of the fast gate

These mutational results suggest that opening of the fast gate involves the protonation of E166. In agreement with this hypothesis, extracellular acidification increases the open probability of the fast gate (Chen and Chen 2001; Dutzler et al. 2003). Importantly however, it seems that only the "route 2" of opening is affected by external pH, such that at acidic external pH the open probability becomes effectively voltage-independent (Chen and Chen 2001). The apparent pK of the effect was ∼5.3. In contrast, the opening rate constant of route 1 and the closing rate constant were found to be independent from pH_{ext} (Chen and Chen 2001). The interpretation of these results is that extracellular protons can directly protonate E166, leading to channel opening (Dutzler et al. 2003) (Fig. 2b).

Intracellular pH dependence of the fast gate

The pH dependence of ClC-0 had been studied more than 20 years ago by Hanke and Miller using reconstituted Torpedo electroplax channels (Hanke and Miller 1983). The interpretation of the results by Hanke and Miller is complicated by the fact that these authors varied the pH on both sides of the membrane. Nevertheless, the study showed that intracellular pH has a dramatic effect on fast gating. The effect is qualitatively different from that of pH_{ext}; increasing pH_{int} leads to a "shift" of the voltage-dependent activation curve to more positive membrane potentials. The effect of pH_{int} on the cloned ClC-0 expressed in *Xenopus* oocytes was recently studied in detail by our group using high-resolution inside-out patch clamp recording (Traverso et al. 2006; Zifarelli et al. 2008). Initially, we studied the pH dependence of the E166D mutation, in which the critical E166 was changed to an aspartate, that lacks one CH_2 group compared to a glutamate. The conservative mutation E166D had a dramatic effect on the channel properties. In particular, compared to WT, the open probability was drastically reduced. Furthermore, noise analysis indicated that the open states that can be reached by the different opening routes, had a different single-channel conductance in the mutant. The mutant could be strongly activated by acidic intracellular pH, with pH "shifting" the activation curve along the voltage axis. The pH_{int} effect was consistent with the idea that the relatively large voltage dependence of route 1 opening was caused by voltage-dependent protonation of the D166 residue from the intracellular side (Traverso et al. 2006). A similar idea was independently proposed by Miller (2006). This proposal was quite speculative and contrasted with the previously established hypothesis that the voltage dependence of fast gating opening (route 1) arises from a voltage-dependent binding and/or translocation of a Cl^- ion (Chen and Miller 1996; Engh et al. 2007; Pusch et al. 1995). These findings generated the necessity to investigate the mechanism by which protons can reach E166 from the intracellular side. The first working hypothesis suggested the presence of a titratable group acting as proton acceptor.

Intrapore water dissociation as a proton-delivery mechanism in ClC-0

More recently, we investigated the pH_{int} dependence of wild-type ClC-0 in further detail (Zifarelli et al. 2008). Based on several lines of evidence, we advanced a radically different view of the mechanism of regulation of the fast gate by intracellular pH. First, using extensive mutagenesis experiments, we were unable to identify an amino acid residue that could reasonably act as an intracellular proton sensor. We next performed a detailed kinetic analysis of the combined pH_{int} and Cl_{int} dependence of gating. Three important results emerged from this analysis:

1. The closing rate is strongly dependent on pH_{int}.
2. The closing rate is strongly dependent on Cl_{int}.
3. The opening rate is practically completely independent from pH_{int} and Cl_{int}.

These findings, and in particular the independence of the opening rate from the intracellular factors, are in qualitative disagreement with most kinetic models that incorporate a proton delivery from the bulk free proton pool via a titratable group. An alternative mechanism, illustrated in Fig. 3a, proposes a completely different mechanism of proton delivery, the dissociation of a water molecule. This is a feasible process; bulk water dissociation has been shown to be the fastest mechanism of proton delivery to a membrane bound weak acid (Kasianowicz et al. 1987). The proton originating from water dissociation would directly protonate E166, whereas the OH^- ion is stabilized in the channel because of a favorable electrostatic environment for an anion. This mechanism is in qualitative agreement with all of the three above-mentioned experimental findings. In particular, the generation of a proton from water dissociation is pH independent. The closing rate is dependent on pH_{int} via the concentration of free $[OH]^-$, and its dependence on Cl_{int} is explained by a competition with OH^- ions. We were able to provide further experimental support for the model using D_2O substitution experiments (Zifarelli et al. 2008). First of all, we found that the opening rate constant is ~ 3-fold smaller in D_2O than in H_2O. This large effect is in agreement with the idea that the translocation of a free proton is rate-limiting for the opening process (DeCoursey and Cherny 1997). Furthermore, the closing rate constant was ~ 7-fold smaller in D_2O than in H_2O. This finding is in very nice agreement with the model shown in Fig. 3a, because the closing rate constant depends directly on the OH^- concentration (and not the H^+ concentration, i.e., pH). Since the dissociation constant of D_2O is ~ 7-fold smaller than that of H_2O [0.14×10^{-14} compared to 1.0×10^{-14} M^2

(Glasoe and Long 1960)] the free OD^- concentration is sevenfold smaller in D_2O than in H_2O at equivalent pD/pH.

Figure 3b illustrates schematically the different ways E166 can be protonated: from the outside protons can arrive directly, from the inside protonation occurs via water dissociation.

Relevance for CLC Cl^-/H^+ exchangers

It will be interesting to explore the relevance of water dissociation for the functioning of CLC transporters. Handling of protons by the CLC Cl^-/H^+ exchangers is intrinsically more complex because of the necessity to couple the H^+ movement stoichiometrically to Cl^- movement. For the exchangers, an additional glutamate residue (E203 in ClC-ec1) has been implicated in the uptake of protons from the intracellular side (Accardi et al. 2005). It is not located in the Cl^- ion permeation pathway. At least in ClC-5, it can be replaced by other titratable residues, like histidine, indicating that not its charge, but only its proton-delivery capability is important for transport (Zdebik et al. 2008). An unresolved question is how protons can travel the ~ 14-Å distance between E203 and E148. In this respect, it is important to note that Y445, located approximately halfway between those glutamate residues and coordinating the central Cl^- ion in ClC-ec1, has been shown to play a crucial role. Several mutations of this tyrosine lead to uncoupling of the proton transport and interestingly, the degree of uncoupling correlates (in a negative way) with the occupancy of the central binding site (Accardi et al. 2006; Walden et al. 2007). Recently, Y445 has been suggested to function as an internal gate that controls the accessibility of the ion at the central binding site to the intracellular side (Jayaram et al. 2008). However, mutating the corresponding Y512 in ClC-0 did not produce any major change in channel properties and

Fig. 3 a Kinetic model incorporating water dissociation as the process underlying the internal pH dependence of fast gating. See text for details. **b** Schematic illustration of different ways of protonation of E166 from the outside and from the inside
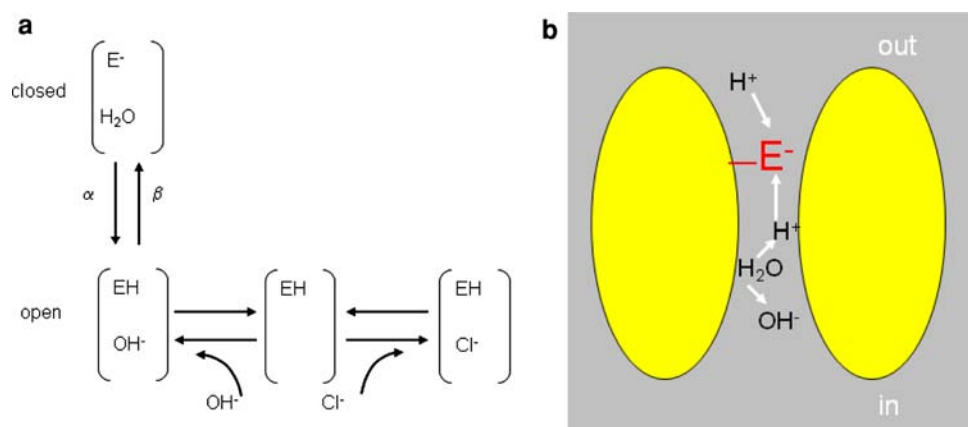
therefore the role of this residue in CLC channels appears to be different compared to the one in ClC-ec1. Nevertheless, the possibility that a hydroxide anion is involved in H^+ shuttling in the CLC transporters as well is worth testing.

Irreversible slow gating transitions driven by proton transport in ClC-0

Richard and Miller (1990) described in 1990 that the gating of ClC-0 is not in thermodynamic equilibrium. At the single-channel level they observed that bursts of the double-barreled channel often started with both pores open but often ended with only one of the protopores open. Thus, events of Type 1 (Fig. 4a) outnumbered events of Type 2. Such a time-asymmetry reveals that the gating is not in equilibrium. The irreversibility must be driven by some free energy difference, ΔG, in a way that $\frac{N_1}{N_2} \leq e^{-\Delta G/(RT)}$ (Hill 1977), where N_1 is the number of Type 1 events and N_2 the number of Type 2 events.

Initially, Richard and Miller speculated that the source of free energy was provided by the electrochemical gradient of chloride (Richard and Miller 1990). However, recently Lísal and Maduke provided compelling evidence that the irreversibility is independent from the chloride gradient, but instead powered by the proton electrochemical gradient across the membrane (Lísal and Maduke 2008). This result demonstrates indirectly, but unequivocally, that protons permeate the ClC-0 channel and, in doing so, favor slow gate opening. The elucidation of such a role for protons in ClC channel function suggests an evolutionary link with the transporters and further blurs the separation previously assumed between ClC channels and transporters. The rate of proton permeation, however, is probably linked to the (slow) gating events and thus extremely small; undetectable by direct methods.

A hypothesis of proton translocation driving the irreversible slow gating transitions

The pathway of proton permeation that drives the irreversibility of the gating is unknown. Our results on the proton dependence of fast gating suggest that protons can traverse the same ion conducting pathway as the permeating Cl^- ion. A very simple mechanism incorporating these findings and producing a certain degree of irreversibility is illustrated in Fig. 4b. Only one of the protopores is shown in Fig. 4b. This simplification does not alter the qualitative nature of the argument. The basic assumption of the model is that protons can traverse the pore once the slow gate is open (states on the right side), but not so once the slow gate is closed (states on the left side). The mechanism of transfer likely involves an intrapore OH^- ion as discussed above, but the details of the transfer are irrelevant. With this assumption, the model shown in Fig. 4b automatically produces net anticlockwise cycling under conditions of an inwardly directed gradient (see "Appendix"). In Fig. 4b, the side-chain of E166 serves as a necessary proton shuttling element for proton transfer. Several experimental pieces of evidence point to a crucial involvement of E166 in the mechanism of slow gating. First, mutants E166A and E166D lack any slow gating transitions having apparently a "locked-open" slow gate (Traverso et al. 2006). Secondly, in heterodimeric WT-E166A/D constructs, the slow gating could be partially recovered (Traverso et al. 2006). Closure of the slow gate is strongly favored in low external (Chen and Miller 1996) and internal (Pusch et al. 1999) chloride, pointing again to a rearrangement inside the ion conducting pore. The conformational change underlying the slow gate remains, however, elusive. The fact that mutations in different parts of the protein affect the slow gate might imply that the mechanism underlying slow gating relaxations involve several channel structures or that very subtle changes are sufficient to disturb those relaxations.
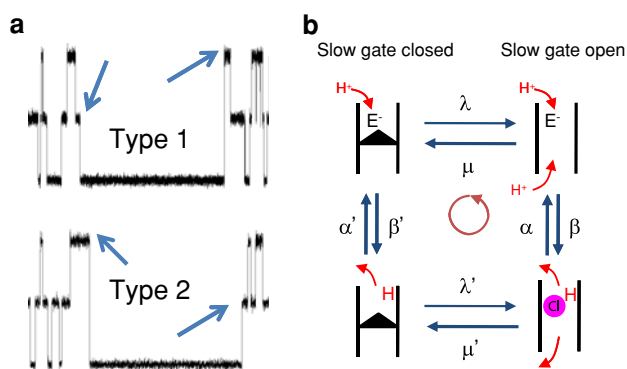
Conclusions

We are steadily getting closer to a detailed understanding of the intricate biophysical mechanism underlying the proton dependent gating of CLC channels and the transport coupling in CLC transporters. Nevertheless, we are still far from a complete picture of gating. In particular, we still do not understand how Cl^- ions and protons interact in the

Fig. 4 Irreversible gating transitions of ClC-0. **a** Schematic example single-channel traces. Type 1 events outnumber Type 2 events, demonstrating the irreversible nature of the gating transitions. **b** Simple model that could explain at least part of the irreversibility of gating. *Red arrows* indicate proton access, E^- stands for the side-chain of E166, H indicates a protonated E166 and is treated as equivalent to an open fast gate

regulation of the fast gate. In the Cl^- dependent gating models, a Cl^- ion competes with the negative side chain of E166 being able to displace it from its blocking position (Dutzler et al. 2003). In the proton dependent gating models, E166 has to be protonated before channel opening can occur (see Fig. 2b). If external Cl^- ions could directly open the channel one may expect that, at a given voltage, channels open fully if the external Cl^- concentration rises high enough. However, experimentally, the open probability saturates at concentrations ~ 200 mM (Chen and Miller 1996). Further experiments are clearly needed to nail down the precise role of Cl^- ions for fast gate opening.

Equivalent questions arise regarding the pumping mechanism of CLC exchangers. The bacterial ClC-ec1 is amenable to X-ray structure analysis; however, protons are practically invisible to X-rays. Additionally, it appears that the transport mediated by CLC exchangers is associated only with minor conformational changes that are difficult to define using X-ray crystallography. Therefore, functional analysis, combined with mutagenesis, and alternative structural methods are necessary to achieve further advances.

Appendix: irreversible slow gating of ClC-0 driven by proton flux

We use the simple model show in Fig. 4b, assuming that protons have access to E166 from both sides of the membrane if the slow gate is open (right states) but only from the outside if the slow gate is closed (left states). For simplicity, we neglect the transmembrane voltage. H_i denotes the intracellular proton concentration, H_e the extracellular proton concentration. We assume that opening rate constants are proportional to the H^+ concentration:

$$\alpha' = H_e K_e'$$

$$\alpha = H_e K_e + H_i K_i$$

with respective association constants K_e', K_e, and K_i. These equations are, of course, not strictly valid; they only serve to illustrate the general, qualitative point. Invoking the OH^- mediated process discussed for the access of protons from the inside, leads to slightly more complicated equations but does not alter the qualitative argument. For $H_i = H_e$, the system is in equilibrium, and the rate constants have to obey microscopic reversibility leading to

$$\frac{\beta \lambda \mu' K_e'}{\beta' \lambda' \mu} = K_e + K_i$$

At any H^+ gradient, the ratio of the product of rate constants in the anticlockwise direction and the product of rate constants in the clockwise direction provides the net cycling ratio, i.e.,

$$\frac{N_1}{N_2} = \frac{K_e + K_i}{K_e + \frac{H_i}{H_e} K_i}$$

Thus, for $H_e > H_i$, and if $K_i \gg K_e$, the simple model in Fig. 4b predicts net anticlockwise cycling, close to the maximal limit.

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