

Charge Movement of a Voltage-Sensitive Fluorescent Protein

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ABSTRACT The N-terminus of *Ciona intestinalis* (Ci-VSP) is a voltage-sensing domain (VSD) controlling the activity of a phosphatase domain on the C terminus. By replacing the phosphatase domain with a tandem of fluorescent proteins, CFP and YFP, a family of fluorescence resonance energy transfer-based, genetically encoded voltage-sensing fluorescent protein (VSFP) was created. VSFP2.3, one of the latest versions of this family, showed large changes in YFP emission upon changes in membrane potential with CFP excitation when expressed in *Xenopus laevis* oocytes. The time course of the fluorescence has two components: the fast component correlates with the time course of sensing current produced by the charge movement, while the slow component is at least one order-of-magnitude slower than the sensing current. This suggests that the tandem of fluorescent proteins reports a secondary conformational transition of the VSD which resembles the relaxation of the VSD of Ci-VSP described in detail for the Ci-VSP. This observation indicates that the relaxation of the VSD of VSFP2.3 is a global conformational change that encompasses the entire S4 segment.

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The voltage-sensitive fluorescent protein (VSFP) is a family of genetically encoded fluorescence resonance energy transfer-based optical probes for membrane potential (1). Unlike organic voltage sensitive dyes (2,3), their expression can be specifically targeted to particular cell populations. The VSFP family have S4-containing voltage-sensing domains (VSD) with a tandem of fluorescent proteins (TFP), CFP and YFP, attached to their C terminus. The TFP has been designed to yield fluorescence resonance energy transfer (FRET) between the fluorescent protein pair with FRET efficiency modulated by membrane potential through the VSD. The latest versions of VSFP are based on the VSD of the voltage-sensitive phosphatase from *Ciona intestinalis* (Ci-VSP) (4, also see 16) with the arginine at position 217 mutated to a glutamine. In Ci-VSP, this mutation shifts the voltage dependence of the fluorescence signal 70 mV toward more negative potentials (5,6). In these proteins, the phosphatase domain of Ci-VSP was replaced for the TFP. Recently, it has been shown that the version 2.3 of the VSFP family displays robust changes in FRET efficiency as a function of the membrane potential (7,8). It has also been shown that, when expressed in PC12 cells, the voltage dependency of both the net charge movement of the VSD and the changes in FRET emission are well correlated (8). However, the kinetic comparison of the electrical with the fluorescence recordings showed a clear discrepancy, displaying time constants differing at least two orders of magnitude. One plausible explanation for this paradoxical observation is that after the sensing charges have moved, a second conformational change takes place producing the main change in the FRET emission from the TFP. We have expressed VSFP 2.3 in *Xenopus laevis* oocytes and simultaneously re-

corded sensing currents (Fig. 1 A) and fluorescence emission (Fig. 1 B) using the cut-open fluorimetry technique (9). The fluorescence recorded corresponds to the emission of YFP at 595 ± 30 nm as a consequence of FRET from the CFP, which was excited at 440 ± 15 nm. As expected, these recordings showed slow kinetics in the optical recording for the entire range of potentials tested (-60 mV to $+40$ mV), for a holding potential (HP) of -90 mV. Detailed analysis of the kinetics of the FRET emission revealed that the optical records (Fig. 2 A, black trace) clearly displayed two components, where the fast component is isochronous with the charge movement (Fig. 2 A, red trace). An extended analysis of the kinetics of both the fluorescence emission and sensing currents revealed that the time constants of the fast component of the fluorescence emission and the sensing charge movement matched. This was observed for all potentials tested above -50 mV (HP = -90 mV), while the slow component of the fluorescence emission was at least one order-of-magnitude slower than the charge movement.

During the repolarization of the membrane, the fluorescence recording also showed two kinetic components. As observed during depolarization, only the fast component of the FRET emission (Fig. 3 A, black trace) is temporally correlated with the charge movement (Fig. 3 A, red trace) of VSFP 2.3. For depolarizing pulses of 200 ms, we found a strong correlation between the time constant of the fast component of the FRET emission and that of the sensing

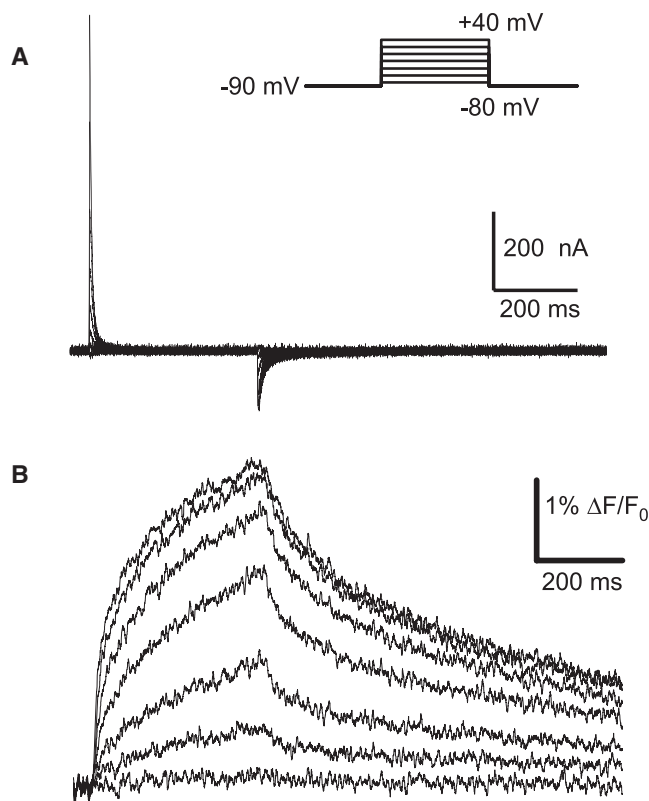


FIGURE 1 Simultaneous recordings of sensing currents (**A**) and emission of YFP (**B**) while exciting CFP (**B**) from VSFP 2.3 expressed in *Xenopus laevis* oocytes. The holding potential (HP) was -90 mV and depolarizing pulses were from -80 mV to $+40$ mV.

charge movement for all potentials tested above -40 mV (Fig. 3 B). The time constant of the slow component of the fluorescence was at least two orders-of-magnitude greater than the time constant of the charge movement in the same range of potentials (Fig. 3 B). Since the slow component of the fluorescence does not correlate with the sensing charge movement, we propose that this component corresponds to a voltage-independent transition. The rate of this transition increases with voltage because the rearrangement of the VSD occurs after the movement of the sensing charges, inheriting its voltage dependence.

Recently, it was shown that the VSD of Ci-VSP relaxes into a new conformation during prolonged depolarization (5). The relaxation of the VSD causes the voltage dependence of the sensing charge movement to shift toward negative potentials (5). We have also shown that VSFP 2.3 displays the same phenomenon (Fig. 3 D) (10). The shift of the relationship between the movement of the sensing charge (Q) and the membrane potential (V) (QV curve), toward negative potential as a consequence of prolonged depolarization, has been described for Voltage Gated Channels and has been correlated with the slow inactivation of the conductance of those channels (11–15). Using Ci-VSP, a protein that has no pore domain, Villalba-Galea et al. (5) showed that the QV curve for this protein is likewise shifted by prolonged

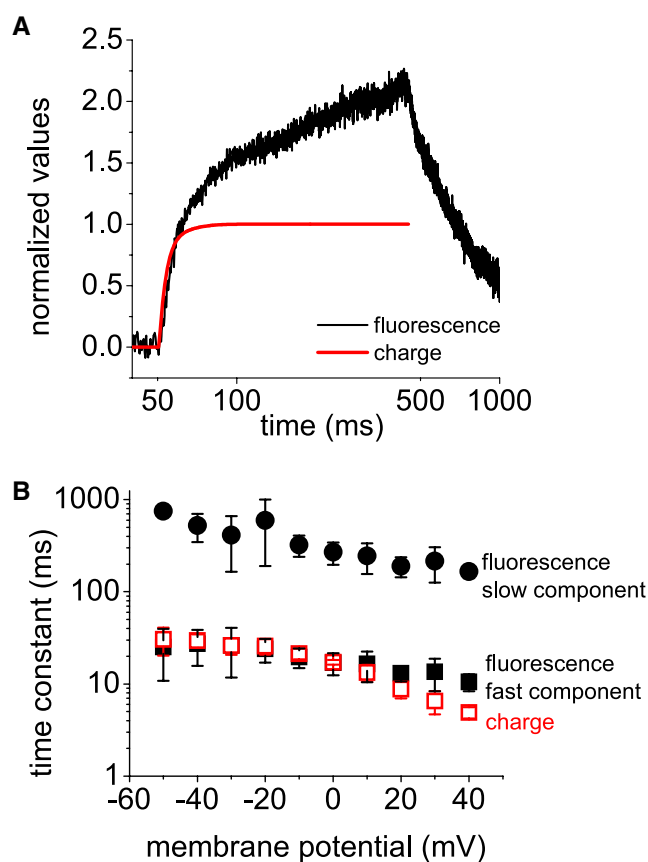


FIGURE 2 Time course of the charge movement (sensing current integral **A**, red trace) during depolarization (to 60 mV from HP = -90 mV) matches the fast component of the fluorescence (**A**, black trace). The time constant of the fast component of the fluorescence (**B**, black square) correlates with the average time constant of the charge movement (**B**, red square) for $V > -50$ mV. HP = -90 mV.

depolarization and that this is an intrinsic property of the VSD. In that work, the depolarization-induced relaxation of the VSD of Ci-VSP was characterized by simultaneous electrophysiological and fluorescence recordings with tetramethylrhodamine-5-maleimide (TMRM) probe in the extracellular portion of the S4 segment. The close similarity of the electrophysiological and fluorescence results presented here for VSFP 2.3 and those of the TMRM-labeled Ci-VSP strongly suggest that the conformational changes during the depolarization-induced relaxation are also followed by the FRET between the fluorescent proteins in VSFP. Since the TMRM tracks local conformational changes, the slow conformational change reported by the fluorophore is likely to be the consequence of a local rearrangement of the S4 segment after the initial voltage-driven transition upon depolarization. However, the fact that the mechanisms of fluorescence changes differ in the two cases and that in Ci-VSP takes place extracellularly while that in VSFP takes place intracellularly, strongly suggests that the conformational change during the relaxation is a global change. In this view, the movement of

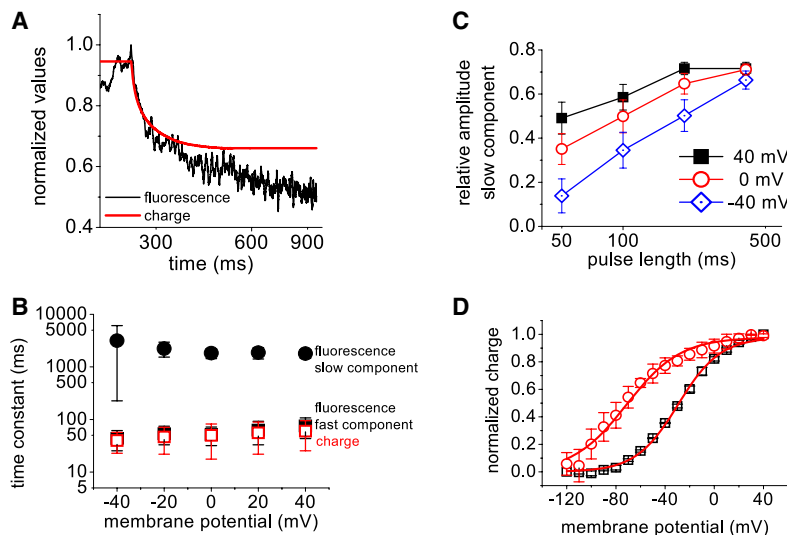


FIGURE 3 (A) Time course of the charge movement of VSFP 2.3 during repolarization from a +40 mV step potential correlates with the fast component of the FRET emission. (B) The time constant of the charge movement matches the fast component of the fluorescence for all potentials tested during repolarization to -90 mV. (C) The relative amplitude of the slow component of the fluorescence increases with the length and amplitude of the depolarizing pulse. (D) The VSFP 2.3 Q-V curve shifts -42 ± 3 mV when the HP is changed from -90 to +20 mV.

the S4 sensed by TMRM on the outside is also felt on the intracellular end of the transmembrane segment by TFP indicating that the relaxation of the VSD is transmitted along the entire S4 segment and suggests a global change involving the entire S4 segment.

As a voltage-sensing probe, VSFP 2.3 tracks fast voltage changes via its fast component but the majority of the fluorescence change is the slow component (see Fig. 3 C). This result indicates that this probe will be most useful when detecting slow voltage changes and suggests that, if there were a way to prevent the relaxation of the VSD, the probe would become a fast probe. However, some other changes would be required to increase the size of the fast fluorescence signal.

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