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Direct observation of proton pumping by a eukaryotic P-type ATPase

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Abstract

In eukaryotes, P-type ATPases generate the plasma membrane potential and drive secondary transport systems; however, despite their importance, their regulation remains poorly understood. Here we monitored at the single-molecule level the activity of the prototypic proton pumping P-type ATPase *Arabidopsis thaliana* isoform 2 (AHA2). Our measurements combined with a physical non-equilibrium model of vesicle acidification, revealed that pumping is stochastically interrupted by long-lived (~100 s) inactive or leaky states. Allosteric regulation by pH gradients modulated the switch between these states, but not the pumping or leakage rates. The autoinhibitory regulatory domain of AHA2 reduced the intrinsic pumping rates, but increased the dwell time in the active pumping state. We anticipate that similar functional dynamics underlie the operation and regulation of many other active transporters.

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Main text

Electrochemical gradients across cellular membranes control many essential biological processes. These gradients are generated by primary active transporters and are used to drive the exchange of other solutes through secondary active transporters and to facilitate signaling through ion channels (1). Patch clamp recording has made it possible to observe the functional dynamics of single ion channels revealing discrete on and off states, subconductance states, and other mechanistically important features that macroscopic experiments cannot probe (2). However, despite extensive structural and biochemical efforts (3), we currently lack a similar depth of understanding of transporters because they in general do not produce electrically detectable single-molecule transport signals (4–8). Here we monitored at the single-molecule level the functional dynamics of a eukaryotic primary active transporter, *Arabidopsis thaliana* H⁺-ATPase isoform 2 (AHA2, referred to as the proton pump), which is responsible for energizing the plasma membrane of plants and fungi (Fig. S1–S2) (3, 9). This provided insights into how the activity of P-type ATPases is modulated by autoregulatory terminal domains (R domains) and pH gradients (10, 11).

We used total internal reflection fluorescence (TIRF) microscopy to image with high throughput single nanoscopic lipid vesicles tethered to a solid support (Fig. 1A, 1B, S3, S4). Tethering was accomplished with a biotin/neutravidin protocol (12), which maintains the native function and diffusivity of reconstituted transmembrane proteins (13) and the vesicles' spherical morphology (14) and low passive ion permeability (15). The fluorescence intensity of all single vesicles was quantitatively converted to pH (Fig. S5) and tracked over periods of up to 30 minutes.

Initial studies were carried out on the well-studied activated form of AHA2, which lacks the flexible C-terminal auto-inhibitory R domain (AHA2^R) (Fig. 1A, Fig. S1–S3) (9). Initialization of H⁺ pumping into the vesicle lumen was triggered by the addition of ATP and Mg²⁺, which are non-membrane permeable and thus only activate proton pumps with an outward facing ATP binding domain (Fig. 1A) (12). Consistent with this, we never observed luminal alkalinization (Fig. 1C). Acidification kinetics reached a plateau of well-defined pH (pH_{max}) as a result of a dynamic steady state in which active pumping (influx) of protons matched the passive leakage (efflux) of protons through the membrane due to the build up of a proton motive force (16). As expected, addition of the protonophore CCCP collapsed the H⁺ gradients (Fig. 1C), while controls performed without Mg²⁺, ATP or AHA2^R showed no response (Fig. S6D). Furthermore, the activity of the pump was blocked by the addition of the specific inhibitor vanadate (11) and decayed after flushing out ATP and Mg²⁺ (Fig. S7). To control for potential artifacts arising from the surface-tethering of vesicles, we performed a side-by-side comparison with vesicles suspended in solution, which proved indistinguishable within experimental uncertainties (Fig. 1C, S6). Taken together, these results demonstrate that we are able to observe the AHA2^R-mediated and ATP-fuelled pumping of protons against their concentration gradient into the lumen of single vesicles. The single-vesicle experiments revealed a remarkable heterogeneity of acidification rates and pH_{max} values between vesicles (Fig. 1C) that remain masked in the ensemble averages (16).

At the low protein-to-lipid molar ratio (1:12,000) used in our experiments, 84% of vesicles exhibited no detectable pH changes (Fig. 1C and Fig. 2A top trace) indicating the absence of active pumps and thus suggesting that there are only a few active pumps in each of the remaining vesicles whose pH changed over time (hereafter termed 'active vesicles'). We inspected the pH changes in the 16% of active vesicles and indeed found that all of them exhibited the hallmark of single molecule behavior, i.e. stochastic changes between discrete states (Fig. 2A). Since the passive leakage rates of the vesicles are constant over time (Fig. S10), these data demonstrate that the individual proton pumps are stochastically transitioning between active and inactive states. This behavior is termed 'functional dynamics' (17–24) and is key to the function and regulation of ion channels (25).

Further examination of all active vesicle traces revealed that ~60% of them reverted back to the zero pH baseline after switching off, strongly suggesting the presence of only one molecule since it is improbable for many molecules to switch off simultaneously (Fig. 2A). In the remaining traces (~40%) we observed two or three discrete plateaus, a feature that has been observed in all studies of single channels to date and has been interpreted to demonstrate that the activity of multiple single molecules can be discretely resolved. The latter conclusion was further supported by experiments where titration of the protein to lipid ratio modulated the percentage of multiple plateaus (Fig. S5G), excluding the possibility that multiple plateaus represent multiple single-molecule activity states. These observations allowed us to unambiguously identify those traces resulting from a single active proton pump, which we then selected for further analysis. Importantly, the activity of single proton pumps is amplified and reported by $\sim 10^3$ pH-sensitive fluorophores (Fig. S3, S4) (16) circumventing the issue of photobleaching that fundamentally restricts most fluorescence studies of single molecules.

Dynamic transitions between active/inactive states were also observed in experiments with the wild type AHA2 (Fig. 2C), demonstrating that they are not solely a property of the truncated version. Here ~80% of all vesicles were inactive, while ~73% of those that showed activity had a single plateau indicating a single molecule (Fig. 2D). With wild type AHA2 we succeeded in using a SNAP tag to fluorescently label the protein and count directly the number of proteins per vesicle (Fig. 2E). This allowed us to observe activity dynamics and directly count the number of labeled proteins at the same time on the same vesicles (Fig. 2C and 2E). We then estimated the labeling efficiency and the probability that a proton pump was active (12). We were thus able to convert quantitatively the bleach step distribution to a distribution of active molecules per vesicle, and demonstrate that $70 \pm 15\%$ of proteoliposomes carried one active molecule (Fig. 2F). This was in quantitative agreement with the distribution of activity plateaus (~73%) (Fig. 2D) providing an additional demonstration that we can resolve and record the functional dynamics of the proton pump at the single molecule level.

The activity of the proton pump, and likely other active transporters, is thus not constant in time (Fig. 2). Therefore, for transporters (just like ion channels), the rates measured in macroscopic experiments are the product of the active state probability and the intrinsic pumping rate. To quantitatively analyze the kinetics and dynamics of pumping, we constructed a physical model of a single vesicle (12) which accounts for several parameters

that affect the acidification kinetics, including passive and active ionic fluxes across the membrane, proton buffering in the lumen, vesicle size, and build up of membrane potential (Fig. 3A) (26). Proton pumping is modeled with a fixed rate I_P , a lifetime (t_{on}) and time between pumping events (t_{off}), Fig. 3A, B. The vesicle is assumed to have a passive membrane permeability to protons (P_{leak}) which is constant over time as revealed by control experiments (Fig. S10). The stochastic switching of the pump between active and inactive states was extracted directly from the traces and used as time-dependent input to the model. The model is constrained to fit the entire trace, it provides a realistic description of the full electrochemical gradient and a direct estimation of the absolute numbers of pumped and leaked ions.

Initially, all experimental traces were fit with the model by varying two parameters: I_P and P_{leak} . This provided a good quantitative description of the majority of AHA2^R traces (~65% of 126 counts, Fig. S8); however, it systematically underestimated the observed leaking rates for the remaining traces (Fig. 3B, blue line) suggesting the existence of an additional proton leaking route apart from passive leakage through the membrane (Fig. S8). Indeed, leakage of ions through transporters has been reported before e.g. for P-, and V-type ATPases (27, 28). To test this hypothesis, we collected all lifetimes of exponential fits to the leakage kinetics from traces transitioning between active and inactive states. The histogram of leakage lifetimes (Fig. S9C) had two clearly separated peaks: one that according to control experiments corresponded to passive leakage through the membrane (transmembrane leak), and another that was approximately 20-fold faster (Fig. S9, S10). The latter peak was specifically inhibited by addition of the vanadate which locks the pump in the E2 state (11) (Fig. S9D), demonstrating that the leak is not passively mediated by the membrane (or the protein/membrane interface), but the pump itself. Since vanadate is membrane-impermeable we can exclude the possibility that the fast leak component originated from pumps with the opposite orientation, because they would not be blocked by vanadate. We thus modified the model to include a time-dependent transprotein proton leak (P_{AHA2}), which turns on once pumping stops and turns off at the beginning of the next pumping cycle (Fig. 3C, blue dotted line). As expected, the revised model considerably improved the fits of the remaining traces (Fig. 3B red line, and Fig. S8).

Next, we quantitated proton permeabilities by fitting the kinetics with the model. The average transmembrane leak P_{leak} (Fig. 3D and Fig. S11) was $\sim 7 \times 10^{-5}$ cm/s, in line with previous measurements and estimates (26), and the average transprotein leak P_{AHA2} had a similar value ($\sim 46 \times 10^{-5}$ cm/s) (Fig. 3D). However, when normalized for surface-area the transprotein proton current was greater than the transmembrane by a factor of $\sim 10^4$.

The inhibitory R domain of AHA2 has been shown to reduce the net macroscopic proton transport rate by ~2-fold (10, 11). In order to elucidate the mechanisms underlying this regulation, we characterized the activity of the proton pump with and without the R domain. Counter-intuitively, the autoinhibitory R domain increased the total time the transporter spent in the active state, both by increasing t_{on} ~3-fold (from 337 to 951 s, $p = 10^{-19}$, and by decreasing t_{off} ~0.5-fold (from 121 to 65 s, $p = 0.05$) (decay constants of exponential fits to the distributions in Fig. 3F, & 3G; unless otherwise stated, p is a Kolmogorov-Smirnov test of statistical similarity between two distributions). Thus the probability to find the pump in

an active state $P_{on} = t_{on}/(t_{on} + t_{off})$, increased ~200% for AHA2 (from 0.35 ± 0.05 to 0.76 ± 0.06). Importantly, 100% of AHA2^R and ~60% of AHA2 molecules switch on/off during our observation period highlighting that functional dynamics is a dominant property of this system (Fig. 3F). The R domain also had a pronounced effect on the overall intrinsic transport rates of the pump that were reduced by ~10-fold compared to AHA2^R (from 928 to 85 protons/s, average values, $p = 10^{-20}$) (Fig. 3E). In addition, the R domain promoted an overall decrease in the transprotein leak (~1.4-fold, $p = 0.005$) (Fig. 3D).

The activity of the pump was regulated also by the pH gradients established across the membrane during proton pumping. Increasing pH_{max} decreased by >2-fold the lifetime of the active state, however only for the wild type (Fig. 4A, B). This regulation seems to be transmitted allosterically across the bilayer since the R domain of AHA2 is facing the vesicle exterior where the pH remains constant. In addition, traces with larger pH_{max} had a dramatic 8-fold increase (from 0.1 to 0.8) in the probability of a transprotein leak for both forms of AHA2 (Fig. 4C). Thus, regulation by pH gradients can manifest through two mechanistically distinct processes that reduce the net average proton transport, reduction of the pumping lifetime and increase of the probability of a transprotein leak, whereby only the former is encoded in the R domain.

Our observations of proton transport and leakage dynamics at the single molecule level also provide critical insights to the ATP/H⁺ stoichiometry (27, 28). Ensemble average experiments have reported that the build up of pH gradients can in general alter the stoichiometry of transport and therefore pumping rates (27, 28). Contrary to expectation however, we found that the intrinsic (single molecule) pumping rate remained constant for gradients as large as 2 pH units (Fig. 3B, 3C, S12C). As discussed above, pH gradients did reduce the net proton transport but primarily by increasing the probability of a downhill transprotein leak (Fig. 4C). Importantly, since the transprotein leak takes place once the pump has switched to the inactive state (Fig. 3C, S9), it does not affect the actual stoichiometry of active transport. In contrast, the R domain reduced the intrinsic pumping rates by ~10-fold (Fig. 4E). Since the R domain that does not significantly affect the ensemble average ATPase activity (10, 29), our measurements suggest the R domain can reduce the stoichiometry of active transport by a factor of ~10 (or 20 if we correct for the change in P_{on}) (11). We note that our measurements of proton transport integrate over thousands of Post-Albers catalytic cycles per second per single molecule, a better mechanistic understanding of these processes would ultimately require direct measurement of stoichiometry of single turnover cycles or careful molecular simulations.

Here we developed a single-molecule technique to observe in a highly parallel manner uphill substrate transport into single nanoscopic lipid vesicles. Our measurements revealed the existence and the dynamics of several distinct functional states (active, inactive and leaky) that together defined the activity and the regulation of the proton pump, and that we anticipate underlie the operation of many other primary and secondary active transporters. The assays introduced here render these processes accessible to direct experimental observation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Imaging proton pumping into the lumen of single surface-tethered vesicles using TIRF microscopy

A) Illustration of AHA2^R reconstituted vesicles tethered to a passivated glass surface and imaged on individual basis with TIRF microscopy. Zoom: extra-vesicular addition of both ATP and Mg^{2+} activated exclusively outward facing AHA2^R molecules triggering H^+ pumping in the vesicle lumen. We quantified changes in the vesicular H^+ concentration by calibrating the response of the lipid-conjugated pH sensitive fluorophore pHrodoTM. Valinomycin was always present to mediate K^+/H^+ exchange and prevent the build up of a transmembrane electrical potential. **B)** TIRF image of single vesicles tethered on a passivated glass slide. **C)** Acidification kinetics of single vesicles upon addition of ATP and Mg^{2+} . Red traces highlight three representative signals from single vesicles showcasing: absence of transport activity, continuous pumping of protons and fluctuations in proton-transport activity. The black trace is the average of 600 single vesicle traces. As expected, addition of the protonophore CCCP collapsed the proton gradient established by AHA2^R.

Fig. 2. Single-molecule observation of proton pumping reveals active and inactive states

A) Typical examples of pH changes inside individual AHA2^R reconstituted vesicles. ATP and Mg²⁺ (2 mM) were added to initiate proton pumping and CCCP (5 μM) was added to collapse the pH gradients. Traces show $-\Delta\text{pH}$ defined as a difference between the initial and final pH. Images of each respective liposome at different time points are shown below each trace. At the right-hand side of the traces we plotted histograms of pH plateaus numbered to indicate the number of active pumps per vesicle. The pH inside the majority of vesicles showed no changes indicating the absence of functional transporter molecules (top panel). For a small fraction of vesicles we observed intermittent H⁺ pumping indicating the presence of single-molecules (middle panels). The observation of two discrete steady-state pH plateaus in single vesicle traces indicated the occasional presence of two active pumps per single vesicle (bottom panel). **B)** Population histogram of pH plateaus for AHA2^R-reconstituted vesicles ($n = 3$, where hereafter n is the number of independent experiments). **C, D)** Same as in **A, B** but for full length AHA2. For **D** $n = 2$. Labeling of AHA2 with Alexa Fluor[®] 647 enabled counting on the same vesicles of both the number of labeled AHA2 proteins (**E**), and of the respective activity dynamics (**C**). **F)** The histogram of active proteins per vesicle was calculated from step-bleaching analysis of the data in **E** that was corrected for labeling efficiency and the probability that a proton pump is active (12). The two independent methods for estimating the number of active molecules agreed that ~70% of vesicles containing a protein have one active proton pump.

Fig. 3. Modeling active, inactive and leaky states, and their role in autoinhibiting proton pumps
A) Main parameters of the physical model we used to fit changes in the vesicular pH were pumping rate (I_p), protein associated leak (P_{AHA2}), membrane leak (P_{leak}), valinomycin induced K^+ permeability (P_{K^+}), buffering capacity in the interior of the vesicle (β), electrical potential across the membrane (Ψ) (12). **B)** Example of a typical proton pumping trace and respective fits without (blue) and with (red) a transprotein proton leak. A threshold in the first derivative of the pH kinetics (12) was used to define the lifetime of the active state t_{on} and the time between pumping events t_{off} . **C)** Temporal evolution of the proton pumping rate (grey) and the proton efflux rates due to passive membrane (red) leakage and transprotein back-flow (blue) for the pH trace shown in **B**. **D)** Histogram of proton permeability associated with the membrane, AHA2 and AHA2^R. Respective counts were 95, 37 and 45. **E)** Histogram of pumping rates for AHA2^R and AHA2. Respective counts were 126 and 95. **F)** Histogram of t_{on} for AHA2^R and AHA2. Respective counts were 241 and 134. The bar at >1200 s shows the number of traces that did not switch in the duration of the experiment. **G)** Histogram of t_{off} for AHA2^R and AHA2. Respective counts were 69 and 39. For AHA2^R and AHA2 respectively: the number of independent experiments was 3 and 2, while the number of individual proteoliposomes analyzed was 126 and 95.

Fig. 4. Regulation of proton pumping by pH gradients

A, B) Relation between t_{on} and the maximal pH gradient for AHA2^R and AHA2, respectively. Each data point represents the average of 3 consecutive values. Error bars represent corresponding standard deviations. The decay constants from the error weighted exponential fits to the data are 6.9 ± 2.0 s and 0.4 ± 0.1 s for (A) and (B), respectively, where uncertainties represent 95% confidence intervals from the fits. **C)** Probability to observe transprotein leak as a function of pH gradient proton pumping. For AHA2^R and AHA2 respectively: data were binned with 0.25 and 0.5 pH units; number of independent experiments and individual proteoliposomes analyzed was the same as in Fig. 3. Spearman's rank order correlation coefficients $\rho(126) = 0.40$ $P = 10^{-4}$ and $\rho(95) = 0.30$ $P = 0.03$ indicated a strong positive correlation between leakage probability and pH_{max} for both AHA2^R and AHA2.