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Inhibition of the Gastric H,K-ATPase by Clotrimazole†

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ABSTRACT: The antimycotic drug clotrimazole inhibits the function of the gastric H,K-ATPase in a manner similar to that observed for the Na,K-ATPase. Because of the high hydrophobicity of the compound, the interaction between clotrimazole and the ion pump occurs at the membrane domain in the apolar core of the membrane. The enzymatic activity was inhibited with a half-saturating concentration of 5.2 μM. Various partial reactions of the pump cycle were analyzed with the electrochromic styryl dye RH421 that has been widely used to study the transport mechanism of P-type ATPases. We discovered that the interaction of clotrimazole with the H,K-ATPase introduces a single "dead-end" branch added to the Post-Albers scheme in the E_1 state of the pump. In this inhibiting state, the ion binding sites have a significantly enhanced affinity for protons and bind up to two protons even at pH 8.5. Inhibition of the pump can be reversed by a decreased pH or increased K^+ concentrations. The mechanistic proposal that allows an explanation of all experiments presented is similar to that published for the Na,K-ATPase.

In the late 1960s, the imidazole derivative clotrimazole (CLT)[1] was developed as a potent antimycotic compound (*1*) and has been widely used in the treatment of fungal infections for more than 30 years. The compound inhibits the biosynthesis of ergosterols (*2*) but also produces various side effects, such as inhibition of cell proliferation (*3*, *4*) and inhibition of steroid metabolism (*5*), which may be attributed to an inhibitory effect on cytochrome P-450 (*6*). Inhibition is also observed with numerous transport proteins of cellular membranes, including Ca^{2+}-dependent potassium channels (*7*, *8*), multidrug resistance proteins (*9*, *10*), the SR Ca-ATPase (*11*, *12*), and the Na,K-ATPase (*13*).

In case of the Na,K-ATPase, a member of the P_2-type ATPase family, detailed mechanistic analyses of the effects of CLT on the ion pump cycle revealed the existence of a single CLT-inhibited state that evolves from the ion-occluding E_2 state of the so-called Post-Albers cycle, as shown in Figure 1 (*13*). We can assume that a similar inhibitory mechanism also holds for the SR Ca-ATPase (*11*). A third P_2-type ATPase is the closely related gastric H,K-ATPase (*14*, *15*), and numerous data demonstrate that the transport mechanisms of the Na,K-ATPase and the H,K-ATPase are similar (*16*−*18*). Here, we examine whether the interaction of CLT with the H,K-ATPase follows the same scheme.

Such a proposal is not necessarily self-evident. In the case of the Na,K-ATPase (and similarly in the SR Ca-ATPase), the rate-limiting step of the pump cycle at low ATP concentrations is the conformational transition, $E_2(K_2) \rightarrow K_2E_1$, and $E_2(K_2)$ is the preferentially populated state under turnover conditions. Therefore, in the presence of Na^+ and ATP, K^+ titration experiments generate the K^+ occluded state $E_2(K_2)$, and as a result, the same binding affinity is measured for K^+ activation of enzyme activity or the K^+ dependence of RH421 signals associated with K^+ occlusion (*19*). In contrast, the K^+-occluded state of the H,K-ATPase is not stabilized at low K^+ concentrations (*16*, *20*), and as a consequence, the conformation transition, $E_2(K_2) \rightarrow K_2E_1$, is not rate-limiting (*21*, *22*). Therefore, under turnover conditions, the enzyme does not accumulate appreciably in the state with two K^+ ions occluded, $E_2(K_2)$. Only in the presence of high K^+ concentrations (> 10 mM) does "reverse" K^+ binding predominate to give $E_1 + 2K^+ \rightarrow ... \rightarrow E_2(K_2)$, and turnover is inhibited. This process is reported correctly by both the RH421 experiments and the enzyme activity (*20*, *23*). In the case of the Na,K-ATPase, CLT is bound to $E_2(K_2)$ at equilibrium (Figure 1) to produce inhibition. Since the corresponding $E_2(K_2)$ state is hardly populated under physiological conditions in the gastric H,K-ATPase, the effect of CLT on the H,K-ATPase and its mechanism were studied in this ion pump. The experiments were performed by using a fluorescence technique that makes use of the electrochromic styryl dye RH421 whose spectral characteristics are affected by the proximity to charge in the membrane. The results expand on previous investigations of the Na,K-ATPase and H,K-ATPase (*13*, *20*, *24*, *25*).

MATERIALS AND METHODS

Chemicals. NADH and ATP (disodium salt, special quality) were obtained from Boehringer (Mannheim, Germany). The ionophore nigericin was ordered from Sigma (Steinheim, Germany) and the fluorescent dye RH421 from MoBiTec (Göttingen, Germany). SCH28080 was provided by Altana Pharma Germany. All other reagents were of the highest grade commercially available.

Enzyme Preparation. The gastric H,K-ATPase was derived from hog gastric mucosa by previously published methods, which involve differential and density gradient centrifugation (*26*). The crude gastric mucosal membranes were collected from the stomach and homogenized in a solution of 0.25 M sucrose, 5 mM

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[1]Abbreviations: CLT, clotrimazole; RH421, *N*-(4-sulfobutyl)-4-{4-[*p*-(dipentylamino)phenyl]butadienyl}pyridinium inner salt; NADH, nicotinamide adenine dinucleotide; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, 3,12-bis(carboxymethyl)-6,9-dioxa-2,12-diazatetradecanedioic acid.

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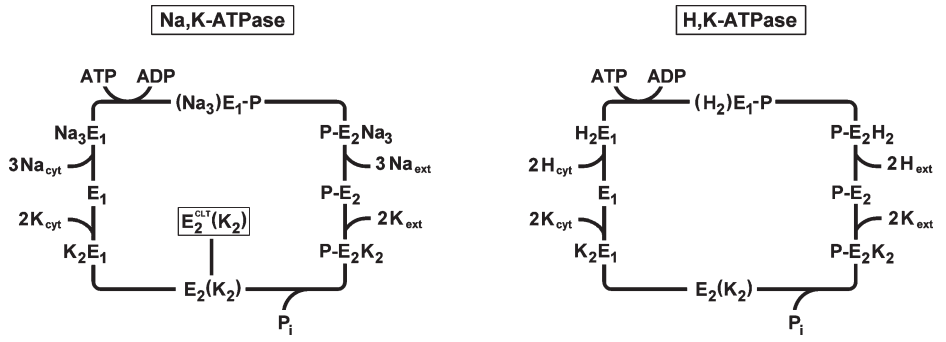

FIGURE 1: Post-Albers pump cycle of Na,K-ATPase and H,K-ATPase. In the ATP-driven half-cycle, three Na^+ ions are extruded from the cytoplasm in the case of the Na,K-ATPase, while in the H,K-ATPase, only two protons are transported out of the cytoplasm. In the second half-cycle, both ion pumps transfer two K^+ ions to the cytoplasm. A specific property of the P-type ATPase is the existence of so-called occluded states, in which the ions are encased in their binding sites so that they cannot exchange with the aqueous phase on either side of the membrane. This condition is indicated by the use of parentheses [$(Na_3)E_1$-P, $(H_2)E_1$-P, and $E_2(K_2)$]. The investigation of the interaction of clotrimazole (CLT) with the Na,K-ATPase revealed that CLT inhibits the Na,K-ATPase by a single (dead-end) reaction step from $E_2(K_2)$ to $E_2{}^{CLT}(K_2)$ (*13*).

PIPES/Tris (pH 6.8), and 1 mM EGTA. The homogenate was centrifuged at 11000 rpm in a Sorvall GSA rotor for 45 min. The supernatant was centrifuged at 30000 rpm in a Beckman type 30 rotor for 1 h. The microsomal pellet was resuspended in a solution of 0.25 M sucrose in buffer A [5 mM PIPES/Tris (pH 6.8) and 1 mM EGTA]. The microsomal suspension was separated by sucrose step gradient centrifugation, and the membranes between layers of 0.25 M sucrose containing 7.5% ficoll and 34% sucrose, both in buffer A, were collected, pelleted, and resuspended in 0.25 M sucrose and 10 mM PIPES/Tris (pH 6.8). The resulting vesicle preparation has ~90% of the H,K-ATPase oriented as in the parietal cell with the cytoplasmic side outward. The experiments were performed with two different preparations of H,K-ATPase, which had different protein concentrations, 7.9 mg/mL (G1a) and 5.8 mg/mL (G1b). Before any experiments, 10 μg of H,K-ATPase (in the form of purified vesicles) was incubated at room temperature for 30 min in 43 μL of standard buffer [25 mM imidazole, 1 mM EDTA, and 5 mM $MgCl_2$ (pH 8.5, adjusted with NaOH)] and 0.5 mM nigericin to allow effective exchange of K^+ and H^+ across the vesicle membrane.

The K^+-stimulated ATPase activity was measured by determination of the amount of P_i with malachite green (*27*). The membrane fractions were incubated at 37 °C for 1 h in Na^+ free buffer [25 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$ (pH 7.2), 0.03 mM ouabain, 0.02 μM bafilomycin, and 0.03 μM thapsigargin] with 4.8 mM Tris-ATP and the indicated K^+ concentration. Specific activities of 151 and 120 μmol of P_i (mg of protein)$^{-1}$ h^{-1} were found in the presence of 5 mM KCl for the G1a and G1b preparations, respectively.

Detection of Partial Reactions by the Electrochromic Styryl Dye RH421. Fluorescence measurements were performed with a self-constructed setup using a HeNe laser with a wavelength of 594 nm (Laser2000, Wessling, Germany) to excite the fluorescence of the electrochromic styryl dye RH421 as described previously (*28*). In short, the emitted light was collected at an angle perpendicular to the incident light, filtered by a narrow-band interference filter (λ_{max} = 663 nm, half-width of 18 nm), and detected by a head-on photomultiplier (R2066, Hamamatsu Photonics, Hamamatsu, Japan). The photo current was amplified by a Keithley current amplifier 427 (Keithley Instruments, Cleveland, OH) and collected with a data-acquisition board of a personal computer (PCI-T112, Imtec, Backnang, Germany) with a sampling frequency between 1 and 10 Hz. The experimental data were displayed on the monitor, stored, and analyzed on the personal computer. The temperature in the cuvette (2 mL) was maintained by a thermostat at 20 °C. To allow a comparison between different fluorescence experiments, relative fluorescence changes [$\Delta F/F_0 = (F - F_0)/F_0$] were calculated with respect to the initial reference level, F_0 (*20*). The specific fluorescence changes depend on protein density in the membranes and on the lipid composition (*29*) and, therefore, also exhibited some minor differences between both preparations. The principal response in the standard experiments was the same. Nevertheless, in the figures displaying the results of RH421 experiments, results from both preparations were not mixed.

RESULTS

Enzyme Activity. Enzyme activity of the H,K-ATPase in the vesicular membrane preparations was determined as described in Materials and Methods in the absence and presence of various KCl concentrations between 10 μM and 40 mM (Figure 2A). In the absence of K^+ ions, an apparent unspecific ATPase activity was detected on the order of 15−20% of the maximum activity which was subtracted to determine the H,K-ATPase specific activity. The background activity may have its origins in the color reaction of the malachite green test since it was also observed in the absence of protein. In the absence of CLT, a maximum specific enzyme activity of 151 μmol of P_i (mg of protein)$^{-1}$ h^{-1} was found at 5 mM KCl and a half-maximum enzyme activity, $K_{1/2}$, was obtained at 0.3 mM KCl, which is in fair agreement with previously published results (*20*, *23*). The decrease in specific activity at KCl concentrations higher than 5 mM is caused by reverse K^+ binding to the E_1 conformation which traps the pumps in the K_2E_1 state.

When the dependence of the enzyme activity on the K^+ concentration was repeated in the presence of various concentrations of CLT, the specific activity was reduced (Figure 2A). The shape of the KCl dependence was, however, not significantly changed in the presence of 5 μM CLT ($K_{1/2}$ = 0.3 mM) and 10 μM CLT ($K_{1/2}$ = 0.27 mM). At 15 μM CLT, the enzyme activity was nearly completely blocked. To study the concentration dependence of the CLT-induced inhibition of the ATPase activity, the specific activity was determined in the presence of 5 mM KCl and different CLT concentrations up to 20 μM. A reference without CLT was taken in each series of experiments and used to normalize the activities (Figure 2B). Each data point is the average of at least three individual measurements; error

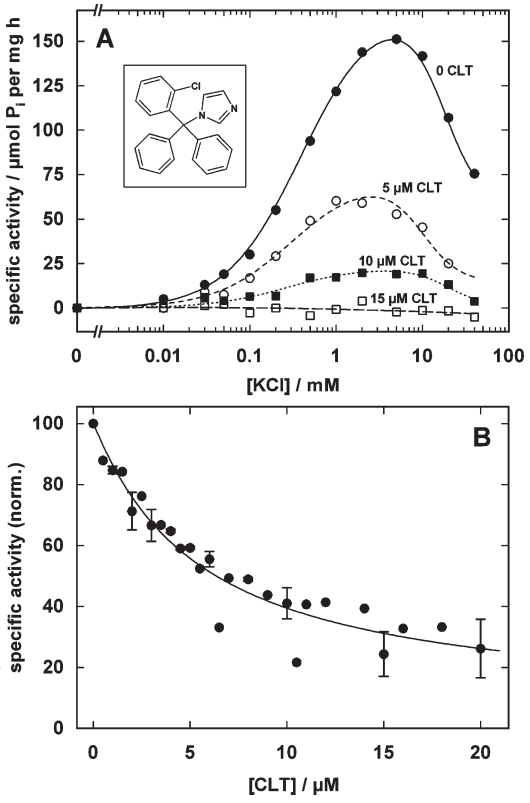

FIGURE 2: Dependence of the specific enzyme activity on K^+ concentration and CLT concentration. The activity was measured by determination of the amount of P_i with the malachite green test at 37 °C. (A) The presence of 5 and 10 μM CLT reduced the specific activity in a K^+-independent manner; at 15 μM CLT, the activity was blocked completely. The inset shows the structure of CLT. (B) In the presence of 5 mM KCl, the CLT dependence of the enzyme activity can be described by a noncompetitive mechanism with a half-inhibitory concentration of 5.2 μM (—).

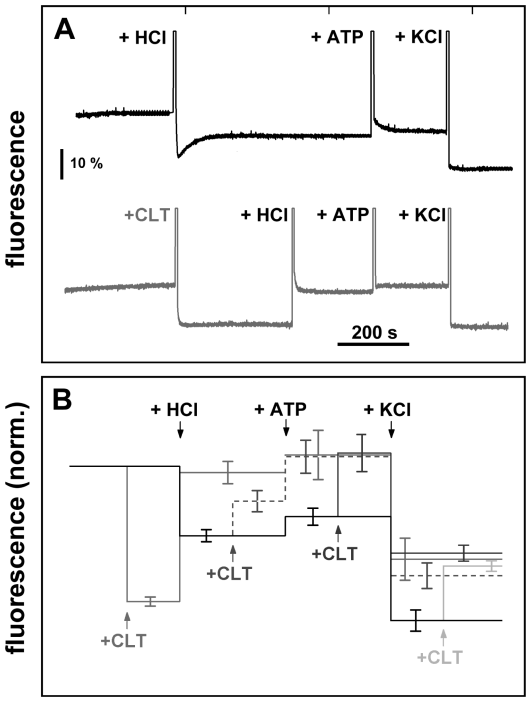

FIGURE 3: Standard experiments performed in the absence and presence of 15 μM CLT. (A) In the top trace, the H,K-ATPase was initially present at pH 8.3 in its E_1 state with virtually empty binding sites. Addition of HCl to lower the pH to 6.3 induced binding of a proton to yield HE_1 as reflected by the RH421 fluorescence decrease. Addition of ATP promoted the conformational transition to the P-E_2 conformation by the $HE_1 \rightarrow H_2E_1 \rightarrow (H_2)E_1\text{-}P \rightarrow P\text{-}E_2H_2 \rightarrow P\text{-}E_2H$ reaction sequence, again with approximately one proton bound to the binding sites (*13*, *20*). Addition of a saturating concentration of KCl allowed further reaction steps of the pump cycle [$H_2E_1 \rightarrow P\text{-}E_2K_2 \rightarrow E_2(K_2) \rightarrow K_2E_1$]. In the bottom trace, the experiment was repeated after addition of 15 μM CLT in the initial E_1 state. This addition caused a fluorescence decrease corresponding to an uptake of two protons (no other monovalent cations present), and this decrease was almost completely reversed when the pH was decreased to 6.3 by addition of HCl. Subsequent additions of ATP and KCl produced fluorescence changes comparable to those in the absence of CLT. (B) Standard experiments were also performed with CLT additions in states H_2E_1, H_2E_1, and K_2E_1. The fluorescence values determined in three or more experiments were averaged and plotted as a schematic representation of the respective fluorescence levels.

bars are shown when different series of the same CLT concentration were performed. The concentration dependence was fitted by a binding isotherm with a half-inhibiting CLT concentration [K_I(CLT)] of 5.2 ± 0.7 μM and an unspecific background activity of 17.2 ± 3.9% (which was not subtracted in these experiments).

Fluorescence Standard Experiments. The molecular mechanism of the inhibitory effect can be studied by steady-state fluorescence experiments with the electrochromic styryl dye RH421. To identify the part of the Post-Albers cycle in which CLT affects the pump function, standard experiments were performed in which the inhibitory compound was added at the various states of the pump cycle. After incubation of the H,K-ATPase preparation with nigericin, the standard experiment begins with the ion pump in the E_1 state. This is maintained by equilibration of the H,K-ATPase-containing membrane vesicles in a buffer of 25 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$, and 200 nM RH421 in the absence of ATP and KCl at pH 8.5. This condition should maintain the H,K-ATPase in its E_1 state with the ion binding sites (almost completely) empty (*20*). In Figure 3A, the experiment starts with a constant fluorescence level before the first substrate addition. Then 17 mM HCl is added to produce a pH decrease to ~6.3, which corresponds according to Diller et al. (*20*) to an average occupation of the binding sites with at least one proton. Subsequent addition of 2 mM ATP promotes the transition into the P-E_2 state with a somewhat lower (average) occupation of the binding sites with protons, and finally, addition of 210 mM KCl leads to the K_2E_1 state, with the lowest-fluorescence state corresponding to an occupation of the binding sites with two ions. This is represented by the top trace in Figure 3A.

The second experiment, shown as the bottom trace of Figure 3A, contains a modification in which 15 μM CLT was added to the E_1 state, before addition of HCl, ATP, and KCl. Upon addition of CLT, a fluorescence decrease that represents an average occupation of the binding sites with two protons was observed since no other monovalent cations are present. (In the second enzyme preparation used, ~1.5 protons bound under the same conditions.) Upon addition of HCl to produce a pH of 6.5 in the buffer, the fluorescence increased and reversed the effect of CLT, returning to near the baseline level. This suggested almost no protons were bound as compared to the same conditions in the absence of CLT. Addition of ATP led to a further minor increase, while addition of K^+ produced a fluorescence decrease similar to that seen in the absence of CLT.

Correspondingly, additions of 15 μM CLT were made to other substrate-induced states of the H,K-ATPase, and the results are summarized in Figure 3B. To present a better overview, all

experiments used the same substrate addition pattern, and the steady-state fluorescence levels were averaged and are plotted schematically with the respective standard error of the mean at each level. Each trace is the result of at least five independent experiments. After addition of CLT, the fluorescence levels of the different experiments seem to merge, independent of the state at which CLT was added. The fluorescence amplitude changes upon addition of ATP and KCl were the same in the absence and presence of CLT. The size of the error bars (standard error of the mean) indicates the poor reproducibility of the response upon CLT addition, probably caused by the poor solubility of CLT in the electrolyte. The only significant difference was observed when CLT was added to the E_1 conformation of the H,K-ATPase at pH 8.3 and 6.3. These findings indicate that CLT affected preferentially cytoplasmic H^+ binding.

CLT Concentration Dependence. A recent study using Na,K-ATPase membranes showed that high CLT concentrations generate fluorescence artifacts (*13*). The origin of these effects is a direct interaction between CLT and RH421 (*13*). CLT is poorly soluble in water and enters the membrane because of a high partition coefficient favoring the hydrophobic phase. This is likely to affect RH421 fluorescence because of a different change in the polarizability of the lipid alkyl chains, especially in the presence of high concentrations of CLT. Therefore, fluorescence amplitudes were tested as a function of CLT concentration in different states of the pump (Figure 4A). CLT titration experiments were performed first in buffer containing 25 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$, and 200 nM RH421 (pH 8.5). This buffer composition maintains the H,K-ATPase in its E_1 state with empty binding sites. Under this condition, the fluorescence level decreased 12% at 15 μM CLT, while higher concentrations increased fluorescence linearly. The second enzyme state, in which CLT titrations were performed, was HE_1. This state was maintained by addition of HCl to lower the pH. In this case, low concentrations of CLT induced a small fluorescence decrease of 2% at 10 μM CLT before an increase in the CLT concentration again led to a linear fluorescence increase. The third series of experiments was performed in a buffer with 25 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$, 200 mM KCl, and 200 nM RH421 (pH 8.5). Under this condition, the H,K-ATPase is trapped in its K_2E_1 state. As shown in Figure 4A, CLT induced no significant fluorescence change up to a concentration of 10 μM. At >10 μM CLT, there was only the linear fluorescence increase as before.

In the presence of 200 mM KCl, the ion binding sites are more than 99% saturated with K^+. The enzyme activity under this condition is near zero, and we can assume that the pumps are trapped in the K_2E_1 state and that the fluorescence increase with increasing CLT concentrations represents a CLT-induced artifact only. When control experiments were performed with pure lipid vesicles, a CLT dependence of the RH421 fluorescence was found to be similar to that detected in the presence of a saturating concentration of KCl (data not shown).

On the basis of this conclusion and the observation that the linear slope in all three CLT concentration dependencies is the same, we subtracted a shifted artifact signal (dashed lines in Figure 4A) from the fluorescence signals to isolate the effect of CLT on the ion binding equilibrium in the E_1 and HE_1 states of the H,K-ATPase (Figure 4B). The resulting CLT-dependent fluorescence decrease indicates an uptake of positive charge inside the protein when CLT interacts with the ion pump at low concentrations. This is interpreted as additional H^+ binding,

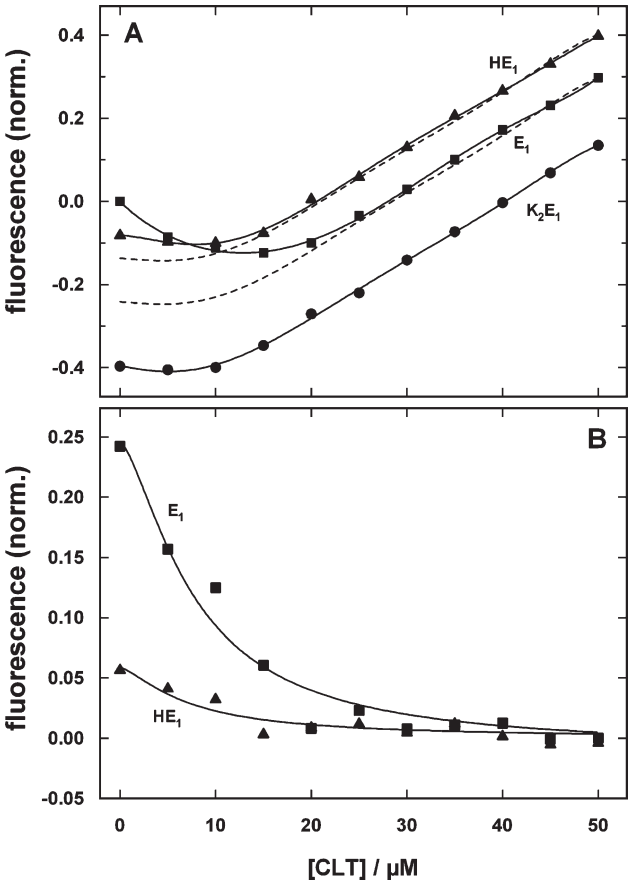

FIGURE 4: Effect of CLT on the RH421 fluorescence in H,K-ATPase-containing vesicle preparations. (A) CLT titrations were performed in three different enzyme states which were induced by corresponding buffer compositions, E_1 with empty binding sites (pH 8.5, 0 M KCl), HE_1 (pH 6.2, 0 M KCl), and K_2E_1 (pH 8.5, 200 mM KCl). In the K_2E_1 state, the enzyme is trapped with both ion binding sites occupied by K^+. As a consequence, the fluorescence changes occurring at concentrations above 10 μM CLT can be assumed to be an artifact induced by modification of the apolar core of the membrane by an increasing amount of the strongly hydrophobic CLT. In the case of the E_1 and HE_1 states, fluorescence decreases were observed at low CLT concentrations that indicate an uptake of positive charge, i.e., H^+, since no other monovalent cations were present. (B) When the CLT-induced fluorescence artifact was subtracted (dashed lines in panel A) from the CLT-dependent fluorescence signals of states E_1 and HE_1, the difference signal was interpreted as specific H,K-ATPase reaction on CLT additions. In both states, the net fluorescence decrease is interpreted as H^+ binding. The half-saturating CLT concentration of this process was $\sim$7.4 μM in both cases, and the different amplitudes correspond to an uptake of approximately two protons (E_1) and fewer than one proton (HE_1).

which comes close to a state with two protons bound at saturating concentrations. Both concentration dependencies were fitted with the Hill function. The half-saturating CLT concentrations are comparable, 7.7 $\pm$ 0.9 μM (E_1) and 7.15 $\pm$ 1.7 μM (HE_1) with a Hill coefficient of 1.5. This value is similar to the half-inhibiting CLT concentration of the enzyme activity [K_I(CLT) = 5.2 μM (see above)].

As a consequence of the CLT-induced fluorescence artifacts, the following experiments were performed preferentially at CLT concentrations of $\leq$15 μM.

Proton Binding in the E_1 Conformation. Effects of CTL on the standard experiments suggest that the cytoplasmic H^+ binding will be affected significantly by the inhibitory compound. Therefore, pH titrations were performed in the absence and

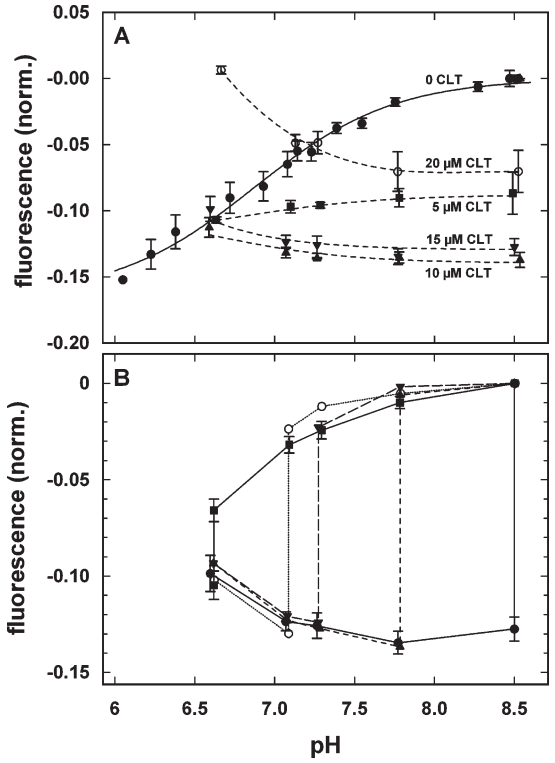

FIGURE 5: Proton binding in the E_1 conformation in the absence and presence of CLT was studied by RH421 fluorescence changes. (A) The fluorescence decrease with a decrease in pH reflects proton binding to the ion binding sites from the cytoplasmic side. In the absence of CLT, the concentration dependence could be fitted by a binding isotherm with a pK of 6.8 ± 0.2. Upon addition of CLT, an initial fluorescence decrease was observed. A nonsignificant pH dependence of the fluorescence amplitude was found at ≤15 μM CLT. The data recorded at 20 μM CLT are mainly controlled by CLT-induced artifacts (see the text). (B) The CLT-induced fluorescence signals are independent of the pH at which 15 μM CLT is added. The fluorescence amplitudes always merge in the presence of the compound.

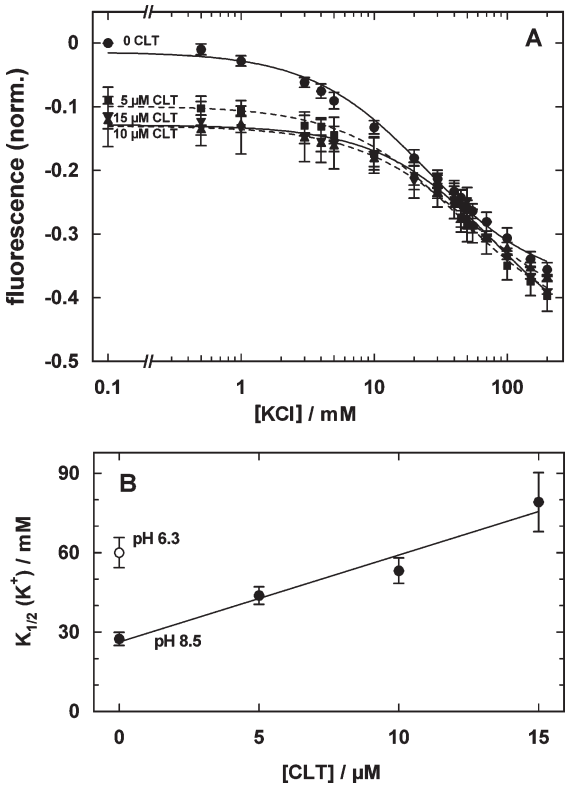

FIGURE 6: Effect of CLT on K^+ binding in the E_1 conformation of the H,K-ATPase. (A) KCl titration experiments were performed in the absence and presence of the indicated concentrations of CLT. The concentration dependence of the fluorescence signals can be fitted satisfactorily by a binding isotherm (lines). (B) The respective half-saturating KCl concentrations ($K_{1/2}$) are plotted vs the CLT concentration and increase linearly. The line is a fit through the data (see the text). For the sake of comparison, the $K_{1/2}$ value determined at pH 6.3 in the absence of CLT is included (○).

presence of various CLT concentrations (Figure 5A). In the absence of CLT, the known proton binding as detected by RH421 was reproduced (*20*) with a pK of 6.8 ± 0.2. When CLT was added at concentrations up to 20 μM at pH 8.5, a fluorescence decrease was recorded as shown in Figure 3A. Subsequently, pH titrations were performed in four steps to reach a pH of 6.6. The conspicuous increase observed in the presence of 20 μM CLT has to be attributed to artifacts as demonstrated in the experiments presented and discussed with the data shown in Figure 4. In the presence of 5 μM CLT, a minor, if at all significant, fluorescence decrease was found when the pH was lowered to 6.6. At 10 and 15 μM CLT, minor fluorescence increases were observed. When compared to Figure 4, small modifications may also be traced back to effects of CLT on the RH421 fluorescence. When the fluorescence levels at 10 and 15 μM CLT are corrected according to Figure 4B, it would correspond to a condition in which the ion pumps contain in the average of ~1.5−1.8 protons in the binding sites in a manner independent of the pH of the buffer in the range between 8.5 and 6.6.

To check whether such a condition can be obtained when only CLT is added to the H,K-ATPase at pH 8.5, series of experiments were performed in which 15 μM CLT was added at various buffer pH values (Figure 5B). These experiments show the fluorescence levels, and therefore, the number of protons bound to the H,K-ATPase, is independent of pH and the initial amount of H^+ bound.

K^+ Binding in the E_1 Conformation. The second ion species that can bind to the H,K-ATPase in the E_1 conformation is K^+ that competes with H^+. Therefore, the effect of CLT on K^+ binding was determined. K^+ titration curves were measured at pH 8.5 in the absence and presence of CLT up to a concentration of 15 μM (Figure 6A). In agreement with Figure 3A, the presence of CLT leads to a fluorescence decrease at 0 M KCl and a slight fluorescence increase at 200 mM KCl. The concentration dependence of K^+ binding is reflected by the fluorescence decrease and can be fitted by a binding isotherm characterized by a half-saturating K^+ concentration ($K_{1/2}$). In Figure 6B, the dependence of $K_{1/2}$ on CLT concentration is plotted. It increases linearly with the CLT concentration present in the buffer from 27.4 ± 2.5 mM in the absence of CLT to 79.1 ± 11.1 mM at 15 μM CLT. For a comparison, $K_{1/2}$ was also determined at pH 6.3. Under this condition, the ion pump is present primarily in the HE_1 state, and K^+ binding therefore requires a displacement of the proton. This leads to an apparently higher $K_{1/2}$ of 60 ± 5.7 mM. The decreasing K^+ binding affinity in the presence of CLT may be produced by an allosteric effect that affects the structure of the ion sites. Alternatively, CLT binding may prevent K^+ entry and show competition with K^+ binding. This alternative will be discussed below.

H^+ Binding in the P-E_2 Conformation. For this set of experiments, pH titrations have to be performed in the presence of 2 mM ATP to maintain the pumps preferentially in their P-E_2

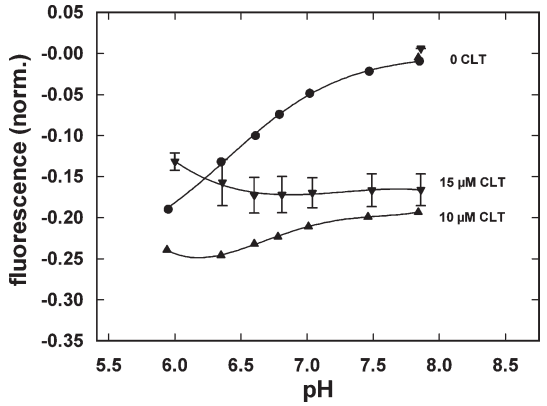

FIGURE 7: Effect of CLT on proton binding in the P-E_2 conformation of the H,K-ATPase. In the absence of CLT, proton binding was observed as a decrease in the RH421 fluorescence with a pK of 6.4. As in the E_1 conformation, an initial fluorescence decrease was observed upon addition of CLT. In the presence 10 μM CLT, the pumps were not completely inhibited and a small but significant fraction still performed H^+ binding in the pH range of 6.3−7.8. At 15 μM CLT, no further H^+ binding was found except for a minor fluorescence increase below pH 6.5.

conformation. The experiments were performed as in the case of the pH titrations in the E_1 conformation with the single difference that ATP was added before aliquots of HCl were applied. Because the ATP solution was unbuffered, the pH decreased from 8.5 to 7.8 as the starting point for the pH titration. When the experiment was performed as a control in the absence of CLT, the pH dependence was reproduced as described previously (*30*). Upon addition of 10 and 15 μM CLT, initial fluorescence decreases of 19 and 17% were observed and a pH dependence of the steady-state fluorescence levels was found (Figure 7) comparable to that in the E_1 conformation of the H,K-ATPase (Figure 5A). A significant difference was observed in the time course of the fluorescence change upon addition of CLT between the E_1 and P-E_2 conformations. In E_1, the transition into the new steady state was completed within the time of mixing of the cuvette contents. When CLT was added in the P-E_2 conformation, the relaxation into the new steady state was visible still after the mixing period of 5 s. It exhibited an exponential decay with a time constant τ of 3.3 s. In those H^+ addition steps in which fluorescence changes could be observed (pH <6.5), even slower relaxations were found [11 s (at pH 5.5) $< \tau <$ 30 s (at pH 7.8) (data not shown)]. In the presence of 15 μM CLT, the addition of H^+ hardly affected the fluorescence level between pH 7.8 and ~6.5. At pH <6.5, an increase was observable. In the presence of 10 μM CLT, a slight fluorescence decrease was visible between pH 7.8 and ~6.5, with a $K_{1/2}$ comparable to the pH dependence observed in the absence of CLT. At pH <6.5, the beginning of a fluorescence increase is indicated. Such a behavior would match the assumption that the P-E_2 conformation has a decreased affinity for CLT.

DISCUSSION

The function of the H,K-ATPase is affected by clotrimazole (CLT) as has been shown previously for other members of the same class of P-type ATPases, the Na,K-ATPase (*13*) and SR Ca-ATPase (*11*, *12*). Although the molecular mechanism of inhibition has not yet been identified, it is likely CLT interferes with the ion pumps near the interface of the lipid phase and membrane domain. Because of its high hydrophobicity, CLT accumulates significantly in the membrane at nominally micromolar concentrations, as shown by the changes it induces in the fluorescence properties of RH421. This has been proven with pure lipid vesicles (data not shown). CLT inhibits the enzyme activity and modifies the ion binding kinetics of the H,K-ATPase as shown in the case of the closely related Na,K-ATPase. The results can be summarized in a mechanism that contrasts with that developed for the Na,K-ATPase (*13*).

Inhibition of the Enzyme Activity. The enzyme activity measurements clearly prove that CLT inhibits ATP hydrolysis of the H,K-ATPase (Figure 2). At a CLT concentration of 5 μM, the activity is reduced to ~50% while the shape of the K^+ induction of enzyme activity is not significantly altered. Such a behavior may be caused by two different mechanisms. Either 50% of the enzyme is completely inhibited while the other 50% runs undisturbed, or on average, all pumps progress through the cycle at half-speed. The observation in Figure 2B that the inactivation can be described by a binding isotherm indicates that the mechanism is based on a reversible binding of CLT to the pump. In the lower range of K^+ concentrations, binding of K^+ is rate-limiting with respect to enzyme activity. Since in the absence and presence of 5 and 10 μM CLT K^+ binding kinetics is not affected ($K_{1/2} = 0.29 \pm 0.01$ mM), the presence of CLT has neither affected K^+ binding nor has another step of the pump cycle become rate-limiting. This observation favors the proposal that CLT blocks the H,K-ATPase completely when interacting with the protein. As mentioned above, the compound is highly hydrophobic, and we can assume that the CLT binding site is located inside or close to the surface of the membrane domain of the H,K-ATPase.

The fact that no significant H,K-ATPase-dependent ATP-hydrolysis activity could be detected at 15 μM CLT (Figure 2A) does not seem to fit well with the residual activity at 15 μM CLT when the concentration dependence is determined (Figure 2B). However, when the average unspecific background activity of ~17% present in these experiments is taken into account, a residual activity of 7% remains which can be assumed to be nonsignificant with respect to the error of ±7% (standard error of the mean) at 15 μM (see Figure 2B). The size of the error bars at higher concentrations suggests that poor solubility of the compound gives rise to variations in the CLT concentration in the lipid phase. Depending on individual mixing variations, different actual concentrations may have resulted in the membrane when the experiments were repeated.

While the half-inhibiting CLT concentration for the H,K-ATPase was determined to be 5.2 ± 0.7 μM (K_I), comparable experiments with the Na,K-ATPase gave two different K_I values. In the physiological mode of the Na,K-ATPase, the K_I was ~30 μM, and in the Na-only mode, the K_I was 9.5 μM (*13*). For the SR Ca-ATPase, a K_I of 20−30 μM may be estimated from published data (*11*). From these findings, we can conclude that the H,K-ATPase is inhibited via a mechanism similar to those of both the Na,K-ATPase and Ca-ATPase. The H,K-ATPase, however, shows a greater sensitivity to CLT.

Effect of CLT on Ion Binding Reactions. Experiments were performed to reveal which conformational states of the H,K-ATPase are affected by CLT (Figure 3). The increase in the RH421 fluorescence with a CLT concentration of >15 μM is shown Figure 4A to be independent of the conformational state and therefore only a CLT-induced artifact. The standard experiments in Figure 3B were therefore normalized to give the same fluorescence level after addition of all substrates and 15 μM CLT.

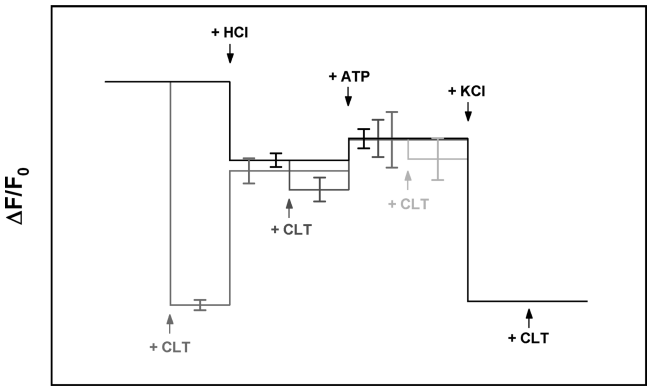

FIGURE 8: Renormalized standard experiments. Assuming that addition of 15 μM CLT induces an artificial fluorescence increase as discussed for Figure 4A, the data in Figure 3B were modified to compensate for this artifact.

The results (see Figure 8) show the largest effect of CLT is on the (empty) E_1 state of the H,K-ATPase, i.e., at pH 8.5, with no K^+ and no ATP present. Addition of 15 μM CLT produced an explicit decrease in the fluorescence level which corresponds to an import of almost two positive elementary charges. Reducing the pH to 6.3 increased the fluorescence to the level given in the absence of CLT. This observation demonstrates that a pH of 6.3 is able to reverse the CLT-induced modification of the H,K-ATPase, and the similarity of the fluorescence intensity after addition of ATP and KCl suggests that CLT does not modify the H,K-ATPase in the other states of the ion pump.

This proposal can be substantiated with pH titration (Figure 5) and K^+ binding experiments (Figure 6) in the absence and presence of various CLT concentrations. In the absence of KCl and ATP, when the only possible physiological reaction steps are proton binding and release, $E_1 \rightleftarrows HE_1 \rightleftarrows H_2E_1$, the observed fluorescence traces reported in Figure 5 indicate that addition of CLT induces H^+ binding. As discussed in the framework of the CLT-induced artifacts (Figure 4), it is clear that the data taken at 20 μM CLT should be ignored for the moment since they would require a correction of the artificial fluorescence increase to allow a mechanistic analysis. The synopsis of the other data from Figure 5 is in agreement with a modified reaction mechanism in which CLT-controlled binding of up to two protons competes with the physiological H^+ binding as described by the reaction scheme

$$(H_2E_1^*[CLT] \rightleftarrows)HE_1^*[CLT] \rightleftarrows E_1^*[CLT] \rightleftarrows E_1 \rightleftarrows HE_1 \rightleftarrows H_2E_1 \quad (1)$$

The transition into state $H_2E_1^*[CLT]$ is presented in parentheses to indicate that the ion pumps are not necessarily occupied quantitatively by two protons at pH 8.5. When it is assumed that in the CLT-modified H,K-ATPase the protons bind to the same binding sites as in the case of the unmodified pump, then the binding affinity of the sites has to be significantly higher when CLT interacts with the pumps so that even at pH 8.5 the sites are occupied by one or two protons. Both pK values of binding of the proton to the unmodified pump are 6.7 and 4.5 (*20*). A decrease in the pH in the aqueous phase leads to an increase in fluorescence which has to be assigned to weakened H^+ binding to unmodified pumps in state E_1. Obviously, reducing the pH induces a depletion of the CLT-modified states $[(H_2E_1^*[CLT] \rightleftarrows) HE_1^*[CLT] \rightleftarrows E_1^*[CLT]]$ and an increased occupation of the unmodified states $(E_1 \rightleftarrows HE_1 \rightleftarrows H_2E_1)$. As a molecular mechanism driving this process, we propose the protonation of CLT, which has a pK_a of 6.12. When the buffer pH is lowered from 8.5 to 6.2, ~50% of the CLT will become protonated and either repartitions into the aqueous phase or no longer binds to the H,K-ATPase (or both). As a consequence, the $E_1^*[CLT] \rightleftarrows E_1$ reaction step will be shifted to the unmodified state on the right side. This leads to an overall reduction in the number of bound ions in the membrane domain of the pump and explains the (counterintuitive) increase in the RH421 fluorescence until pH values are attained at which H^+ binding to the unmodified H,K-ATPase becomes significant, as shown in Figure 5B or in the standard experiment (Figure 3B or 7). An alternative (or additional) interpretation could be that a histidine side chain at the membrane domain becomes protonated with a decrease in pH and this modification weakens CLT binding. The experimental data obtained so far do not allow these two possibilities to be distinguished.

The argument about direct competition between protons and CLT for the E_1 state holds also for the CLT dependence of K^+ binding in the E_1 conformation of the H,K-ATPase (Figure 6). The K^+ binding affinity is affected by the interaction of CLT with the pump, and at pH 8.5, the reaction scheme that represents the K^+ titration experiments in the presence of CLT can be written as

$$(H_2E_1^*[CLT] \rightleftarrows)HE_1^*[CLT] \rightleftarrows E_1^*[CLT] \rightleftarrows E_1 \rightleftarrows KE_1 \rightleftarrows K_2E_1 \quad (2)$$

In the experiments depicted in Figure 6A, the buffer pH was kept at 8.5. Therefore, the fractional equilibrium distribution among states $[(H_2E_1^*[CLT] \rightleftarrows) HE_1^*[CLT] \rightleftarrows E_1^*[CLT]]$ was constant, and all three states may be summarized to $E^*[CLT] = E_1^*[CLT] + HE_1^*[CLT] + H_2E_1^*[CLT]$. Binding of K^+ could be fitted satisfactorily by a simple binding isotherm (Figure 6A) with a single half-saturating K^+ concentration, $K_{1/2}(K^+)$. Taking both simplifying assumptions into account, we are able to reduce reaction scheme 2 to

$$E_1^*[CLT] \rightleftarrows E_1 \rightleftarrows KE_1 \quad (3)$$

With this reaction scheme, the effect of the interaction of CLT with the pump ($E_1^*[CLT] \rightleftarrows E_1$), which also can be represented by a binding isotherm (Figure 2B), may be used to formulate an apparent dependence of $K_{1/2}(K^+)$ on the "competitive inhibitor" CLT:

$$K_{1/2}(K^+)^{app} = K_{1/2}(K^+)[1 + [CLT]/K_I(CLT)] \quad (4)$$

where [CLT] is the CLT concentration and K_I(CLT) the half-inhibitory CLT concentration. This linear equation was used to fit the data points in Figure 6B. The $K_{1/2}(K^+)$ is 26.2 mM in the absence of CLT, and the slope of 3.3 mM/μM gives a K_I(CLT) of ~8 μM. This is in good agreement with the effect of CLT on H^+ binding, which gave a $K_{1/2}$ of 7.7 ± 0.9 μM at pH 8.3 (Figure 4B).

These findings suggest CLT does not bind directly to the ion occlusion sites but instead promotes proton binding ($E_1^*[CLT] \rightleftarrows HE_1^*[CLT] \rightleftarrows H_2E_1^*[CLT]$). Therefore, higher K^+ concentrations are needed to replace the protons in the binding sites. As an apparent consequence, the CLT modification of the protein competes with K^+ binding. In the absence of CLT, a competition between K^+ and H^+ in the binding sites was also demonstrated by K^+ titration experiments performed at pH 6.3, when the H,K-ATPase is preferentially in the state with one proton bound, HE_1. An increased $K_{1/2}(K^+)$ of 60 mM also was measured under this condition (Figure 6B).

The effect of CLT on ion binding in the P-E_2 conformation of the H,K-ATPase was investigated for both proton and K^+

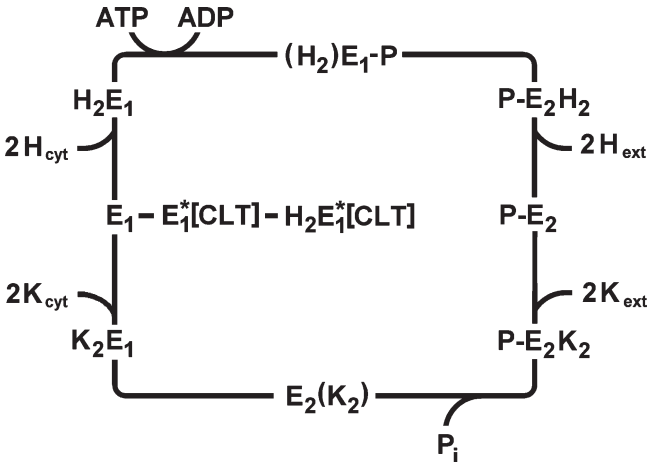

FIGURE 9: Modified Post-Albers scheme for proposing a mechanism of the inactivation of the H,K-ATPase by CLT. Reversible "binding" of CLT to the membrane domain occurs only in the E_1 state of the enzyme and modifies the conformation of the membrane domain in a way that the binding affinity for protons is significantly increased. Depending on the cytoplasmic pH, up to two protons can bind sequentially to their regular ion sites in the CLT-modified form of the pump to form an inactive dead-end state.

binding. In the case of H^+ binding, the RH421 fluorescence showed nearly the same pH dependence as in the E_1 conformation. The slow transition into a new steady state upon addition of CLT and H^+ indicates, however, that a rate-limiting reaction step is included in this ligand-triggered partial reaction. Since CLT as well as H^+ or K^+ binding was found not to be rate-limiting under all other experimental conditions in this study, another ligand-independent step of the pump cycle has to be taken into consideration. With a time constant on the order of many seconds, the noncanonical back reaction to the E_1 conformation is the most promising candidate in the absence of K^+ ions. Two different pathways can be conceived, one involving empty binding sites to give $P\text{-}E_2 \rightarrow E_1$ and the alternative with two protons bound to give $P\text{-}E_2 \rightarrow P\text{-}E_2H_2 \rightarrow H_2E_1 \rightarrow E_1$. The observation that the relaxation time is shorter at low pH would support the second suggestion, with the idea that H^+ binding to the $P\text{-}E_2$ state is the rate-limiting step. In summary, it is plausible to propose that CLT does not interact with the pump in its $P\text{-}E_2$ conformation but interferes only after the enzyme has performed the transition to the E_1 state. Once trapped in E_1 by CLT, the enzyme may not be rephosphorylated, and therefore, the $P\text{-}E_2$ conformation is successively depleted. This concept is compatible with the effect of CLT on K^+ binding in the $P\text{-}E_2$ conformation.

K^+ binding in the $P\text{-}E_2$ conformation can be studied only via K^+-stimulated ATPase activity (*20*). As discussed above, the respective results (Figure 2A) indicate that the presence of CLT does not significantly affect the kinetics of K^+ binding affinity in the $P\text{-}E_2$ conformation. In the E_1 conformation, the value of $K_{1/2}(K^+)$ was more than doubled when 10 μM CLT was added. In contrast, $K_{1/2}(K^+)$ was unaffected in the $P\text{-}E_2$ conformation. This is a clear indication that CLT does not interfere with the ion binding kinetics of H,K-ATPase in the $P\text{-}E_2$ conformation.

This observation of CLT-insensitive ion binding from the luminal side of the H,K-ATPase and the fact that standard experiments do not show significantly different behavior in ligand-stabilized states other than in the E_1 state when CLT is applied suggest that the H,K-ATPase is affected by CLT only in the E_1 state.

Mechanistic Proposal. All experiments presented are in agreement with the modified Post-Albers cycle depicted in Figure 9. Several characteristics of the transport properties were discussed above and have to be taken into account.

(1) The interaction between CLT and the H,K-ATPase affects the pump function in an inhibitory manner and takes place in the hydrophobic core of the membrane. Binding (or adsorption) of CLT to the membrane domain of the protein is reversible. This is demonstrated by the fact that in the CLT-dependent enzyme activity (Figure 2B) the concentration dependence can be represented by a binding isotherm and in the standard experiments the inactivation at pH 8.5 can be reversed at least partially by addition of H^+.

(2) CLT interacts with the protein in the E_1 conformation. The affinity of the binding sites for protons increases significantly in the CLT-modified state so that even at pH 8.5 on average more than one proton is bound in the binding sites. No experimental evidence was found that would support any K^+ binding to the CLT-modified protein.

(3) In the phosphorylated E_2 conformation, ion binding affinities were not affected by the presence of CLT.

(4) When in the E_1 conformation H^+ or K^+ titration experiments are performed with the CLT-modified H,K-ATPase, the modification or inhibition is reversed. In the case of K^+ binding, we found that the dependence of the apparent half-saturating K^+ concentration on CLT can be described by a mechanism of competitive binding of K^+ and CLT to the enzyme (Figure 6).

(5) Combining the experimental findings and their analysis leads to a reaction scheme that is sufficient to explain completely the presented observations:

$$\begin{array}{l} H_2E_1 \rightleftarrows HE_1 \\ \qquad\quad \searrow \\ \qquad\quad E_1 \rightleftarrows E_1^*[CLT] \rightleftarrows HE_1^*[CLT] \left(\rightleftarrows H_2E_1^*[CLT]\right) \\ \qquad\quad \nearrow \\ K_2E_1 \rightleftarrows KE_1 \end{array}$$

When this reaction scheme is merged into the Post-Albers pump cycle of the H,K-ATPase, it is modified in a condensed version as shown in Figure 9.

Interaction of CLT with the H,K-ATPase introduces a single "dead-end" branch added to the Post-Albers scheme. At physiological pH and low K^+ concentrations, the flow through the pump cycle is redirected at least partly into both dead-end states ($H_2E_1^*[CLT] \rightleftarrows HE_1^*[CLT]$) and the ion pump becomes inhibited. In principle, this mechanism should work also at lower pH with higher CLT concentrations. Due to the artifacts of the hydrophobic compound produced with the styryl dye RH421, the range of applicable CLT concentrations is, however, limited.

When this proposal is compared to the respective mechanism that has been suggested for the Na,K-ATPase (Figure 1), an explicit similarity can be seen. For both pumps, a single (dead-end) state with two ions bound has to be introduced to explain the experimental findings. There are, however, two differences in the mode of action between these structurally similar ion pumps. For the Na,K-ATPase in the CLT-inhibited state, two ion species, H^+ and K^+, can be bound, although the inhibited state is more stable when H^+ ions are in the binding sites (*13*). In the case of the H,K-ATPase, only protons were found to occupy the binding sites when the pump was inhibited. A second difference is that the inhibited state branches off from the dephosphorylated E_2 state in the case of the Na,K-ATPase, $E_2(X_2)$, where X is either

H^+ or K^+. In the H,K-ATPase, it is the "empty" E_1 state (Figure 9). Under physiological conditions, the K^+-occluded state of the H,K-ATPase is not stabilized (*16*, *20*, *22*), but instead, proton binding is rate-limiting due to the p*K* of 6.7 for the first and the p*K* of 4.5 for the second proton binding. Therefore, in the case of the H,K-ATPase, E_1 is a long-lived state that provides the chance for CLT to modify the protein. It is possible that for the H,K-ATPase the interaction or binding of CLT is possible only when the protein is in the E_1 conformation with empty binding sites, while for the Na,K-ATPase, this process is possible only in the $E_2(X_2)$ state. The fact that in both proteins the ion binding sites are affected by CLT or by the CLT−protein interaction is obvious by the observations that on the one hand the CLT-modified H,K-ATPase has a strongly enhanced affinity for H^+ and that on the other hand K^+ ions destabilize the CLT-modified state of the Na,K-ATPase.

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