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Temperature Dependence of the Rates of Conformational Changes Reported by Fluorescein 5′-Isothiocyanate Modification of H^+,K^+- and Na^+,K^+-ATPases†

Larry D. Faller,*,‡ Ruben A. Diaz,‡ Georgios Scheiner-Bobis,§ and Robert A. Farley§

Center for Ulcer Research and Education, Department of Medicine, University of California at Los Angeles School of Medicine, and Department of Veterans Affairs Medical Center West Los Angeles, Wadsworth Division, Los Angeles, California 90073, and Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles, California 90033

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ABSTRACT: Stopped-flow fluorometry has been used to measure the forward and reverse rates of the conformational change from E_1 to E_2 in the fluorescein-modified proton and sodium pumps (1) as a function of Na^+ and K^+ concentrations to verify the proposed mechanism of ion interaction with the enzymes and (2) as a function of temperature to gain insight into the nature of the conformational transition. (1) The fluorescence changes caused by Na^+ and K^+ are consistent with rapid competitive binding of the two ions to the E_1 conformations of the enzymes followed by rate-limiting transitions between E_1K and E_2K. (2) Reaction coordinate diagrams for the E_1K to E_2K transitions in the H,K-ATPase and Na,K-ATPase are qualitatively similar. Enthalpy barriers to reaction are partially compensated by increased entropy in the transition states. However, there are striking quantitative differences between the two enzymes. The E_2K to E_1K reaction of the H,K-ATPase is more than 2 orders of magnitude faster ($\tau_{1/2}$ = 6 ms at 22 °C) than the reverse rate of the Na,K-ATPase transition ($\tau_{1/2}$ = 1.6 s), explaining repeated failure to detect a K^+-"occluded" form of the H,K-enzyme. The E_2K conformer of the Na,K-ATPase is 3 orders of magnitude more stable than E_1K, while the E_1K and E_2K conformations of the H,K-ATPase are nearly equivalent energetically.

Enzymes that catalyze active transport via formation of a covalent phosphoenzyme intermediate are classified as E_1E_2-type ATPases, where E_1 and E_2 denote different protein conformations that have been postulated to explain coupling of ATP hydrolysis to the physical translocation of ions. Experimental evidence for a conformational change has been obtained by chemical modification of the enzymes with fluorescein 5′-isothiocyanate (FITC),[1] which reacts specifically

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‡University of California at Los Angeles School of Medicine and Department of Veterans Affairs Medical Center West Los Angeles.
§University of Southern California School of Medicine.

[1] Abbreviations: H,K-ATPase, Mg^{2+}-dependent, H^+-transporting, and K^+-stimulated ATPases (EC 3.6.1.36); Na,K-ATPase, Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase (EC 3.6.1.37); Ca-ATPase, Ca^{2+}- and Mg^{2+}-dependent ATPase (EC 3.6.1.38); SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; DCIP, 2,6-dichloroindophenol; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidene)adenosine 5′-triphosphate; pNPP, *p*-nitrophenyl phosphate; P_i inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; SD, standard deviation.

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with a lysine residue in a conserved region of the primary structure. A pentapeptide containing the lysine covalently labeled by fluorescein is identical in the Na,K-ATPase, H,K-ATPase, and SR Ca-ATPase (Farley & Faller, 1985). The site of modification is thought to be in, or near, the part of the active site that recognizes the adenine ring, because ATP protects against fluorescein incorporation, but the modified enzymes catalyze pNPP hydrolysis and can be phosphorylated by P_i (Karlish, 1980; Pick & Bassilian, 1980; Jackson et al., 1983).

Ions that are transported affect the quantum yield of the fluorescent reporter group. K^+ quenches the fluorescence of fluorescein-labeled Na,K-ATPase. Na^+ prevents or reverses the K^+ quench. To explain evidence for a maximum rate of the K^+ quench, Karlish (1980) proposed a mechanism in which the conformational change between E_1K and E_2K is rate limiting:

$$E_1^F Na \overset{K_{Na}}{\rightleftharpoons} Na^+ + E_1^F + K^+ \underset{K_K}{\rightleftharpoons} E_1^F K \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2^F K \qquad (1)$$

Na^+ reversal of the K^+ quench was attributed to competitive binding of Na^+ and K^+ to the E_1 conformation of the enzyme, because the apparent K^+ and Na^+ dissociation constants estimated from equilibrium titrations with the other ion present depended linearly on the competing ion's concentration. The superscript (F) in eq 1 is a reminder that normal catalytic activity is inhibited in the fluorescein-modified enzyme. Nevertheless, the conformational change appears to be normal, because the rate of the fluorescence intensity change in the FITC-modified enzyme agrees with values obtained by using reversibly bound formycin nucleotides (Karlish et al., 1978) and intrinsic protein fluorescence (Karlish & Yates, 1978) to follow conformational transitions in unmodified Na,K-ATPase. Parentheses, sometimes placed about potassium in the E_2 conformer to indicate that the ion is "occluded", have been omitted, because they imply a molecular interpretation of the slow rate of the reverse reaction (k_{-2}) that goes beyond what can be learned from rate measurements at a single temperature.

In this paper, K^+ and Na^+ are shown to cause analogous changes in the fluorescence of FITC-modified H,K-ATPase. Additional tests of eq 1 are reported that support the proposed mechanism for monovalent cation interaction with E_1E_2-type ATPases. In particular, both the nonzero rate of the K^+ quench as $[K^+]$ approaches zero and the Na^+ dependence of the rate at which Na^+ reverses the K^+ quench predicted by eq 1 have been observed, the former with the H,K-ATPase and the latter with the Na,K-ATPase. Additional evidence that conformational changes reported by fluorescein are normal has been obtained by showing that changes in the fluorescence of another FITC-modified enzyme (H,K-ATPase) are fast enough to be steps in its catalytic cycle.

The model has been extended by measuring the rates of the fluorescence changes reported by fluorescein as a function of temperature. The quasi-thermodynamic parameters derived from the temperature dependence of reaction rates cannot reveal molecular details of the conformational change. However, the transition-state analysis does place constraints on the types of molecular chemical events that may be occurring by resolving the activation energy into enthalpy and entropy changes.

An abstract reporting some of the results of this study has been published (Faller et al., 1990). In Polvani et al., (1989), E.C. Rabon, S. J. D. Karlish, and G. Sachs are reported to have independently shown the feasibility of using fluorescein fluorescence to measure the rate of the E_1E_2 conformational change in the H,K-ATPase.

Experimental Procedures

Materials

H,K-ATPase. Porcine H,K-ATPase was isolated and purified by a published procedure (Faller, 1990), except that a Beckman JCF-Z rotor was used for the zonal centrifugation. The enzyme was purified by sedimenting for 9 h at 14000 rpm on a discontinuous sucrose–Ficoll gradient. The ATPase activity per milligram of protein (Lowry et al., 1951) of the preparations used in the reported experiments ranged from 100 ± 20 μmol h^{-1} (7% Ficoll–34% sucrose fraction) to 170 ± 30 μmol h^{-1} (0.25 M sucrose-7% Ficoll fraction).

Na,K-ATPase. Microsomal vesicles containing the Na,K-ATPase were isolated from the outer medulla of hog kidneys. The enzyme was purified by extracting nonintegral membrane proteins with SDS (Jorgensen, 1974). Preparations that released from 16 to 23 μmol of P_i from ATP per minute per milligram of Lowry protein were used in the reported experiments.

Chemical Modification. Broken vesicles containing the enzymes were reacted with FITC at pH 9.2 using experimental protocols published previously (Carilli et al., 1982; Farley & Faller, 1985). The residual ATPase activity of the modified enzyme used in the stopped-flow experiments described was <10%. Changes reported by fluorescein in the lighter and heavier gradient fractions of the H,K-ATPase were indistinguishable. Both the FITC-modified Na,K-ATPase and the FITC-modified H,K-ATPase could be recycled after being mixed with monovalent cations and used again for kinetic experiments without affecting the measured rates. The enzymes were recovered from the stopped-flow effluent by centrifugation and washed by alternately resuspending in buffer and sedimenting several times.

Reagents. Fluorescein 5′-isothiocyanate (isomer 1) was obtained from Molecular Probes. Highly purified preparations of all the other reagents used in this study are commercially available from a variety of sources.

Methods

Stopped-Flow Measurements. Equal volumes (100 μL) of solutions containing labeled enzyme and monovalent cation were mixed by using a drive pressure of 7 bar in a Hi-Tech PQ/SF-53 sample handling unit. The volume of the observation cell is 40 μL. The dead time (t_d) of the instrument was measured by mixing 185 μM DCIP with 5, 10, and 20 mM ascorbic acid in 20 mM HCl. t_d = 3.1 ± 0.8 ms (n = 3) was obtained from the best fit of

$$A = A_0 e^{-kt_d} \qquad (2)$$

to the measured first-order rate constants (k) and amplitudes (A) with A_0 fixed at the independently determined absorbance of 92.5 μM reduced DCIP.

The Hi-Tech mixer is integrated into an On Line Instrument Systems spectrometer. The fluorescein label was excited at 495 ± 4 nm (1-mm slits) by a 75-W xenon arc lamp. The emitted light is detected, after passage through a 1-mm-thick OG 515 long band-pass optical filter with measured cutoff (50% transmission) at 513.7 nm, as a proportional voltage. The instrument is interfaced to an AST Premium 286 computer. The electronic time constant was always at least an order of magnitude shorter than the reaction half-time. An approximately constant gain setting was used in titrations with metal ion to faciltate comparing the amplitudes of the responses. Fluorescence changes are reported in arbitrary units

(au). Temperature was controlled by circulating water from a Lauda RMS-20 refrigerating bath around the drive syringes and observation chamber.

Analysis. In each experiment at a single ion concentration and temperature, from 3 to 10 kinetic curves of 1000 points were stored and analyzed. The first-order model (eq 2 with t_d generalized to t), or a competing first plus zero-order model, was fitted to the data with the successive integration algorithm resident in the On Line Instrument Systems software. One time, curves differing from the mean in time constant or amplitude by more than two standard deviations were rejected. The concentration dependence of the amplitudes and first-order time constants, and the temperature dependence of the derived rate constants, was analyzed with the derivative-free, nonlinear least-squares program of BMDP Statistical Software, Inc.

The mixing experiments reported in this paper can be thought of as concentration jump, chemical relaxation experiments (Eigen & de Maeyer, 1963). The rate at which the concentrations (c) of the reactants in a unimolecular reaction reequilibrate is proportional to their distance from equilibrium. Comparing the integrated rate law for a step perturbation

$$\Delta c = \Delta c_0 e^{-t/\tau} \quad (3)$$

with eq 2, k is the reciprocal of the relaxation time (τ). The relationship between $1/\tau$ and the individual rate constants depends on the specific mechanism of the reaction. For the mechanism in eq 1:

$$1/\tau = k_2[K^+]/\{[K^+] + K_K(1 + [Na^+]/K_{Na})\} + k_{-2} \quad (4)$$

assuming formation of noncovalent protein–monovalent cation complexes is fast compared to the conformational change. K_K and K_{Na} are the dissociation constants of K^+ and Na^+, respectively, from the E_1 conformation of the enzyme. Equation 4 predicts a hyperbolic dependence of $1/\tau$ on $[K^+]$ ($[Na^+] = 0$) and an inverse relationship between $1/\tau$ and $[Na^+]$ ($[K^+]$ fixed).

In principle, the K^+ quench and Na^+ reversal data can be combined, and k_2, K_K, k_{-2}, and K_{Na} can be estimated by fitting eq 4 to the combined data. In practice, K_K and k_2 for the Na,K-ATPase were estimated from nonlinear least-squares fits of eq 4 to measurements of $1/\tau$ as a function of $[K^+]$ ($[Na^+] = 0$). Estimates of K_{Na} and k_{-2} could then be obtained from the $[Na^+]$ dependence of $1/\tau$ by fitting eq 4 to the data with $[K^+]$, k_2, and K_K fixed. K_{Na} could not be estimated for the H,K-ATPase, presumably because the first term in eq 4 is negligible over the Na^+ concentration range studied. Therefore, the $1/\tau = k_{-2}$ values measured in Na^+ reversal experiments were combined at $[K^+] = 0$ with the K^+ quench data to obtain best estimates of k_2, K_K, and k_{-2} for the H,K-enzyme. The total concentrations of K^+ and Na^+ were used in the calculations, because the enzyme concentration was always much less than the monovalent cation concentrations experimentally.

The measured fluorescence change (ΔF), or amplitude, was corrected for the dead time of the instrument with eq 2. The apparent dissociation constant that would be measured in an equilibrium titration ($K_{0.5}$) was estimated by fitting the equation for binding of a single class of any number of equivalent and noninteracting sites to corrected fluorescence changes (ΔF_0) as a function of metal ion concentration. Equation 1 gives for the K^+ quench:

$$K_{0.5K} = K_K/(K_c + 1) \quad (5)$$

where $K_c = k_2/k_{-2}$ describes the distribution of enzyme between the E_1K and E_2K conformations at equilibrium. The maximum fluorescence change observed experimentally will

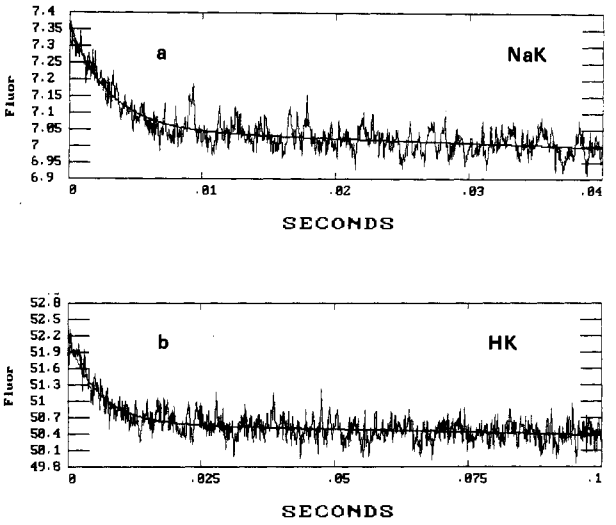

FIGURE 1: Quench of fluorescence by K^+. 100 μL of 140 mM KCl in 60 mM choline chloride was mixed with 100 μL of 300 μg mL^{-1} FITC-labeled enzyme in 200 mM choline chloride and 0.3 mM EDTA–Tris. Both reactant solutions were buffered by 50 mM Tris-HCl at pH 7.4 and thermostated at 22.0 ± 0.5 °C. The excitation wavelength was set between 494.7 and 495.3 nm, the photomultiplier voltage averaged 600 v, and the electronic time constant was 10^{-4} s. Fluorescence in arbitrary units is plotted against time in seconds. (A) Na,K-ATPase: A single trace is shown. $1/\tau = 340 \pm 18$ s^{-1}. The observed amplitude is -0.315 ± 0.008 au. (B) H,K-ATPase: Nine traces are shown summed. $1/\tau = 177 \pm 11$ s^{-1}. The mean amplitude of an individual trace is -0.175 ± 0.006 au.

be less than the theoretical value (ΔF_{max}), if a significant fraction of enzyme with K^+ bound remains in the E_1 conformation at equilibrium. For the Na^+ reversal:

$$K_{0.5Na} = K_{Na}(1 + [K^+]/K_{0.5K}) \quad (6)$$

assuming Na^+ and K^+ bind competitively.

The Arrhenius activation energies (E_a) for the forward and reverse reactions were obtained from semilogarithmic plots of the appropriate rate constant versus the reciprocal of the temperature. Quasi-thermodynamic activation parameters were calculated from the Arrhenius activation energy and the rate constants with equations derived by transition-state theory (Frost & Pearson, 1953). Assuming that the mechanism in eq 1 is correct and k_2 and k_{-2} are rate constants for the same reaction step, the difference between the Gibbs free energies of activation (ΔG^*), enthalpies of activation (ΔH^*), or entropies of activation (ΔS^*) in the forward and reverse directions is the standard Gibbs free energy change ($\Delta G°$), standard enthalpy change ($\Delta H°$), or standard entropy change ($\Delta S°$) for the conformational change between E_1K and E_2K.

Results

K^+ Quench. K^+ quenches the fluorescence of either FITC-labeled Na,K-ATPase (Karlish, 1980) or H,K-ATPase (Jackson et al., 1983) in equilibrium titrations. The rate of this reaction was measured by mixing enzyme covalently modified by FITC with KCl in a stopped-flow spectrofluorometer. The ionic strength was kept constant at 200 mM with choline chloride (Helmich-de Jong et al., 1986). Other experimental details are given in the legend to Figure 1, which compares two of the shortest, and therefore technically most difficult, measurements made with the two enzymes.

Figure 1a is a single relaxation curve obtained with the Na,K-ATPase. An unexpected observation illustrated in the figure is a slow linear decrease in fluorescence following the rapid quench. Possible artifacts that could account for the "drift" are lamp instability, photobleaching of the fluorescent probe, or protein denaturation. None of these explanations

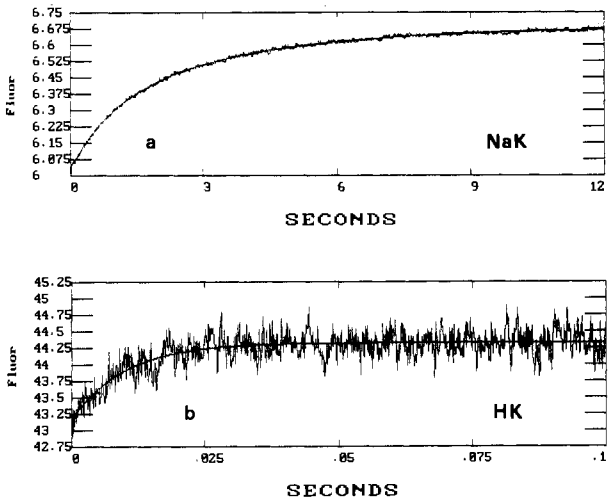

FIGURE 2: Reversal of K^+ quench by Na^+. 100 μL of 200 mM NaCl was mixed with 100 μL of 300 μg mL^{-1} FITC-labeled enzyme in 10 mM KCl, 0.3 EDTA–Tris, and 190 mM choline chloride. Both reactant solutions were buffered by 50 mM Tris-HCl at pH 7.4 and thermostated at 22.0 ± 0.3 °C. The same excitation wavelength and photomultiplier voltages given in the legend to Figure 1 were used. Fluorescence in arbitrary units is plotted against time in seconds. (A) Na,K-ATPase: A single trace is shown. The electronic time constant was 10^{-2} s. $1/\tau = 0.597 \pm 0.004$ s^{-1}. The measured amplitude is +0.532 + 0.002 au. (B) H,K-ATPase: Seven traces are shown summed. The electronic time constant was 10^{-4} s. $1/\tau = 103 \pm 5$ s^{-1}. The average amplitude of a single trace is +0.167 ± 0.005 au.

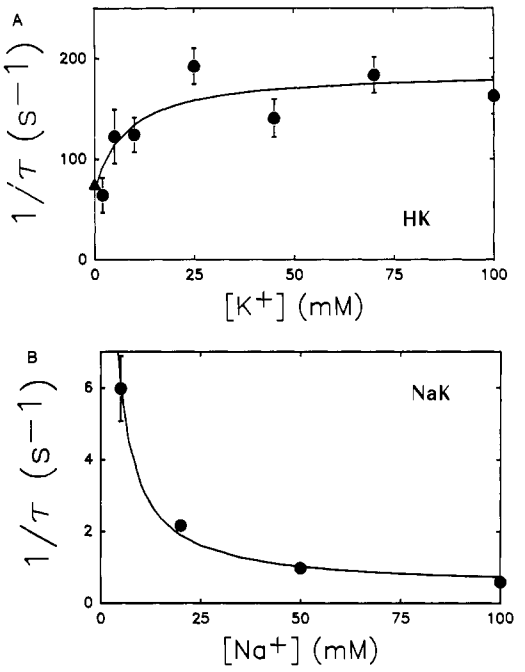

FIGURE 3: Ion dependence of reciprocal relaxation times. (A) $1/\tau$ is plotted againt $[K^+]$ (after mixing) for the H,K-ATPase at 22 °C (●). Each reactant solution contained 1 mM EDTA–Tris, and the KCl and choline chloride concentrations were varied to give the plotted value of $[K^+]$ and maintain the ionic strength constant at 200 mM. Otherwise, the reaction conditions were identical with those quoted in the legend to Figure 1. The value of $1/\tau$ plotted at $[K^+] = 0$ (▲) was measured by reversing the K^+ quench with Na^+ (see legend to Figure 2 and text). The means of from 4 to 16 traces are plotted. Vertical bars indicate the standard deviation in the $1/\tau$ values calculated for the individual traces, when SD exceeded the symbol size. The theoretical curve was calculated with eq 4 for $k_2 = 118$ s^{-1}, $K_K = 8$ mM, and $k_{-2} = 69$ s^{-1} ($n = 69$). (B) $1/\tau$ is plotted against $[Na^+]$ for the Na,K-ATPase at 22 °C. The NaCl and choline chloride concentrations were varied to give the final $[Na^+]$ plotted and keep $\mu = 200$ mM. Otherwise, the reaction conditions were those given in the legend to Figure 2. The theoretical curve was calculated with eq 4 for $k_{-2} = 0.44$ s^{-1} and $K_{Na} = 0.19$ mM ($n = 29$) with $k_2 = 320$ s^{-1} and $K_K = 10$ mM fixed at the values estimated from the 22 °C $1/\tau$ vs $[K^+]$ plot.

seems likely, because the drift is in the opposite direction when the K^+ quench of the Na,K-ATPase is reversed by Na^+ (Figure 2a). Another possibility, that cannot be excluded, is labeling of lysines other than Lys-501 (Xu, 1989) that might be slowly affected by K^+ binding. A competing zero- and first-order model with the base line offset was fit to the data. The zero-order correction is small. In the typical example shown, the zero-order rate (1.4 s^{-1}) accounts for less than 6% of the total fluorescence change. There was no obvious trend in the zero-order rate constants with $[K^+]$ or temperature, so subsequent analysis of K^+ quench data with both enzymes is restricted to the dominant and fast exponential phase of the reaction.

The K^+ quench of the H,K-ATPase under comparable experimental conditions is about half as fast (Figure 1b). The amplitude of the changes observed with the H,K-ATPase was also smaller. Figure 1b illustrates the signal to noise enhancement that can be obtained by summing individual traces. In practice, fitting the summed curve gave a smaller estimate of the error in the reciprocal relaxation time than fitting individual relaxation curves. The larger standard deviation in the mean of the individual estimates was used to draw the error bars in Figure 3. The precision of the reciprocal relaxation times and amplitudes measured with the H,K-ATPase (10–15%) is less than with the Na,K-ATPase (3–5%), because of the smaller fluorescence changes in the fluorescein-labeled H,K-enzyme.

Na^+ Reversal. No change in fluorescence could be detected when Na^+ was mixed with either FITC-labeled enzyme in the absence of K^+. Na^+ does reverse thes K^+ quench of the Na,K-ATPase. Figure 2b demonstrates that Na^+ also reverses the K^+-induced conformational change in the H,K-ATPase reported by fluorescein. The rate of reversal of the K^+ quench by Na^+ was measured by mixing NaCl with labeled enzyme preincubated in 10 mM KCl. Other details of the experiment are given in the figure legend.

Figure 2 shows the dramatic difference between the rates of the E_2K to E_1K transition in the Na,K-ATPase (a) and H,K-ATPase (b). Reversal of the conformational change in the Na,K-ATPase is followed by a relatively small and slow, linear increase in fluorescence. No significant drift was observed when Na^+ reversed the K^+ quench of FITC-labeled H,K-ATPase, but the amplitude of the fluorescence change is smaller and the experiments are shorter. Only the exponential component of the relaxation effect observed with the Na,K-enzyme depends monotonically on $[Na^+]$ and temperature.

Ion Dependence of Relaxation Times. Figure 3 compares the dependence of $1/\tau$ for the K^+ quench on K^+ concentration with the dependence of $1/\tau$ for the Na^+ reversal on Na^+ concentration.

Figure 3A shows that $1/\tau$ for K^+ quench of the H,K-ATPase depends hyperbolically on $[K^+]$ as predicted by eq 4. The estimated ordinate intercept is nonzero and is equal, within experimental error, to k_{-2} measured by reversing the K^+ quench with Na^+. The estimates of k_2, K_K, and k_{-2} recorded in Table I for the H,K-ATPase were obtained by fitting eq 4 to the combined K^+ quench and Na^+ reversal data. A maximum rate is also observed when K^+ quenches the fluorescence of fluorescein-modified Na,K-ATPase. However, the reverse reaction is so slow compared to the forward reaction that the ordinate intercept of $1/\tau$ vs $[K^+]$ plots was experimentally zero, and k_{-2} could not be estimated by fitting eq 4 to K^+ quench data for the Na,K-ATPase. The estimates of

Table I: Parameters Estimated from Rate Data[a]

enzyme	T (°C)	k_2 (s^{-1})	K_K (mM)	k_{-2} (s^{-1})	K_{Na} (mM)
NaK	22	320 ± 8	10 ± 1	0.44 ± 0.24	0.19 ± 0.02
	18.5	165 ± 8	6 ± 1	0.25 ± 0.07	0.11 ± 0.01
	15	165 ± 5	20 ± 3	0.16 ± 0.01	0.09 ± 0.01
	11.5	92 ± 2	10 ± 1	0.09 ± 0.03	0.08 ± 0.01
	8	45 ± 1	9 ± 1	0.04 ± 0.01	0.07 ± 0.01
HK	30	243 ± 26	8 ± 4	120 ± 17	<0.2
	22	118 ± 18	8 ± 5	69 ± 12	<0.2
	15	57 ± 12	17 ± 14	29 ± 6	<0.2
	8	27 ± 7	7 ± 8	11 ± 5	<0.2

[a] Estimate ± SD.

Table II: Parameters Estimated from Amplitude Data[a]

		K^+ quench		Na^+ reversal	
enzyme	T (°C)	$K_{0.5K}$ (mM)	ΔF_{max} (au)	$K_{0.5Na}$ (mM)	ΔF_{max} (au)
NaK	22	0.31 ± 0.05	−0.82	56.7 ± 6.6	0.84
	18.5	0.82 ± 0.09	−0.45	55.6 ± 5.4	0.66
	15	1.39 ± 0.30	−0.67	30.4 ± 4.5	0.56
	11.5	0.23 ± 0.03	−0.70	29.1 ± 4.8	0.42
	8	0.85 ± 0.07	−0.85	22.4 ± 9.7	0.49
HK	30	4.0 ± 1.4	−0.43	<5	0.33
	22	3.2 ± 1.1	−0.32	<5	0.17
	15	3.8 ± 1.1	−0.16	<5	0.08
	8	2.0 ± 1.2	−0.07	<5	0.06

[a] Estimate ± SD.

k_2 and K_K are reported in Table I.

Figure 3B shows the decrease in $1/\tau$ with increasing [Na^+] predicted by eq 4 for Na^+ reversal of the K^+ quench by competitive binding of the two ions to the E_1 form of the Na,K-ATPase. To reduce the number of estimated parameters, k_2 and K_K were fixed at the values derived from K^+ quench data when estimating the values of k_{-2} and K_{Na} reported in Table I from the dependence of $1/\tau$ on [Na^+]. No significant decrease in $1/\tau$ with increasing [Na^+] from 5 to 100 mM could be demonstrated for the H,K-ATPase. An upper estimate of K_{Na} consistent with this observation is given in Table I. The measurements of $1/\tau$ were combined with K^+ quench data at [K^+] = 0 to improve the estimates of k_{-2} reported for the H,K-ATPase in Table I.

Ion Dependence of Amplitudes. Since the maximum fluorescence change observed in a mixing experiment depends on the equilibrium distribution of labeled enzyme between the E_1 and E_2 conformers at the final reactant concentrations, the corrected amplitudes deduced from kinetic experiments contain the same information that would be obtained from a static, fluorometric titration. Figure 4 compares "kinetic" titrations of FITC-labeled enzyme with K^+ and Na^+.

Figure 4A is a kinetic titration of FITC-labeled H,K-ATPase with K^+. The shape of the titration curves observed with the Na,K-ATPase was similar, but the half-maximum amplitude is shifted to lower K^+ concentrations. Values of $K_{0.5K}$ and ΔF_{max} estimated for binding to a single class of specific sites are recorded in Table II.

Figure 4B is a kinetic titration of the Na,K-ATPase with Na^+. The estimates of $K_{0.5Na}$ and ΔF_{max} are given in Table II. No change in the amplitude of the Na^+ reversal of the H,K-ATPase with [Na^+] in the range 5–100 mM could be measured. An upper estimate of $K_{0.5Na}$ for the H,K-ATPase is given in Table II, and the mean amplitude of the Na^+ reversal at each temperature is reported in the ΔF_{max} column.

Temperature Dependence of Rate Constants. Plots of the logarithms of the rate constants against the reciprocal of the absolute temperature were linear. The activation energies

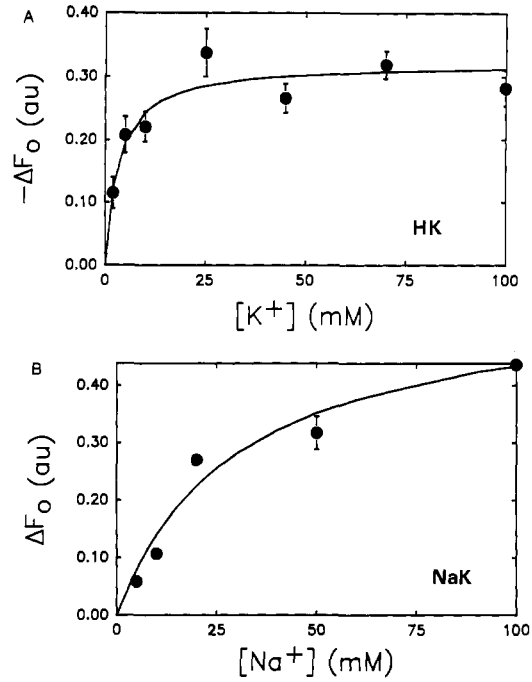

FIGURE 4: Ion dependence of amplitudes. The vertical bars indicate the standard deviation in the mean of the measured amplitudes. (A) Corrected fluorescence change (ΔF_0) is plotted against final [K^+] for the H,K-ATPase at 22 °C. Other details of the experiment are given in the legend to Figure 1. The theoretical line was calculated for binding to a single class of sites with $K_{0.5K}$ = 3 mM and ΔF_{max} = −0.32 au (n = 53). (B) Corrected fluorescence change is plotted against final [Na^+] for the Na,K-ATPase at 15 °C. Other experimental details are given in the legend to Figure 2. The theoretical line was calculated for $K_{0.5Na}$ = 30 mM and ΔF_{max} = 0.56 au (n = 37).

Table III: Derived Thermodynamic Parameters[a]

enzyme	constant	E_a (kcal/mol)	ΔH^* (kcal/mol)	ΔG^* (kcal/mol)	ΔS^* (eu)
NaK	k_2	21.3 ± 3.1	20.7 ± 3.1	13.9 ± 0.0[1]	23.0 ± 10.5
	k_{-2}	28.7 ± 1.9	28.1 ± 1.9	17.8 ± 0.3	34.9 ± 6.5
	K_c		−7.4 ± 3.6	−3.9 ± 0.3	−11.9 ± 12.3
HK	k_2	17.0 ± 0.3	16.5 ± 0.3	14.5 ± 0.1	6.8 ± 1.1
	k_{-2}	18.7 ± 1.8	18.1 ± 1.8	14.8 ± 0.1	11.2 ± 6.1
	K_c		−1.6 ± 1.8	−0.3 ± 0.1	−4.4 ± 6.2

[a] Estimate ± SD. ΔG^* and ΔS^* at 22 °C. The asterisk is (‡) for quasi-thermodynamic parameters derived from rate (k_2, k_{-2}) constants and the asterisk is (°) for standard changes at equilibrium (K_c).

calculated from the slopes of the Arrhenius plots and the thermodynamic parameters derived by transition-state theory are summarized in Table III. The uncertainties in the estimates of E_a result primarily from the use of different FITC-modified enzyme preparations on different days at different temperatures, rather than the precision of the kinetic measurements. The uncertainty in derived thermodynamic parameters was obtained from the total differential of the function, or the square root of the sum of the squares of the standard deviations in the quantities used in the calculation. Figure 5 compares the reaction coordinate diagrams of the two enzymes.

Discussion

Although the working hypothesis in most models for mammalian active transport is that the carrier can assume different conformations in which the ion binding sites are alternately exposed to opposite sides of the membrane, the precise nature of the conformational change that is postulated to explain transport remains largely a matter for conjecture. The principal objective of the study reported in this paper was to obtain

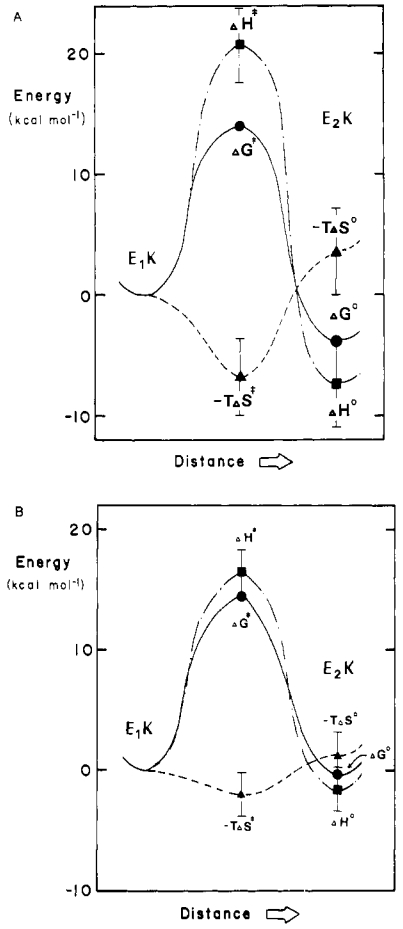

FIGURE 5: Reaction coordinate diagrams for conformational change reported by fluorescein. (A) Na,K-ATPase. (B) H,K-ATPase. Energy in kilocalories per mole is plotted against distance along the reaction coordinate. (—) Gibbs free energy change. (-·-) Enthalpy change. (---) Entropy change.

experimental data on the conformational change by measuring and comparing the temperature dependence of the conformational changes reported by fluorescein in two E_1E_2-type ATPases. Evidence ions cause the observed fluorescence changes by the mechanism in eq 1, evidence the conformational change reported by fluorescein could occur in the catalysis–transport cycle, and the implications of the thermodynamic analysis for the molecular mechanism of the conformational change are discussed in turn.

Mechanism of Ion Interaction with Enzymes. The mechanism by which ions trigger the observed fluorescence changes was reinvestigated, because the method of calculating standard energy changes and the reaction coordinate diagrams in Figure 5 assume k_2 and k_{-2} are the forward and reverse rate constants for the same reaction step.

(*A*) K^+ *Quench.* Figure 3A extends the experimental evidence for eq 1 by demonstrating the nonzero ordinate intercept, as well as the functional dependence of $1/\tau$ on $[K^+]$ with an unequivocal maximum rate, predicted by eq 4. The smaller amplitude of the K^+ quench observed with the H,K-ATPase (Table II, column 4) is qualitatively consistent with dramatically different distributions of the two enzymes between E_1K and E_2K at equilibrium, because ΔF_0 asymptotically approaches $[K_c/(K_c + 1)]\Delta F_{max}$ at high $[K^+]$. The variation of ΔF_{max} for the H,K-ATPase with temperature is consistent with greater sensitivity of the ratio $K_c/(K_c + 1)$ to changes in K_c when K_c is numerically near 1. The observed decrease in amplitude with temperature suggests that the transition from E_1K to E_2K in the H,K-ATPase is endothermic. The sign of ΔH° could not be definitely established from the variation of the rate constants with temperature (Table III). Estimation of k_2 and k_{-2} from eq 4 is complicated by their near-equivalence in the case of the H,K-ATPase.

Table IV: Comparison of Measured and Calculated Parameters[a]

		$K_{0.5K}$ (mM)		$K_{0.5Na}$ (mM)	
enzyme	T (°C)	measured	calculated	measured	calculated
NaK	22	0.3	0.02	57	69
	18.5	0.8	0.01	56	65
	15	1.4	0.02	30	25
	11.5	0.2	0.01	29	38
	8	0.9	0.01	22	48
HK	30	4	3		
	22	3	3		
	15	4	6		
	8	2	2		

[a] See text eq 5 and 6.

(*B*) *Na^+ Reversal.* The conclusion that Na^+ reverses the K^+ quench by binding exclusively to the E_1 conformer is strengthened by the demonstration in Figure 3B that $1/\tau$ for the Na,K-ATPase varies inversely with $[Na^+]$. The faster rate and smaller amplitude of the Na^+ reversal reaction of the H,K-ATPase permitted only upper estimates of K_{Na} and $K_{0.5Na}$. Consistent with failure to observe a Na^+ enhancement when Na^+ was mixed with either labeled enzyme minus K^+, the amplitude of the Na^+ reversal is experimentally less than or approximately equal, but opposite in sign, to the amplitude of the K^+ quench (Table II). Therefore, under the stated experimental conditions, the conformational equilibrium of both enzymes favors E_1 in the absence of K^+.

(*C*) *Consistency of Equilibrium and Kinetic Data.* Table IV compares the $K_{0.5}$ values estimated from equilibrium titrations of the amplitude (Table II) to values of $K_{0.5}$ calculated from kinetic data (Table I) with eq 5 and 6. There is quantitative agreement between equilibrium and kinetic estimates of $K_{0.5Na}$ for the Na,K-ATPase and between measured and calculated values of $K_{0.5K}$ for the H,K-ATPase. The kinetically measured $K_{0.5K}$ values for the Na,K-ATPase are less than the estimates of K_K (Table I) as predicted by eq 5, but the agreement is only qualitative. Measurement of the very tight binding predicted by the calculation of $K_{0.5K}$ from kinetic parameters is inherently difficult. The quantitative inconsistency reported in Table IV may be further exaggerated by the approximate nature of stopped-flow amplitude measurements. However, a comparable discrepancy (3–15-fold) between presumably more accurate static, fluorometric measurements of $K_{0.5K}$ and kinetic estimates of $K_{0.5K}$ for the Na,K-ATPase was reported by Karlish (1980).

Postulating an additional conformation (E_3K) of the Na,K-ATPase in equilibrium with E_2K in eq 1 would provide a reasonable explanation of the slower reverse rate of the sodium pump than the proton pump and might explain quantitative discrepancy between measured and calculated values of $K_{0.5K}$ only in the case of the Na,K-ATPase. However, the derived expressions for $1/\tau$ and $K_{0.5K}$ have the same form as eq 4 and 5 with k_{-2} replaced by $k_{-2}k_{-3}/(k_3 + k_{-3})$ in both equations. The practical consequence of this parallelism is that only an apparent reverse rate constant can be determined experimentally, and the discrepancy between equilibrium and kinetic estimations of $K_{0.5K}$ is numerically the same for the expanded reaction mechanism. Therefore, introducing additional unimolecular steps into the reaction mechanism will not make the amplitude and rate data more consistent. A higher order reaction step is unlikely, because eq 3 satisfactorily describes the fast phase of the observed fluorescence changes.

Comparison of Rate with Steps in the Catalytic Cycle. The best experimental evidence that conformational changes in the Na,K-ATPase are involved in transport comes from studies with fluorescent probes that permit simultaneous measurement of turnover, so the fluorescence changes can be correlated with events in the catalysis–transport cycle. A particularly lucid review of these studies and discussion of their possible significance has been published by Stein (1986). Unfortunately, the relationship between conformational changes reported by fluorescein and ion transport cannot be tested directly, because FITC-modified enzymes are inactive.

However, there is good agreement between the constants deduced in this study for Na^+ and K^+ dissociation from the E_1 form of the fluorescein-modified Na,K-ATPase and values reported for Na^+ and K^+ dissociation from intracellular sites on the erythrocyte Na,K-ATPase (Garay & Garrahan, 1973). At 22 °C, the value of K_K estimated from the rate of the K^+ quench of fluorescein fluorescence is 10 mM, and the value of K_{Na} estimated from the rate of the Na^+ reversal is 0.19 mM (Table I). Neither dissociation constant depends dramatically on temperature. Garay and Garrahan estimate from studies of Na^+ efflux from red blood cells at 37 °C that Na^+ and K^+ compete for binding to identical and noninteracting internal sites with K_{Na} = 0.19 mM and K_K = 9 mM. The remarkable similarity between ion dissociation constants derived from measurements of the rate of fluorescence changes in FITC-modified Na,K-ATPase and from measurements of transport by the sodium pump supports identification of the conformational change reported by fluorescein with a step in the transport mechanism.

Additional evidence that fluorescein reports the conformational change postulated to explain transport can be obtained by showing that fluorescence changes induced in another FITC-modified enzyme by transported ions are fast enough to participate in the transport mechanism. The conformational change from E_2K to E_1K is particularly important for comparisons of the Na,K-ATPase with the H,K-ATPase. In the case of the Na,K-ATPase, this reaction is too slow ($\tau_{1/2}$ several seconds) to be a step in active transport. However, ATP binding with $K_s > 100$ μM increases the k_{-2} reported by intrinsic fluorescence more than 2 orders of magnitude (Karlish & Yates, 1978). Low-affinity nucleotide acceleration of the E_2K to E_1K transition is a common feature of mechanistic proposals for the Na,K-ATPase. A corollary, since $K_m < 5$ μM (Cantley, 1981), is that ATP binds with different affinity to the E_1 and E_2 conformations of the Na,K-ATPase. This conclusion has been used to explain the complex dependence of the hydrolysis rate on ATP concentration (Smith et al., 1980; Moczydlowski & Fortes, 1981b) and is supported by studies of TNP-ATP binding (Moczydlowski & Fortes, 1981a) and vanadate inhibition (Cantley et al., 1978) of the Na,K-ATPase.

No change in intrinsic fluorescence, to compare with the changes reported by fluorescein, could be detected when K^+ was added to unlabeled H,K-ATPase (data not shown). However, the reverse rate, as well as the forward rate, of the conformational change in FITC-modified H,K-ATPase is fast enough to be an intermediate step in the pump mechanism. The forward (k_2 = 986 s^{-1}) and reverse (k_{-2} = 560 s^{-1}) rate constants for the E_1E_2 conformational change at 37 °C, calculated with the van't Hoff equation by using the data in Tables I and III, are both larger than first-order rate constants estimated from medium ^{18}O exchange measurements for terminal steps in the catalytic cycle. At 37 °C, the rate constant for phosphorylation of the H,K-ATPase from inorganic phosphate is 305 s^{-1}, and the rate constant for dephosphorylation of the enzyme is 457 s^{-1} (Faller & Diaz, 1989).

The ion normally exchanged for K^+ by the H,K-ATPase is H^+. However, there have been numerous reports that H^+ and Na^+ compete for the same site(s) on the enzyme (Wallmark & Mardh, 1979; Ljungstrom et al., 1984; Ray & Nandi, 1985; Polvani et al., 1989). The growing number of examples in which active transport can be coupled to either Na^+ or H^+ transport (Skulachev, 1985) has led Boyer (1988) to suggest that enzymes may transport protons as hydronium ions. Therefore, it is likely that fluorescein reports the E_1E_2 conformational change in the H,K-ATPase, as well as in the Na,K-ATPase, and that the rapid rate of the back-reaction of the proton pump is an intrinsic property of the H,K-enzyme.

The much faster rate of the E_2K to E_1K transition in the H,K-ATPase than in the Na,K-ATPase may explain repeated failure to observe a K^+-occluded form of the H,K-ATPase (Rabon et al., 1985; De Pont et al., 1985), and the rapid rate of passive Rb^+–Rb^+ exchange by the H,K-ATPase (Soumarmon et al., 1984) compared to the rate at which the Na,K-ATPase carries out Rb^+–Rb^+ exchange (Karlish & Stein, 1982). It is unnecessary for weak nucleotide binding to speed up the rate of the E_2K to E_1K transition in order for the E_1E_2 conformational change to participate in the mechanism of the H,K-ATPase. Consistent with this observation, no effect of K^+ on the enzyme's affinity for TNP-ATP could be demonstrated (Faller, 1990), and vanadate inhibition of the H,K-ATPase could not be explained by two states of a single site with different affinities for ATP (Faller et al., 1983). The rapid rate of the E_2K to E_1K conformational change in the H,K-ATPase suggests that a relatively stable chemical intermediate, $E_2(K)$, in which the transported ion is "occluded", or physically shut off from solvent, and acceleration of the "occluded" ion's release by nucleotide binding may not be essential features of an E_1E_2-type pump.

Transition-State Analysis. Information about the transition state can provide insight into the mechanism of a chemical reaction. For example, much tighter binding of vanadate than phosphate to the Na,K-ATPase (Cantley et al., 1978) and H,K-ATPase (Faller et al., 1983) is evidence E_1E_2-type ATPases catalyze phosphoryl transfer via a pentacoordinate intermediate (Knowles, 1980). The transition state of the protein conformational change believed to be involved in ion transport by E_1E_2-type ATPases does not appear to have been investigated previously. Since it is not feasible with the information currently available to calculate the energy changes expected for proposed models of the E_1E_2 conformational change and compare them with the observed enthalpy and entropy changes, suspected constituents of the transition state are being varied to find out which activation parameter is affected.

Figure 5 compares reaction coordinate diagrams for the E_1K to E_2K conformational changes in the sodium and proton pumps. Qualitatively, the energetics of the conformational transitions in the two enzymes are similar. First, the barrier to reaction in both cases is an increase in the enthalpy of activation. The magnitude of the enthalpy increase is consistent with net cleavage of several weak bonds accompanying formation of the transition state. Second, an increase in entropy between the ground and transition states of both enzymes reduces the reaction barrier. An indication of the importance this increase in randomness plays in accelerating the reaction rate can be obtained by noticing that if ΔS^* were zero, the forward and reverse rate constants for the Na,K-ATPase would have approximately the same numerical values, but the units

would be day^{-1} instead of s^{-1}. Enthalpy–entropy compensation may be related to changes in solvation and/or a structural change in the protein–metal complex (Etfink et al., 1983).

Quantitatively, there are significant differences between the sodium and proton pumps. Although the measured rates of the K^+ quench differ by only a factor of 2 (Figure 1), the enthalpy barriers in the forward direction (E_1K to E_2K) differ by 4.2 kcal mol^{-1}, or a factor of more than 10^3 in rate, that is compensated by a bigger (3-fold) activation entropy. The conformational change in the H,K-ATPase from E_2K to E_1K is 150 times faster than the corresponding change in the Na,K-ATPase. The initial and final stages of the H,K-enzyme are nearly equivalent energetically ($K_c \approx 2$). In sharp contrast, $K_c \approx 1000$ for the Na,K-ATPase. $\Delta H°$ estimated from rate measurements (Table III) indicates the transition from E_1K to E_2K in the H,K-ATPase is exothermic, but the decrease in amplitude with temperature (Table II) suggests that the reaction may be endothermic. The conformational change from E_1K to E_2K of the Na,K-enzyme is clearly exothermic. The E_2K state of the Na,K-ATPase is more ordered ($\Delta S° < 0$) than the E_1K state, which may be evidence the E_2K to E_1K transition of the Na^+ pump is slow because K^+ is occluded in the E_2K conformation. Formation of weak bond(s) and a more ordered complex could be explained by coordination of K^+ to ligands on the protein in E_2K, or it could mean that there is a change in the enzyme's shape that occludes the ion.

Registry No. Na^+, 7440-23-5; K^+, 7440-09-7; ATPase, 9000-83-3.

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