Effect of Na^+ and Nucleotide on the Stability of Solubilized Ca^{2+}-Free Ca-ATPase from Scallop Sarcoplasmic Reticulum†

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ABSTRACT: In membranous scallop sarcoplasmic reticulum, the alkali metal cations Na^+ and K^+ and nucleotide together promote dimer formation by the Ca^{2+}-free Ca-ATPase and stabilize the enzyme activity [Kalabokis, V. N., Bozzola, J. J., Castellani, L., & Hardwicke, P. M. D. (1991) *J. Biol. Chem. 266*, 22044–22050]. The dependence of stabilization of the Ca^{2+}-free membranous scallop Ca-ATPase on Na^+ concentration does not show saturation and may involve several superimposed effects. In order to assess the contribution of dimer toward stabilization, i.e., determine the relative importance of intra- and intermolecular effects on stabilization, the influence of varying Na^+ concentration and nucleotide on the decay of enzyme activity of the Ca^{2+}-free detergent-solubilized Ca-ATPase was studied. Loss of enzyme activity on removal of Ca^{2+} with EGTA was associated with loss of capacity for phosphorylation by ATP, a Ca^{2+}-dependent function. Stabilization of the soluble Ca^{2+}-free enzyme by Na^+ showed major differences from that seen with the membranous enzyme. The extent of stabilization of the Ca^{2+}-free soluble enzyme by Na^+ showed clear saturation with increasing Na^+ concentration. In contrast to the Ca^{2+}-free membranous enzyme, which is inactivated at pH 7.0 with biphasic first-order kinetics, loss of enzymatic function by the solubilized Ca-ATPase at pH 6.92, 0 °C, followed monophasic first-order kinetics. Investigation of the aggregational state of the Na^+-stabilized, Ca^{2+}-free soluble enzyme by gel permeation chromatography showed that it was monomeric, and this may be related to the differences between the effects of Na^+ on the membranous and soluble systems, since nonsolubilizing levels of $C_{12}E_8$ (below the critical micelle concentration) did not affect the decay of the enzyme activity of the membranous scallop Ca-ATPase observed in the absence of detergent (Kalabokis et al., 1991). By carrying out the incubations at constant ionic strength, it was found that the slowing of the inactivation rate of the Ca^{2+}-free soluble scallop Ca-ATPase by Na^+ was not due to purely ionic strength effects, but was consistent with a simple kinetic model in which binding of Na^+ to a saturable, weak binding site on the Ca^{2+}-free scallop Ca-ATPase of $K_d \approx 3$ mM mediates stabilization by the ion. AMP-PCP decreased the rate constant for inactivation of the Na^+-free, Ca^{2+}-free Ca-ATPase, but did not affect the decay of the Na^+-liganded form or the affinity of the Ca^{2+}-free enzyme for Na^+; i.e., Na^+ and nucleotide act independently. Nucleotide was far less effective in stabilizing the Ca^{2+}-free solubilized enzyme than the Ca^{2+}-free membranous scallop Ca-ATPase, and this is probably explained by the greater opportunity for intermolecular contacts, and therefore dimer formation, by Ca-ATPase located in the SR membrane.

A dimeric arrangement of Ca-ATPase molecules has been observed in sarcoplasmic reticulum from rabbit skeletal and scallop adductor muscles under conditions where the Ca^{2+}-binding sites on the enzyme are empty (e.g., Castellani et al. (1985) and Taylor et al. (1986)). In the native membranes, the Ca-ATPase can also form other types of lattices with only a single Ca-ATPase molecule in each unit cell in the presence of Ca^{2+} or lanthanides or when treated with $Cr^{III}ATP$ (Dux et al., 1985) and in the phosphorylated states (Hardwicke & Bozzola, 1989). Previous work (Kalabokis et al., 1991) has shown that there is an associative relationship between the dimeric form of organization of the Ca-ATPase in membranous vesicles and long-term stabilization of the Ca^{2+}-free state. Saturation of the Ca^{2+}-binding sites of the Ca-ATPase disrupts the dimeric lattice (Dux et al., 1985; Castellani et al., 1989; Kalabokis et al., 1991). Sagara and co-workers (1992) have recently found that thapsigargin, which reacts with the Ca^{2+}-free state and prevents Ca^{2+} binding, allows induction of dimer crystals in rabbit SR by decavanadate even when Ca^{2+} is present in the medium, thereby emphasizing the relationship of the dimer structure to the Ca^{2+}-free (E_2) state of the enzyme.

The dependence of stabilization of the Ca^{2+}-free membranous scallop SR on Na^+ and K^+ concentration shows three phases and is difficult to interpret (Kalabokis et al., 1991). In order to begin an analysis of the mechanism of action of dimer-promoting stabilizing agents, it is necessary to separate out as far as possible effects due to intermolecular interactions between neighboring Ca-ATPase molecules from changes in the intrinsic stability of the isolated molecules produced by ligand binding per se. Thus, a study has been made of the stabilization of the Ca^{2+}-free $C_{12}E_8$[1]-solubilized scallop Ca-ATPase by alkali metal cation and nucleotide, which induce dimer formation in the Ca^{2+}-free membranous scallop enzyme. Replacement of phospholipid by detergent molecules on solubilization of the Ca-ATPase leads to destabilization of the enzyme (e.g., de Foresta et al. (1989)), and detergent-solubilized Ca-ATPase from rabbit SR loses its activity rapidly when the Ca^{2+}-binding sites on the enzyme are empty (see,

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e.g., Moller et al. (1980), Martin et al. (1984), and McIntosh and Ross (1985)).

It was found that, at pH 6.9–7.0 (0 °C), the dependence of inactivation of the solubilized scallop Ca-ATPase system on Na^+ concentration was much simpler than that of the membranous Ca-ATPase and could be fit to a model in which binding sites on the Ca^{2+}-free solubilized Ca-ATPase for Na^+ mediate stabilization by that ion. Under these conditions, loss of enzyme activity was associated with loss of capacity of the Ca-ATPase for phosphorylation by ATP, a function dependent on the integrity of the Ca^{2+}-binding sites. Hydrodynamic experiments indicated that the stabilized soluble Ca^{2+}-free enzyme was monomeric under the conditions of the inactivation studies. Differences in behavior between the soluble and membranous Ca-ATPase were due to disruption of the membrane structure by detergent, since a subcritical micellar concentration of $C_{12}E_8$ that does not solubilize the enzyme did not alter the decay kinetics of membranous Ca^{2+}-free Ca-ATPase seen in the absence of detergent.

EXPERIMENTAL PROCEDURES

Deep sea scallops (*Placopecten magellanici*) were obtained from the Marine Biology Laboratory (Woods Hole, MA). Native scallop FSR vesicles and deoxycholate-extracted vesicles were prepared as described previously (Kalabokis & Hardwicke, 1988). $C_{12}E_8$-solubilized, partially delipidated Ca-ATPase was made by affinity chromatography on Reactive Red as previously reported (Coll & Murphy, 1984; Kalabokis & Hardwicke, 1988), and the solubilized Ca-ATPase typically contained 6–8 mol of phospholipid per mole of protein. The solubilized material was desalted by passage through a column of Sephadex G.50 (fine) run in 0.02% (w/v) (0.37 mM) $C_{12}E_8$, 20% (v/v) glycerol, 1 mM $CaCl_2$, and 5 mM MOPS–Tris, pH 7.0, and concentrated with an Amicon Diaflo apparatus. After spectrophotometric determination of the protein concentration using an extinction coefficient at 280 nm of 1.27 cm^2 mg^{-1} (Hardwicke & Huvos, 1989), DTT was added to 5 mM and the preparation stored on ice. The $C_{12}E_8$ concentration was increased to 2 mM in the subsequent experiments. The starting activity of the preparations was usually ~5 μmol of ATP hydrolyzed min^{-1} mg^{-1} at pH 7.2, 25 °C. Organic phosphorus was determined by the method of Bartlett (1959). When necessary, protein concentrations were determined by the method of Bensadoun and Weinstein (1976), assuming a color yield of 1.08 relative to bovine serum albumin (Hardwicke & Huvos, 1989). Rabbit SR was prepared as described previously (Hardwicke, 1976), and solubilized rabbit Ca-ATPase was made exactly as the scallop enzyme.

Determination of Ca^{2+}-activated MgATPase activity of the solubilized, delipidated enzyme used a coupled assay run at 25 °C, pH 7.0, in the absence of added lipid. The standard assay mixture contained 0.1 M KCl, 2 mM $C_{12}E_8$, 1.05 mM $CaCl_2$, 1 mM EGTA-Na, 5 mM $MgCl_2$, 5 mM ATP, 1 mM phosphoenolpyruvate trisodium salt, and 100 mM MOPS-Na, pH 7.0, plus 0.26 mM β-NADH and the coupling enzymes, pyruvate kinase and lactate dehydrogenase, which were always present in at least 30-fold greater activities than the assayed Ca-ATPase activity (Kalabokis & Hardwicke, 1988). pH–activity profiles were determined at 10 and 25 °C using PIPES, TES, and Tris to cover the pH range 6–9, correcting for the temperature dependence of the ionization constants. In studies of the effect of different conditions on the loss of activity by the Ca^{2+}-free enzyme, the ligands to be tested were added to the desalted enzyme in 2 mM $C_{12}E_8$ on ice, and the inactivation process was started by addition of an equal volume of an ice-cold solution of identical composition containing EGTA–Tris to give a final concentration of 40 mM EGTA, with Ca-ATPase protein at 1.5 mg mL^{-1}. Samples were taken at timed intervals, and the Ca^{2+}-activated ATPase was then assayed. The solubilized enzyme was stable in the presence of 1 mM Ca^{2+} at all of the pH values examined for periods of time comparable to the duration of the inactivation experiments examined, and loss of activity during the incubations was due to the low concentration of Ca^{2+} produced by the addition of EGTA. In order to determine the type of inactivation kinetics, the decay process was usually followed for at least 4 half-lives. Data were fit to multiexponential first-order decays by the simplex algorithm (Nelder & Mead, 1965), using the MEDAS program from EMF Software (Baltimore, MD).

Phosphorylation of the Soluble Ca-ATPase. Steady-state levels of the phosphorylated intermediate formed from ATP in the presence of Ca^{2+} were determined. Samples (60 μL) of the solubilized enzyme were taken from the inactivation incubation mixture containing EGTA at timed intervals and added to 400 μL of ice-cold 0.2 mM [γ-^{32}P]ATP, 0.1 M KCl, 2 mM $C_{12}E_8$, 5 mM $MgCl_2$, 16 mM $CaCl_2$, and 50 mM MOPS-Na, pH 7.0. The reaction was halted after 30 s by the addition of 460 μL of ice-cold 1 M trichloroacetic acid and 8 mM H_3PO_4. The precipitated protein was then washed four times with ice-cold 125 mM perchloric acid and 4 mM H_3PO_4 and dissolved in 4% (w/v) SDS, 0.2 M NaOH, 5% (w/v) Na_2CO_3, and 5 mM Na_2HPO_4 at 50 °C (Lund & Moller, 1988) for scintillation counting and protein determination. For controls for the effectiveness of the washing procedure (blanks), the protein was added immediately after the trichloroacetic acid had been mixed with the phosphorylation medium. Phosphorylation levels before incubation with EGTA were similar to those obtained by Coll and Murphy (1984) with purified rabbit solubilized Ca-ATPase made by the Reactive Red procedure (5–6 nmol of P (mg of protein)$^{-1}$).

Gel permeation HPLC was used to examine the aggregational state of the solubilized enzyme under conditions as close as possible to those used in the inactivation experiments. The runs were carried out in the cold room using a 600 × 7.5 mm TSK SW 3000 column. All column media contained 2 mM $C_{12}E_8$ in 20% (v/v) glycerol and 20 mM MOPS–Tris, pH 7.0, with or without additional NaCl, $CaCl_2$, or EGTA. The column was calibrated with thyroglobulin, ferritin, aldolase, bovine serum albumin, ovalbumin, and carbonic anhydrase run with 0.1 and 0.4 M NaCl present. The eluant was monitored at 222 nm. V_t and V_0 for the column were determined from the elution positions of EGTA and blue dextran.

CD spectra were recorded with an Aviv 62DS circular dichroism spectropolarimeter fitted with a thermostated cuvette holder. Data were obtained at 0.5-nm intervals and stored in an AT&T 63WGS computer.

RESULTS

Preliminary Observations. The time course of the coupled assay for Ca^{2+}-activated MgATPase activity carried out at

[1] Abbreviations: FSR, fragmented sarcoplasmic reticulum; TES, 2-[*N*-[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, 5′-adenylylimidodiphosphate; $C_{12}E_8$, dodecyl-octa(ethylene glycol)monoether; DTT, dithiothreitol.

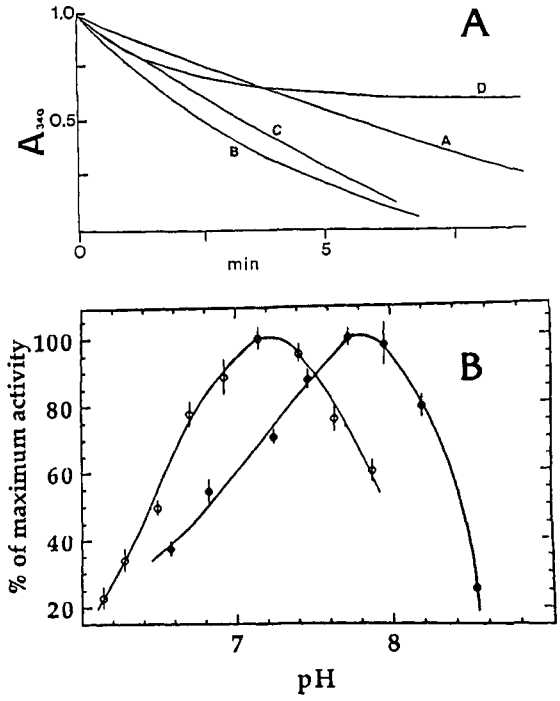


FIGURE 1: Effect of pH on the time course of the ATPase assay and dependence of Ca^{2+}-activated ATPase activity on pH for the solubilized ATPase. (A) Assays of the enzyme activity were carried out at 25 °C as described in the Experimental Procedures. The same amount (5 μg) of $C_{12}E_8$-solubilized Ca-ATPase was used for each assay. Curve A, pH 6.4; curve B, pH 6.9; curve C, pH 7.4; curve D, pH 7.7. (B) At 25 °C (○), the temperature of the routine enzyme assays, a pH optimum at pH 7.2 was obtained. Each point is the average of four determinations. (There was a shift in the pH optimum from 7.2 to 7.8 when the temperature of the assay was changed from 25 to 10 °C (●), near the ambient temperature for deep sea scallops.)

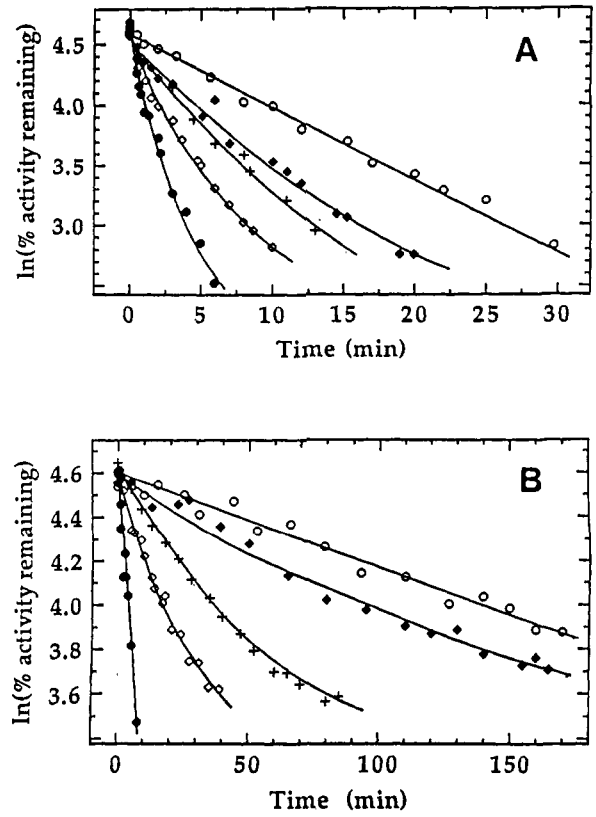


FIGURE 2: Effect of pH on the stability of Ca^{2+}-free solubilized scallop Ca-ATPase. This was examined over the pH range 6.9–8.3 at 0 °C in the absence (A) and presence (B) of 0.1 M NaCl. The zero time activity was 4.8 μmol min^{-1} (mg of protein)$^{-1}$. Note the longer time scales for inactivation in the presence of 0.1 M Na^+. The pH values have been corrected for temperature dependence of the ionization constants (pH of neutrality is 7.47 at 0 °C): (○) pH 6.92; (◆) pH 7.28; (+) pH 7.45; (◇) pH 7.95; (●) pH 8.3.

pH 7.4 at 25 °C showed a loss of linearity after approximately 1 min, similar to the effect reported by Martin et al. (1984) with rabbit Ca-ATPase and ascribed to the formation of an inactive Ca^{2+}-free intermediate that accumulates during the cycling of the enzyme (Figure 1A). At higher pH (e.g., 7.7), the lack of linearity was even more pronounced. However, at lower pH the linearity improved dramatically. At pH 6.9, the assay time course was more linear than at pH 7.4, and lowering the pH of the assay medium further to 6.4 gave time courses that were essentially linear for the first 5 min of the reaction. The pH dependence of the Ca^{2+}-activated ATPase activity of the solubilized enzyme is shown in Figure 1B. Using the initial slopes of the assay plots as the measure of ATPase activity, a symmetrical profile was obtained at 25 °C, with a pH optimum at 7.2 (Figure 1B). The enzyme has a higher activity at pH 6.9 than 7.7, and the greater linearity of the time course at the lower pH cannot be explained by the enzyme passing less frequently through the Ca^{2+}-free intermediate and probably reflects a greater inherent degree of stability at the lower pH.

Effect of Na^+ on the Stability of the Ca^{2+}-Free Solubilized Scallop Ca-ATPase. (1) Stability in the Absence of Na^+. The stability of the Ca^{2+}-free solubilized Ca-ATPase was first examined in the pH range 6.9–8.3 at 0 °C in the absence of NaCl (Figure 2A). Choline chloride was added to 0.4 M to make the experiments comparable with studies where the Na^+ concentration was varied at a constant total salt concentration of 0.4 M (see below). EGTA was added to empty the Ca^{2+}-binding sites on the enzyme. At pH 6.92, in the absence of Na^+, the inactivation process followed monophasic first-order kinetics for a substantial time (3–4 half-lives). As the pH of the inactivation incubation medium was raised, the first-order plots became increasingly nonlinear (Figure 2A) and the overall stability of the Ca^{2+}-free enzyme decreased, indicating that additional deprotonated and more unstable forms of the Ca^{2+}-free active enzyme were contributing to formation of the inactive enzyme: i.e., there was a complex situation, with multiple labile species in the incubation medium. Overall, these results were consistent with the increased linearity of the ATPase assay time courses at lower pH described above.

Samples were taken from the inactivation incubation medium in the absence of Na^+ or K^+ at pH 6.9 at timed intervals and tested for the steady-state level of phosphorylated intermediate that could be formed from ATP in the presence of Ca^{2+} (Figure 3). The level of phosphorylation obtained declined during the course of the incubation at a rate comparable to that of the loss of enzyme activity. Thus, the inactivation mechanism involves loss of capacity for phosphorylation by ATP.

(2) Stability in the Presence of Na^+. Data from inactivation time courses at the different pH values were collected in the presence of 0.1 M NaCl, using choline chloride to balance the total salt concentration to 0.4 M, and plotted according to first-order kinetics (Figure 2B). Note the much longer time scales for inactivation in the presence of 0.1 M NaCl compared to the corresponding decays in the absence of Na^+. Thus, Na^+ stabilized the Ca^{2+}-free enzyme at all of the pH values used and improved the degree of stabilization produced by lowering the pH; for example, in the absence of Na^+, the time needed to lose 50% of the activity at pH 8.3 was about one-seventh that at pH 6.92, while in the presence of 0.1 M Na^+

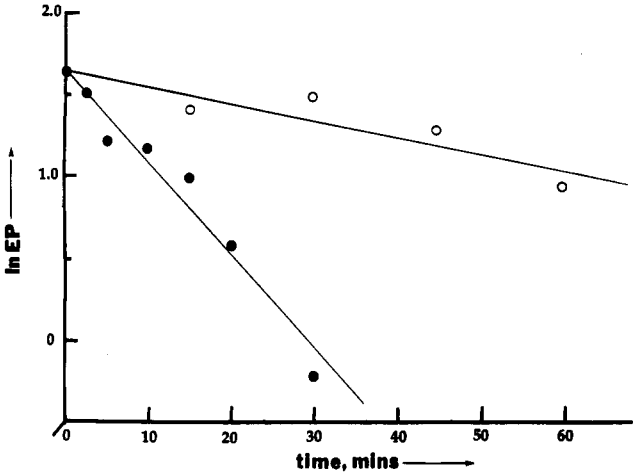
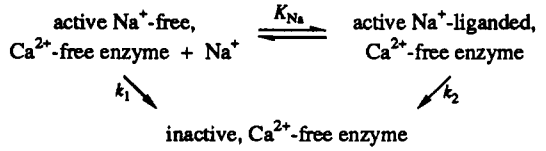


FIGURE 3: Dependence of phosphorylation level obtained with ATP on time of exposure to EGTA in the absence and presence of 0.1 M NaCl at pH 6.9. The change with time of incubation in EGTA in the steady-state phosphorylation level of solubilized scallop Ca-ATPase that could be obtained with ATP and Ca^{2+} was followed exactly as for time courses of the Ca^{2+}-activated Mg-ATPase activity, except that the samples taken at timed intervals from the incubation mixture in EGTA were added to phosphorylation medium containing [γ-^{32}P]ATP, as descirbed in Experimental Procedures. Data fit to a first-order plot by linear least mean squares. The phosphorylation level (EP) is in nmol of P (mg of protein)$^{-1}$. (●) No NaCl in EGTA incubation medium; (○) 0.1 M NaCl in EGTA incubation medium.

the enzyme was approximately 30 times more stable at pH 6.9 than at pH 8.3. At pH 6.9, the decline in the capacity of the Ca^{2+}-free enzyme for phosphorylation from ATP was slowed by 0.1 M NaCl (Figure 3).

The dependence of stabilization on Na^+ concentration was studied at several different pH values. Stabilization of the Ca^{2+}-free solubilized Ca-ATPase by Na^+ was a saturable phenomenon in the pH range 6.9–8, the degree of stabilization as measured by the time taken to lose a given fraction of the starting activity tending toward a maximum value, which depended on the pH and the presence or absence of other ligands. The decay of enzyme activity of the Ca-ATPase at pH 6.92 following chelation of Ca^{2+} showed monophasic first-order kinetics at all Na^+ concentrations studied. While first-order plots of decays at higher pH values tended to become more linear as the Na^+ concentration was raised, the kinetics were usually equivocal. The data at pH 6.92 were fit to a very simple kinetic model in which Na^+ mediates its stabilizing effect by binding to a single site on the Ca^{2+}-free Ca-ATPase, with active Na^+-free, Ca^{2+}-free Ca-ATPase in rapid equilibrium with active Ca^{2+}-free enzyme with bound Na^+. Both species decay irreversibly to give inactive enzyme, the form with bound Na^+ decaying more slowly than the Na^+-free form, and inactivation is overall a monophasic first-order process:



It can be shown that (see the Appendix)

$$\frac{1}{\tau} = \frac{1}{\tau_{min} K_{Na}[Na^+]} + \frac{1}{\tau_{max}} \quad (1)$$

where $\tau_{max} = (\ln 2)/k_2$ corresponds to the half time for inactivation at saturating Na^+ concentration, $\tau_{min} = (\ln 2)/k_1$ is the half time for inactivation in the absence of Na^+, and $\tau = (\ln 2)/k$ is the observed time for one-half inactivation.

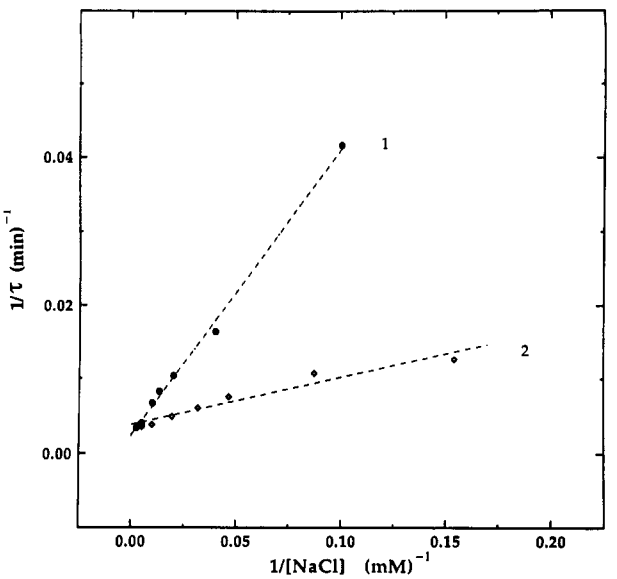


FIGURE 4: Analysis of data. Decays in the absence of AMP-PCP at pH 6.92 were monophasic first-order processes at all Na^+ concentrations, as were inactivations in the presence of 0.75 mM AMP-PCP even at alkaline pH values. Thus, the simple kinetic scheme described in the text could be applied to the data obtained under such conditions. $\tau_{0.5}$ values for decays at varying Na^+ concentrations were determined from inactivation time courses, and the data were fit to eq 1 by least mean squares analysis. (1) At pH 6.92 (correlation coefficient 0.9986); (2) in the presence of 0.75 mM AMP-PCP at pH 7.45 (correlation coefficient 0.9710).

While more complex schemes involving multiple binding sites can be considered, the data at pH 6.92 fit the simple model well (Figure 4). The rate constant for inactivation of the Ca^{2+}-free Ca-ATPase lacking bound Na^+, k_1 (0.0726 min^{-1}), was ~40-fold greater than k_2 (0.0017 min^{-1}), the rate constant for loss of activity when Na^+ was bound, indicating a substantial stabilization of the Ca^{2+}-free enzyme by Na^+. On the basis of this model, the affinity of the site mediating stabilization by Na^+ was ~0.3 mM^{-1}. It should be noted that because of the difference in the magnitudes of k_1 and k_2, the concentration of Na^+ giving one-half saturation does not in general correspond to the concentration giving one-half the maximal half-life (see the Appendix). This is why Na^+ concentrations well above 3 mM are needed for significant stabilization of the soluble Ca^{2+}-free Ca-ATPase.

When incubations of scallop FSR with EGTA were carried out as described previously (Kalabokis et al., 1991) but in the presence of 10 μM $C_{12}E_8$, which is below the critical micelle concentration (Tanford et al., 1977) and does not solubilize the membranes, the time courses for inactivation at pH 6.9 with and without 0.1 M NaCl were the same as those in the absence of detergent. Thus, the differences between the inactivation kinetics of the $C_{12}E_8$-solubilized and membranous enzymes are primarily due to disruption of the normal membrane structure in the solubilized system.

Effect of Na^+ on the CD Spectrum of the Solubilized Ca^{2+}-Free Scallop Ca-ATPase. The effect of Na^+ on the changes in the secondary structure content of the solubilized scallop Ca-ATPase caused by lack of occupation of the Ca^{2+}-binding sites was investigated using circular dichroism (Figure 5). Averaged CD spectra of the solubilized scallop Ca-ATPase (curve A) indicated that the enzyme possessed 63% α-helix, 16% β-sheet, 3% turn, and 18% random coil in a medium of 20% (v/v) glycerol, 2 mM $C_{12}E_8$, 1 mM $CaCl_2$, and 20 mM MOPS–Tris, pH 7.0, at 20 °C, as estimated by the method of Chang et al. (1978). A lower value for the α-helical content

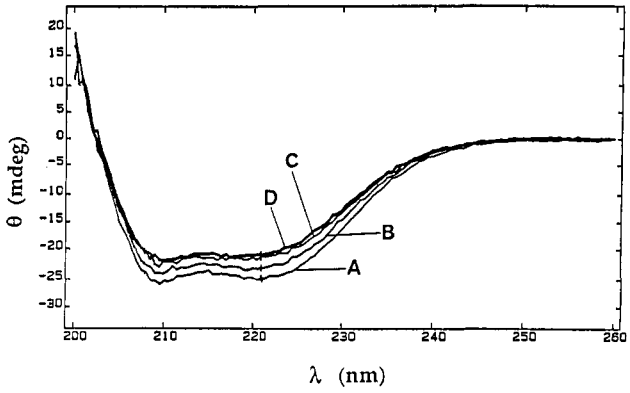


FIGURE 5: Circular dichroic spectra of the solubilized scallop Ca-ATPase. All spectra were recorded at 20 °C with 0.14 mg mL^{-1} Ca-ATPase protein. Each curve represents the average of three spectra after subtraction of the solvent base lines. Bars show the standard deviation at 222 nm. In addition to 20% (v/v) glycerol, 2 mM $C_{12}E_8$, and 20 mM MOPS–Tris, pH 7.0, the solutions contained the following: (A) 1 mM $CaCl_2$ and 0.1 M NaCl; (B, C) 10 mM EGTA and 0.1 M NaCl; (D) 10 mM EGTA and 0.1 M choline chloride. Spectrum A remained unchanged throughout the experiment. Spectra B and C were recorded 15 min and 4 h, respectively, after the Ca-ATPase was mixed with EGTA. Spectrum D was recorded immediately after addition of EGTA to the Ca-ATPase (within ~3 min).

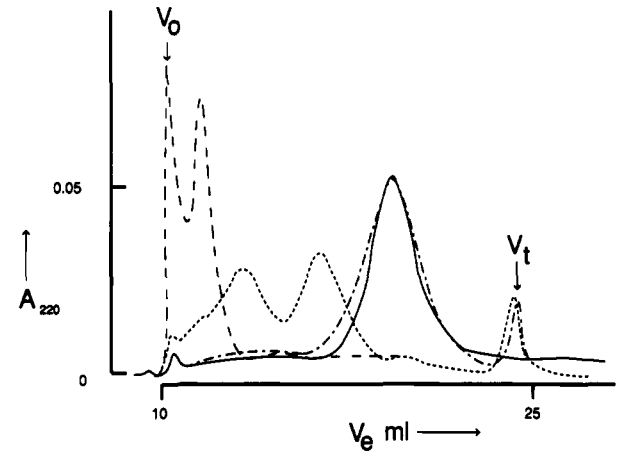


FIGURE 6: HPLC size exclusion chromatography of the solubilized scallop Ca-ATPase. Chromatography was carried out on a 600 × 7.5 mm TSK SW 3000 column at 4 °C. All media contained 20% (v/v) (2.74 M) glycerol, 2 mM $C_{12}E_8$, and 20 mM MOPS–Tris, pH 7.0. (—) Chromatography of 6 μL of 2 mg mL^{-1} (18.2 μM) Ca-ATPase in the presence of 1 mM Ca^{2+} and 0.4 M NaCl. (·····) EGTA was added to the sample to 40 mM in the presence of 0.5 M NaCl, 8 μL of the sample (1.6 mg mL^{-1}) was injected, and the column was run in a medium containing 0.4 M NaCl as well as 1 mM EGTA. The peak eluting at V_t represents the excess EGTA in the sample over the EGTA in the elution buffer. (···) Sample treated with EGTA in the presence of 0.5 M NaCl and allowed to incubate overnight (18 h). (- - -) 40 mM EGTA was added to chelate Ca^{2+}, and the sample was immediately loaded and run on the column equilibrated with a medium containing 1 mM EGTA and no Na^+.

(50%) was obtained by the method of Greenfield and Fasman (1969). This lower value is close to the α-helical content of 52% predicted from the amino acid sequence of the rabbit skeletal muscle protein (MacLennan et al., 1985; Brandl et al., 1986) and close to the α-helical content of 51% found for the rabbit Ca-ATPase by Williams et al. (1986) using Raman spectroscopy.

The presence of 0.1 M NaCl in addition to 1 mM $CaCl_2$ did not modify the CD spectrum seen with 1 mM $CaCl_2$ alone. Spectra in the presence of Ca^{2+} remained unchanged for several hours. When solubilized scallop Ca-ATPase was placed into a medium containing 0.1 M NaCl and 10 mM EGTA, the spectrum shifted from A in the course of 15 min to a form B, indicating decreased helical content. The helical content continued to decrease, until after 4 h there was no further change (Figure 5, curve C). The total change was ~5%. In the absence of Na^+ at the same ionic strength with 0.1 M choline chloride replacing the NaCl, the entire loss of secondary structure produced by chelation of Ca^{2+} with 10 mM EGTA took place within 4 min (curve D). Thus, the presence of even moderate amounts of NaCl (0.1 M) greatly slows the structural changes caused by removal of Ca^{2+} from its binding sites on the Ca-ATPase.

Effect of Na^+ on the Aggregational State of the Scallop Ca-ATPase under the Conditions of the Inactivation Experiments. The aggregational state of the solubilized enzyme was examined by HPLC gel permeation (size exclusion) chromatography under conditions close to those used in the kinetic studies of the inactivation process described above. le Maire et al. (1986) found that it is not possible to use water-soluble proteins to accurately calibrate size exclusion columns run in nonionic detergent for use with integral membrane proteins, such as the Ca-ATPase. The range of integral membrane proteins available for calibration is extremely restricted, and even with those the R_s vs $erf^{-1}(1 - K_d)$ plots are not linear (le Maire et al., 1986). The rabbit and scallop enzymes migrate with identical mobilities on SDS gels (Castellani & Hardwicke, 1983), and the size and shape of the rabbit and scallop molecules from image reconstruction studies of electron micrographs are very similar (Castellani et al., 1985; Taylor et al., 1986). Thus, we directly compared the solubilized scallop enzyme with the rabbit Ca-ATPase, which has been very well characterized by both the sedimentation and gel filtration hydrodynamic methods (Dean & Tanford, 1978; le Maire et al., 1978; Andersen et al., 1980; Martin, 1984; le Maire et al., 1986; Moller et al., 1988). In the presence of 1 mM Ca^{2+} and 0.4 M NaCl, gel permeation chromatography (Figure 6) showed the active enzyme preparation eluting as a single symmetrical peak. Solubilized rabbit Ca-ATPase chromatographed in the same solvent also eluted as a single peak at the same position. Several studies have shown that the rabbit Ca-ATPase is monomeric under similar conditions (Dean & Tanford, 1978; le Maire et al., 1978; Andersen et al., 1980; Martin, 1984; Moller et al., 1988). The above results therefore strongly suggest that the solubilized Ca^{2+}-saturated $C_{12}E_8$-solubilized scallop enzyme is also monomeric in 2 mM $C_{12}E_8$. [It should be noted that Ca^{2+} is extremely effective in disrupting the dimer structures seen in stabilized Ca^{2+}-free rabbit and scallop FSR (Dux et al., 1985; Kalabokis et al., 1991).] After addition of 40 mM EGTA to the solubilized scallop enzyme in 1 mM $CaCl_2$ and 0.5 M NaCl, followed by chromatography in a medium containing 1 mM EGTA and 0.4 M NaCl, the Ca-ATPase eluted with the same partition coefficient as when 1 mM $CaCl_2$ was present. Little aggregated material was formed, and the sample lost only 11% of its activity during the chromatography, on the basis of the rate constants. Therefore, the active solubilized Na^+-stabilized Ca^{2+}-free scallop Ca-ATPase is monomeric. Under essentially identical conditions in the membrane, the Ca-ATPase subunits are very efficiently converted into the dimer ribbon form (Kalabokis et al., 1991). Thus, there is a major difference between the behavior of the Ca^{2+}-free Ca-ATPase when it is embedded in the membrane with its full lipid complement and the solubilized, partially delipidated enzyme with bound detergent. When the sample in 40 mM EGTA, 1 mM $CaCl_2$, and 0.5 M NaCl was allowed to incubate further on ice for 18 h, the elution profile was

altered, with the appearance of 2–3 peaks of larger Stokes radius (R_s) than the monomeric ATPase, while the preparation lost 85% of its activity. Lastly, 40 mM EGTA was added to the solubilized enzyme in 1 mM $CaCl_2$ and 0.1 M NaCl, and the sample was then immediately chromatographed in a medium containing 1 mM EGTA but no NaCl, so that the Na^+ concentration in the medium surrounding the Ca-ATPase fell to very low values soon after the sample entered the column. This material became rapidly and extensively aggregated and lost 99% of its enzyme activity by the time that it eluted from the column. Thus, loss of activity is associated with aggregation of the solubilized Ca^{2+}-free scallop Ca-ATPase. Tests of material from the peaks of aggregated Ca-ATPase showed that it was inactive.

It is important to point out that the dimeric Ca-ATPase previously described in membranous scallop Ca-ATPase (Castellani et al., 1985) represents an extremely specific structure, with well-defined, precise contacts between the subunits within the lipid bilayer region and in the form of a bridge or arch on the outer (cytoplasmic) surface of the membrane. Material eluting with a Stokes radius (R_s) compatible with a complex of two associated Ca-ATPase molecules does not necessarily represent the dimer observed in the stabilized Ca^{2+}-free membranes, and any such identification requires structural confirmation. Inactive species of large R_s eluting ahead of the monomer position in the gel permeation studies are likely to represent nonspecifically associated ATPase. Thus, stabilization of the Ca^{2+}-free solubilized scallop Ca-ATPase by Na^+ does not involve dimer formation, which may be related to the very significant differences in kinetics and dependence on Na^+ concentration from those observed in the membranous system, where formation of stabilized dimers of the Ca^{2+}-free Ca-ATPase is possible.

Effect of AMP-PCP on the Stability of the Ca^{2+}-Free Scallop Ca-ATPase in the Presence and Absence of Na^+. The membranous and solubilized rabbit Ca-ATPase binds ATP with a K_d of 2–4 μM (e.g., see Martonosi (1984) and Bishop et al. (1986)), and the nonhydrolyzable ATP analogue AMP-PNP binds very similarly (Inesi, 1985). The first-order inactivation plot for the Ca^{2+}-free, soluble Ca-ATPase in saturating AMP-PCP (0.75 mM) was linear for several half-lives in the absence and presence of Na^+/K^+, even at relatively alkaline pH values (e.g., pH 7.45, Figure 7). Thus, occupation of the nucleotide binding site inhibits the formation of multiple unstable species at higher pH. AMP-PCP alone slowed the decay of activity by a factor of only ~4-fold, in contrast to the very significant stabilization of the Ca^{2+}-free membranous scallop enzyme of ~100-fold observed with nucleotides (Kalabokis et al., 1991). Because the inactivation process in AMP-PCP followed monophasic first-order kinetics under all conditions, the simple kinetic model for stabilization by Na^+ could be used when AMP-PCP was present, when a good fit was obtained (Figure 4). Comparison of the data (k_1 = 0.0145 min^{-1}, k_2 = 0.0027 min^{-1}) with those obtained at pH 6.92 in the absence of AMP-PCP indicates that K_{Na}, the affinity of the Ca^{2+}-free enzyme for Na^+, is essentially the same under both conditions (~0.3 mM^{-1}); i.e., the binding of Na^+ to the Ca^{2+}-free enzyme is not affected by the occupation of the nucleotide binding site. The rate constant for inactivation of the Na^+-free, Ca^{2+}-free enzyme, k_1, is significantly decreased in the presence of AMP-PCP, while k_2, the rate constant for inactivation of the Na^+-occupied, Ca^{2+}-free enzyme, is little affected by AMP-PCP in the medium. Thus, AMP-PCP primarily affects the stability of the Na^+-free, Ca^{2+}-free Ca-ATPase, and AMP-PCP and Na^+ stabilize the Ca^{2+}-free enzyme independently. Saturation of the Na^+ site is more effective in stabilizing the Ca^{2+}-free enzyme than occupation of the nucleotide binding site.

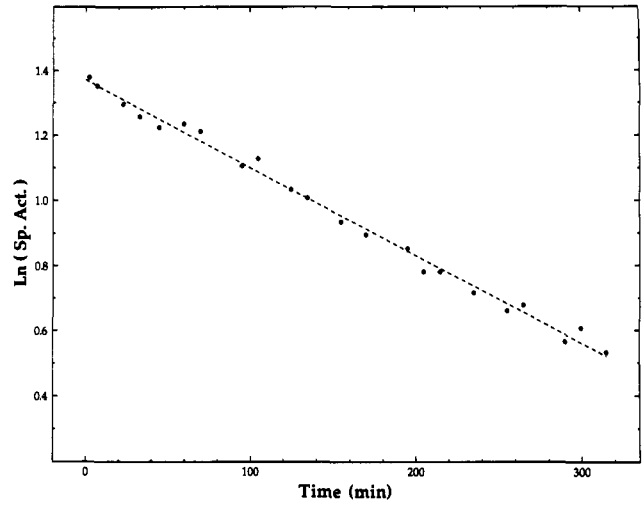


FIGURE 7: Effect of AMP-PCP on the inactivation process. Data from the inactivation of the Ca^{2+}-free solubilized scallop Ca-ATPase at pH 7.45 at 0 °C in the presence of 0.75 mM AMP-PCP and 0.1 M NaCl fit to first-order kinetics. Activity in μmol min^{-1} (mg of Ca-ATPase)$^{-1}$.

DISCUSSION

Solubilized Ca-ATPase retains many of the key functions necessary for ion translocation seen with the membranous enzyme (Andersen et al., 1985; McIntosh & Ross, 1988; Heergaard et al., 1990). Thus, loss of a characteristic property of the enzyme on solubilization implies a strong dependence on the native membrane structure.

Stabilization of the Ca^{2+}-free, $C_{12}E_8$-solubilized scallop Ca-ATPase by Na^+ showed major differences from stabilization of the Ca^{2+}-free membranous scallop Ca-ATPase by the same ligands. Stabilization of the Ca^{2+}-free soluble enzyme by Na^+ displays a clear saturation, while stabilization of the Ca^{2+}-free membranous enzyme does not. In the case of the membranous Ca^{2+}-free enzyme, Na^+/K^+ may act through several superimposed mechanisms (Kalabokis et al., 1991). There was no suggestion of any multiple or cooperative effects in the stabilization of the solubilized Ca^{2+}-free Ca-ATPase by Na^+, and the data were consistent with a simple model directly dependent on the occupancy of the enzyme by Na^+. There was also a difference in the kinetics of inactivation. At pH 7.0, 0 °C, the membranous enzyme is inactivated with biphasic first-order kinetics, implying two non-interconverting populations of Ca^{2+}-free Ca-ATPase molecules in the membrane. In contrast, under very similar conditions (pH 6.92, 0 °C), the solubilized enzyme decays with monophasic first-order kinetics. Since the Na^+-stabilized, soluble Ca^{2+}-free enzyme is monomeric, the characteristic effects of Na^+ and K^+ on the stability of the Ca^{2+}-free membranous Ca-ATPase associated with dimer formation that were previously reported must be dependent on the presence of the normal membrane structure. The presence of a full lipid complement does not protect the Ca^{2+}-free membranous scallop Ca-ATPase from inactivation in the absence of stabilizing agents such as Na^+, K^+, or nucleotide (Kalabokis et al., 1991), and the structure and organization of the protein are likely to be critical factors.

It is of interest that it may be possible to account for stabilization of the Ca^{2+}-free, solubilized scallop Ca-ATPase

by Na^+ by binding of the ion to a single site (although more sophisticated schemes are possible), since this suggests a specific effect of Na^+ on the protein. K^+ behaves identically to Na^+ in stabilizing the Ca^{2+}-free membranous Ca-ATPase (Kalabokis et al., 1991) and the associated formation of dimer ribbons, and it is likely that Na^+ and K^+ mediate their stabilizing effects in that system through binding to a common site. The Ca^{2+}-activated Mg-ATPase activity of the membranous scallop enzyme is half-maximally activated at pH 7.0 by ~40 mM Na^+ (Kalabokis et al., 1991) and the solubilized enzyme shows a similar dependence of activation on Na^+ concentration (data not shown), while the apparent dissociation constant for Na^+ mediating stabilization of the Ca^{2+}-free, solubilized scallop Ca-ATPase as found in these studies is ~3 mM at 0 °C. There are also major differences between the effect of the alkali cations on the stabilization of the Ca^{2+}-free membranous enzyme as opposed to their effect on the *activation* of the Ca^{2+}-activated Mg-ATPase activity. Activation of Ca^{2+}-activated Mg-ATPase enzyme activity and stabilization of the Ca^{2+}-free enzyme by alkali cations may thus occur at different sites, or the site involved in activation is modified or absent in the Ca^{2+}-free enzyme.

Inactivation of the enzyme produced by the absence of Ca^{2+} from its binding sites is most likely due to the loss of the enzyme to accept the terminal phosphoryl group from ATP, as opposed to inhibition of turnover of the phosphorylated enzyme. Since phosphorylation of scallop Ca-ATPase by ATP, as with rabbit Ca-ATPase (Inesi, 1985), is completely dependent on the occupation of the Ca^{2+}-binding sites, it is possible that the inactivation mechanism involves irreversible loss of the integrity of the Ca^{2+}-binding sites in the intramembrane segments of the Ca-ATPase (Clarke et al., 1989). Addition of high levels of Ca^{2+} to the inactivated Ca-ATPase does not restore enzyme activity. There is good evidence in the case of the membranous rabbit enzyme that K^+ and Na^+ can bind to the Ca^{2+}-binding domain and modify the binding of Ca^{2+} (Scofano et al., 1985; Moutin & Dupont, 1991; Timonin et al., 1991). K^+ and Na^+ may thus stabilize the Ca^{2+}-free Ca-ATPase by interacting with the empty Ca^{2+}-binding domain. Three glutamic acid and one aspartic acid side chains are thought to participate in the binding of Ca^{2+} (Clarke et al., 1989), and since the Ca^{2+}-free enzyme is much more labile at alkaline pH, this may suggest that mutual repulsion between neighboring ionized carboxyl groups (carboxylate) at the empty binding site could be involved in the inactivation mechanism and the associated loss of helical content. Na^+ or K^+ bound at or near the Ca^{2+}-binding sites might attenuate the strength of this destabilizing interaction.

Deprotonation of the enzyme is likely to be the key factor in the loss of linearity in the first-order plots at higher pH values seen with the scallop enzyme and in the overall loss of stability as the pH is raised. Lund et al. (1989) found that at 20 °C slightly acidic pH values slowed loss of activity by the Ca^{2+}-free, $C_{12}E_8$-solubilized rabbit Ca-ATPase, but in that system the decays fit monophasic first-order kinetics at all pH values. There is now a substantial amount of evidence indicating interactions of H^+ with the Ca^{2+}-binding sites (Inesi et al., 1992). The Ca-ATPase catalyzes the exchange of an average of one proton for every Ca^{2+} transported by the pump (Chiesi & Inesi, 1980; Levy et al., 1990), probably through direct replacement of Ca^{2+} at the binding site (Inesi & Hill, 1982, 1983). Thus, H^+ is effectively a substrate of the Ca-ATPase, and stabilization of the Ca^{2+}-free ATPase by moderately low pH values probably reflects binding of H^+ to the Ca^{2+}-free site, producing what amounts to substrate stabilization of the enzyme. Although $C_{12}E_8$ does not possess an ionizable head group, it is also possible that pH effects on the protein–detergent interaction might play a role.

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APPENDIX

Derivation of the Expression Relating $\tau_{0.5}$ to $[Na^+]$.

$$\text{rate of loss of activity} = k[E_{act,tot}]$$

$$= k([E_{Na}] + [E_{free}]) = k_1[E_{Na}] + k_2[E_{free}] \quad \text{(A1)}$$

where $[E_{act,tot}]$ is the total concentration of active Ca^{2+}-free enzyme, and $[E_{Na}]$ and $[E_{free}]$ are the concentrations of the active Ca^{2+}-free Ca-ATPase in its Na^+-liganded and Na^+-free forms, respectively. k is the observed rate constant, and k_1 and k_2 are the rate constants for the inactivation for the Na^+-free and Na^+-liganded enzymes, respectively.

$$K_{Na} = \frac{[E_{Na}]}{[E_{free}][Na^+]}$$

Substitution of $[E_{Na}] = K_{Na}[E_{free}][Na^+]$ into eq A1 yields

$$k = \frac{k_1 + k_2K_{Na}[Na^+]}{1 + K_{Na}[Na^+]}$$

which becomes

$$k = \frac{k_1 + k_2K_{Na}[Na^+]}{K_{Na}[Na^+]}$$

when $K_{Na}[Na^+] \gg 1$. This can be rearranged to give

$$\frac{1}{\tau} = \frac{1}{\tau_{min}K_{Na}[Na^+]} + \frac{1}{\tau_{max}}$$

In terms of the fractional saturation, ϕ, using $[E_{Na}] = \phi[E_{act,tot}]$ and $[E_{free}] = (1 - \phi)[E_{act,tot}]$,

$$k = k_1(1 - \phi) + k_2\phi$$

When $\phi = 0.5$,

$$k = (k_1 + k_2)/2$$

When $\tau = \tau_{max}/2$,

$$k = 2k_2 \quad \text{and} \quad \phi = (k_1 - 2k_2)/(k_1 - k_2)$$

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