Acad. Sci. U.S.A. 71, 2300.
Martinson, H. G. (1973), *Biochemistry 12,* 139.
McKnight, G. S., Pennequin, P., and Schimke, R. T. (1975), *J. Biol. Chem. 250,* 8105.
Monahan, J. J., Harris, S. E., Terrel, G. R., and O'Malley, B. W. (1976b), *Anal. Biochem.* (in press).
Monahan, J. J., Harris, S. E., Woo, S. L. C., Robberson, D. L., and O'Malley, B. W. (1976a), *Biochemistry 15,* 223.
Munford, R. E. (1963), *J. Endocrinol. 28,* 1.
Packman, S., Aviv, H., Ross, J., and Leder, P. (1972), *Biochem. Biophys. Res. Commun. 49,* 813.
Rabbitts, R. H. (1976), *Nature (London) 260,* 221.
Rosen, J. M. (1976), *Biochemistry 15* (preceding paper in this issue).
Rosen, J. M., and Comstock, J. P. (1976), *Proc. 58th Annu. Meet. Endocr. Soc.,* 123.
Rosen, J. M., Harris, S. E., Rosenfeld, G. C., Liarakos, C. D., and O'Malley, B. W. (1974), *Cell Differ. 3,* 103.
Rosen, J. M., Liarakos, C. D., and O'Malley, B. W. (1973), *Biochemistry 12,* 2803.
Rosen, J. M., Woo, S. L. C., and Comstock, J. P. (1975a), *Biochemistry 14,* 2895.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., and O'Malley, B. W. (1975b), *Biochemistry 14,* 69.
Ross, J., Gielen, J., Packman, S., Ikawa, Y., and Leder, P. (1974), *J. Mol. Biol. 87,* 697.
Russel, D. H., and McVicker, T. A. (1972), *Biochem. J. 130,* 71.
Schechter, I. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2511.
Shiu, R. P. C., and Friesen, H. G. (1976), *Science 192,* 259.
Sober, H. A. Ed. (1970), Handbook of Biochemistry, Selected Data for Molecular Biology, 2nd ed, 1970, Cleveland, Ohio, The Chemical Rubber Publishing Co., p H-112.
Socher, S. H., and Rosen, J. M. (1976), *Proc. 58th Annu. Meet. Endocr. Soc.,* 180.
Studier, W. F. (1965), *J. Mol. Biol. 11,* 373.
Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M., Bishop, J. M., and Schimke, R. T. (1973), *J. Biol. Chem. 248,* 7530.
Taborsky, G. (1974), *Adv. Protein Chem. 28,* 91.
Turkington, R. W. (1971), *Biochem. Actions Horm. 2,* 55.
Velez, R., Kantor, J. S., Picciano, D. J., Anderson, W. F., and Nienhuis, A. W. (1975), *J. Biol. Chem. 250,* 3193.
Venetianer, P., and Leder, P. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3892.

A Slow Interconversion between Active and Inactive States of the (Na-K)ATPase†

Lewis C. Cantley, Jr., and Lee Josephson*,‡

ABSTRACT: We have examined slow changes in the rate of ATP hydrolysis for purified dog kidney Na^+ and K^+ stimulated adenosine triphosphatase [(Na-K)ATPase] at various concentrations of free Mg^{2+}, Mg-ATP, K^+, and Na^+. The effect of these ligands on the rate of ATP hydrolysis is explained by a rapid binding step determining the initial rate of turnover followed by a slow conformational change. Inactivation of enzyme stored in the presence of ethylenediaminetetraacetic acid occurs upon adding free Mg^{2+}, Mg-ATP, and K^+; reactivation may be achieved if the concentration of these ligands is reduced. Because of the slow conformational change, the affinities for ligands affecting inactivation are time dependent. A model is presented to explain the effects of free Mg^{2+} and Mg-ATP on (Na-K)ATPase activity.

Studies of the Na^+ and K^+ stimulated ATPase [(Na-K)-ATPase][1] have provided important information about the mechanism of ion transport by examining the pre-steady-state and steady-state kinetics of the enzyme (see Glynn and Karlish, 1975b, for references). Hysteretic changes in enzyme activity have not been described, although there are suggestions that the properties of the enzyme depend not only on the composition of the assay media, but also on the handling of the enzyme prior to the assay. Kanazawa et al. (1970) employ a washing procedure prior to their transient kinetic studies, while Post et al. (1975) find that washing alters the ability of the enzyme to be phosphorylated by inorganic phosphate (P_i). Another possible example of hysteretic effects may be the slow increase in enzyme activity observed by some workers when (Na-K)-ATPase microsomes are aged (Barnett and Palazzotto, 1974; Cantley and Josephson, unpublished observations).

In this communication we have investigated hysteretic changes in ATPase activity for the (Na-K)ATPase prepared from dog kidney by the method of Jorgensen (1974). Our results indicate that the rate of ATP hydrolysis depends on a slow interconversion between forms of the enzyme with drastically different catalytic activities and that the equilibrium between forms depends on the presence of ligands required for (Na-K)ATPase activity. (Na-K)ATPase is a hysteretic enzyme as defined by Frieden (1970).

† From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138. *Received June 28, 1976.* This work was supported by grants from the Massachusetts Heart Association, Grant HL 08893 from the National Institutes of Health, and Grant GB36827 from the National Science Foundation.

‡ National Institutes of Health Postdoctoral Fellow (AM 00899-02).

[1] Abbreviations used: (Na-K)ATPase, Na^+ and K^+ stimulated adenosine triphosphatase; P_i, inorganic phosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PNPase, *p*-nitrophenylphosphatase; NADH, reduced nicotinamide adenine dinucleotide.

Experimental Section

(Na-K)ATPase Preparation. The (Na-K)ATPase was prepared from fresh dog kidney outer medulla by the procedure of Jorgenson (1974) and stored frozen (−70 °C) at 2 mg/ml in a solution of 25 mM imidazole, 1 mM EDTA, pH 7.5. The purified enzyme migrated as two bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with apparent molecular weights of ~105 000 and 58 000. The specific ATPase activity was approximately 12 μmol mg^{-1} min^{-1} using the "coupled" assay described below with the following assay conditions: 10 mM Hepes-triethylamine (pH 7.4), 100 mM NaCl, 20 mM KCl, 2.5 mM ATP, 2.5 mM $MgCl_2$, ca. 5 μg/ml (Na-K)ATPase, 37 °C. The ATPase activity dropped to less than 1% upon adding 50 μM ouabain or removing the NaCl. Protein concentrations were determined by the method of Lowry (1951) using bovine serum albumin as a standard.

Kinetic Measurements. In order to explore the change in ATPase activity with time, two assays were used which record the release of product vs. time. The "coupled" assay of Barnett (1970) which links ADP production to NADH oxidation that can then be monitored at 340 nm on a Cary 15 recording spectrophotometer was used for most experiments. With this assay a nearly constant ATP concentration is maintained even at low ATP concentrations, and ADP accumulation is prevented. The assay was started by adding about 5 μg of enzyme to 1 ml of 10 mM Hepes-triethylamine (pH 7.4), 10 μg/ml lactic dehydrogenase, 10 μg/ml pyruvate kinase, 1.4 mM phosphoenolpyruvate, 0.26 mM NADH, and the stated concentrations of ATP, NaCl, KCl, and $MgCl_2$. A thermostated cell holder was used to regulate temperature.

In experiments where the effects of varying KCl or $MgCl_2$ concentrations on ATPase activity were explored, the pH stat technique (Green and Mommaerts, 1953) was used. The assay mixture contained the stated concentrations of NaCl, KCl, $MgCl_2$, and Na_2–ATP in a final volume of 3 ml. Approximately 20 μg of enzyme was added, and the pH was maintained at 7.4 by titrating with 2 mM NaOH using a Radiometer Type TTT1a titrator. Dry nitrogen was bubbled through the solution to reduce CO_2 absorption, and the cell was thermostated. In all experiments the change in ATP concentration was less than 10% and the change in Na^+ concentration was less than 5% during the course of the assay.

The *p*-nitrophenylphosphatase activity (PNPase) of the (Na-K)ATPase was assayed in the Cary 15 spectrophotometer by monitoring the change in absorbance at 410 nm. The assay solution contained 20 mM Tris-HCl (pH 7.4), 8 μg/ml of enzyme, and the stated concentrations of $MgCl_2$, KCl, and ATP.

Instantaneous velocities at some time, t, following enzyme addition to the assay mixture (V_t) were calculated by drawing tangents to the recorded curve of absorbance or volume of NaOH added vs. time. In cases of rapid change in V_t, the chart speed was increased to expand the time scale. The velocities were corrected for any drift that occurred in the absence of enzyme.

Incubation Experiments. In incubation experiments where the enzyme concentration was high (0.1 to 0.5 mg/ml), a washing procedure was used to ensure removal of contaminating ions. The stock solution (~2 mg/ml) was diluted 20:1 with 20 mM Tris, 10 mM EDTA, pH 7.4 (adjusted with HCl), and centrifuged at 200 000g for 30 min in an IEC A-321 rotor at 4 °C. The pellet was resuspended in 20 mM Tris (pH 7.4) to a final protein concentration of about 0.1 mg/ml using a glass homogenizer and again centrifuged. The washing procedure was repeated a third time with 20 mM Tris, pH 7.4, and the final suspension was either used immediately or quickly frozen for later use.

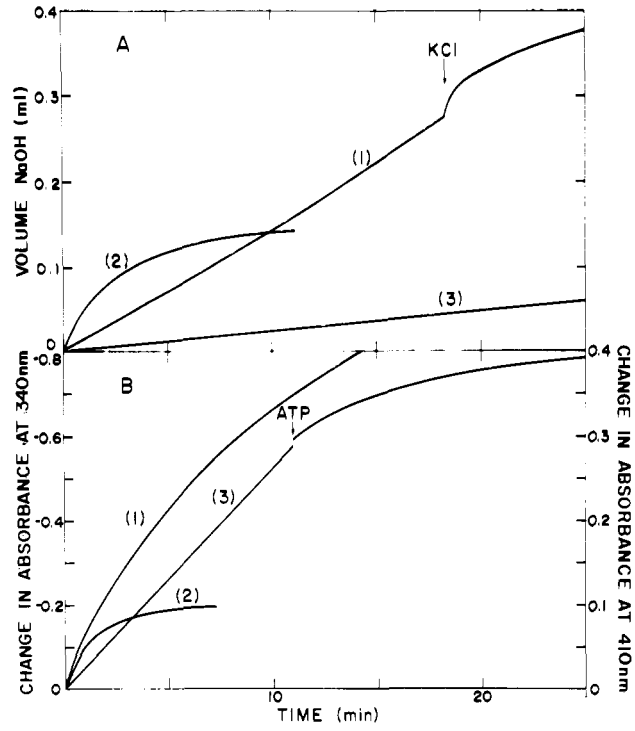

FIGURE 1: (A) The time course of ATP hydrolysis as measured by the pH stat technique. Assay 1 contained 100 mM NaCl, 10 mM $MgCl_2$, 2.5 mM ATP, 25 μg/ml enzyme (pH 7.4, 25 °C); assay 2 contained 100 mM NaCl, 10 mM $MgCl_2$, 15 mM KCl, 2.5 mM ATP, 25 μg/ml enzyme (pH 7.4, 25 °C). Assay 1 was made 15 mM in KCl at the arrow. The NaOH concentration was 2 mM, and curve 3 is the background due to CO_2 absorption. (B) The time course of ATP hydrolysis in the presence of (1) 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 2.5 mM ATP, 10 μg/ml enzyme; (2) 100 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 2.5 mM ATP, 20 μg/ml enzyme, at pH 7.4, 25 °C, using the "coupled" assay (340-nm absorbance). The *p*-nitrophenylphosphatase assay (3) contained 20 mM Tris-Cl, 25 mM KCl, 20 mM $MgCl_2$, 2.5 mM *p*-nitrophenyl phosphate, 8 μg/ml enzyme, pH 7.4, 25 °C (410-nm absorbance). Assay 3 was made 0.24 mM in Tris-ATP at the arrow. See Experimental Section for description of assays.

Data Analysis. The parameters presented in the Results section were determined from visual fits to the data and the uncertainties were determined by propagating the error in the data using differential error analysis.

Results

Conditions Producing Inactivation of the (Na-K)ATPase. The time course of the Na^+-stimulated ATPase activity [(Na)ATPase], the Na^+ and K^+ stimulated ATPase activity [(Na-K)ATPase], and the K^+ stimulated *p*-nitrophenylphosphatase activities (PNPase) are shown in Figure 1. Assay systems are described in the Experimental Section. The (Na)-ATPase activity (Figure 1A) is linear over long periods, while the (Na-K)ATPase activity decreases with time in the presence of 10 mM $MgCl_2$. Adding KCl to the (Na)ATPase assay actually decreases the ATPase activity following an initial burst. The initial velocity of the (Na)ATPase is approximately 20% of the (Na-K)ATPase activity using the pH stat technique. Figure 1B shows that the (Na-K)ATPase activity also decreases with the "coupled" assay and that the rate of change of velocity is sensitive to the $MgCl_2$ concentration. PNPase activity is linear over long periods at high $MgCl_2$ and KCl concentrations, but the addition of 0.24 mM Tris-ATP causes

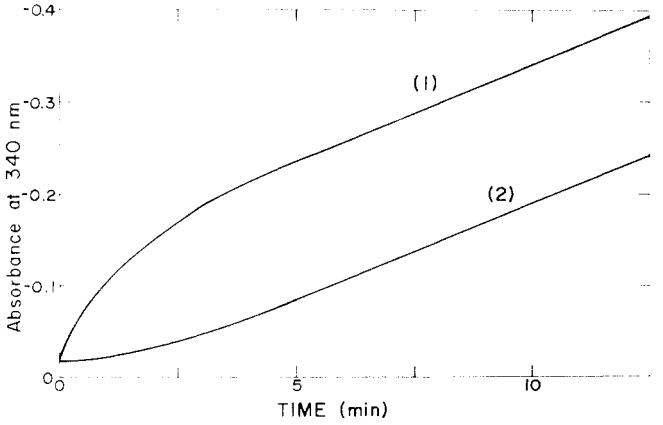

FIGURE 2: The time course of ATP hydrolysis of "active" (1) and "inactive" (2) enzyme using the "coupled" assay system with 100 mM NaCl, 25 mM KCl, 6 mM $MgCl_2$, 5 mM Na_2ATP, pH 7.4, 37 °C. The preparation of "active" and "inactive" enzyme is described in the text.

TABLE I: Reactivation of Inactive Enzyme.

Enzyme[a]	Incubation[b] Mixture (25 °C, pH 7.4)	Incubation Time (min)	Spec Act.[c] (μmol mg^{-1} min^{-1})
Active	Tris-HCl	0	4.35
		35	4.24
	Tris-HCl, EDTA, NaCl	15	5.23
		32	5.45
Inactive	Tris-HCl	0	0.20
		22	0.28
	Tris-HCl, NaCl	10	3.53
		24	3.37
	Tris-HCl, EDTA	4	3.40
		26	3.49
	Tris-HCl, EDTA, NaCl	1.5	4.28
		12	4.62
		30	4.38

[a] The "inactive" enzyme was prepared by incubating active enzyme (0.16 mg/ml) for 30 min in 25 mM Tris, 20 mM $MgCl_2$, 20 mM KCl, 4 mM Tris-ATP, pH 7.4 (25 °C). The solution was diluted tenfold with 20 mM Tris-HCl, pH 7.4 (0 °C), centrifuged as described in the Experimental Section, and resuspended in 20 mM Tris-HCl, pH 7.4 (0 °C). The "active" enzyme was treated identically, except that Tris-ATP was omitted from the first incubation step. [b] The Tris-HCl was 20 mM, the NaCl was 100 mM, and the EDTA was 1 mM where present. Protein concentration was 0.14 mg/ml for the "active" enzyme and 0.21 mg/ml for the "inactive" enzyme. [c] Specific activities were determined using the "coupled" assay as described in the Experimental Section. Twenty microliters of the incubation mixture was added to 1 ml of assay buffer containing 100 mM NaCl, 25 mM Tris-HCl, 4 mM $MgCl_2$, 3 mM ATP, pH 7.4, 25 °C, and initial velocities were measured.

an initial decrease in activity followed by a slow decay of the remaining activity.

The stability of the (Na)ATPase and PNPase activities suggests that inactivation of the (Na-K)ATPase is related to the presence of Mg^{2+}, K^+, and ATP. Indeed, when the enzyme was preincubated for 20 min at 0.1 mg/ml 20 mM Tris-HCl (pH 7.4, 25 °C) in the presence of all possible combinations of 20 mM KCl, 20 mM $MgCl_2$, and 4 mM Tris-ATP, it was found that all three ligands were required for inactivation. Activity was determined by a 50:1 dilution using the "coupled" assay system. In the absence of any one of the three ligands, no detectable loss of activity occurred with incubations up to 60 min (data not shown). Incubations with ADP, KCl, and $MgCl_2$ produced inactive enzyme, indicating that phosphorylation is not essential for inactivation. The results are in agreement with a more extensive study of the nucleotide requirements for enzyme inactivation performed by Fagan and Racker (1976).

Reversibility of the Inactivation of (Na-K)ATPase. The inactivation of the (NA-K)ATPase produced by incubation with KCl, $MgCl_2$, and ATP may be completely reversed if the concentrations of ligands are appropriately changed. The experiment described by Table I shows that inactive enzyme may be prepared which will not immediately reactivate following a mild washing procedure. Addition of NaCl or EDTA produces a rapid reactivation of enzyme activity to levels similar to those of enzyme which has not been inactivated. The requirement for NaCl or EDTA presumably reflects the need to remove trace amounts of ligands remaining after the washing procedure. The EDTA acts through its ability to chelate Mg^{2+}, while Na^+ acts by increasing the concentration of free Mg^{2+} required to maintain inactive enzyme (Figure 7) or by displacing K^+.

The reversibility of the inactivation process is further demonstrated by the two assays presented in Figure 2. Prior to both assays, the enzyme (50 μg/ml) was incubated for 90 min in 20 mM Hepes, 20 mM KCl, 20 mM $MgCl_2$, pH 7.4, 25 °C. The incubation prior to assay 2 also contained 0.66 mM ATP to cause "inactivation". Both enzymes were then assayed by the "coupled" assay method at 37 °C. In assay 2, the initial rate of ATP hydrolysis was less than 2% of the initial rate obtained with assay 1 but, regardless of the initial rates of ATP hydrolysis, similar steady-state rates were eventually reached. Final steady-state rates of Na^+ and K^+ stimulated ATP hydrolysis remained linear up to 30 min, the longest period investigated, and were reduced approximately 98% by the addition of 100 μM ouabain.

Affinities of Ligands Affecting the Rate of Inactivation. Studies of the mechanism of inactivation were performed at 25 °C since at this temperature the reactivation rate is slow and the equilibrium lies on the side of inactive enzyme over a wide range of ligand concentrations. Data were analyzed in the following fashion. Plots of ln V_t, the natural logarithim of the instantaneous rate of hydrolysis, vs. time were constructed and were linear over a wide range of decay rates and times. Because of the linearity of these plots, rates of ATP hydrolysis could be described in terms of a rate of inactivation ($1/\tau$), determined from the slope of the plots, and an initial velocity (V_0), determined by extrapolation to zero time. In experiments where the final steady-state velocity (V_∞) was greater than 10% of V_0, $1/\tau$ was determined from the slope of ln ($V_t - V_\infty$) vs. time.

The variation in V_0 and $1/\tau$ with free Mg^{2+} at three different ATP concentrations is shown in Figures 3A and 3B. The free Mg^{2+} concentrations were calculated from the total concentrations of ATP and $MgCl_2$ using a stability constant of 1.1×10^4 M^{-1} for the Mg-ATP complex. This constant was calculated from the data of Sullivan and Perrin (1964) and Burton (1958) taking into account temperature, pH, and ionic strength effects. Changes in V_0 and $1/\tau$ as a function of free Mg^{2+} behave as normal saturation curves in double-reciprocal plots. (Values of V_0 were omitted at high ATP and low Mg^{2+}, where a significant decrease in the substrate, Mg-ATP, occurred.) The apparent dissociation constant for free Mg^{2+} was 2.5 ± 0.7 mM for both effects and was independent of the total ATP concentration. The maximum inhibition of V_0 by free

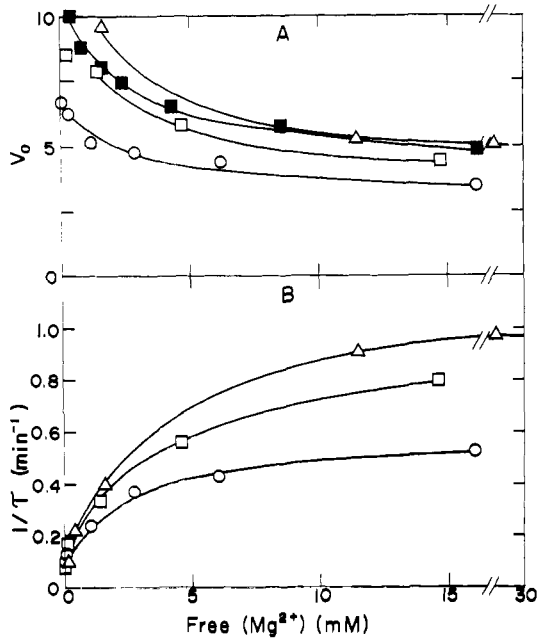

FIGURE 3: (A) The initial velocity of ATP hydrolysis (V_0 (relative units)) and (B) the rate of inactivation ($1/\tau$) as a function of free Mg^{2+} concentration. The V_0, $1/\tau$, and free Mg^{2+} were determined as described in the text. All assays were done by the pH stat technique with 100 mM NaCl, 20 mM KCl, 0.33–34 mM $MgCl_2$ and 0.5 mM (○), 2.0 mM (□), 2.5 mM (■), or 5.0 mM (△) Na_2ATP, pH 7.4, 25 °C.

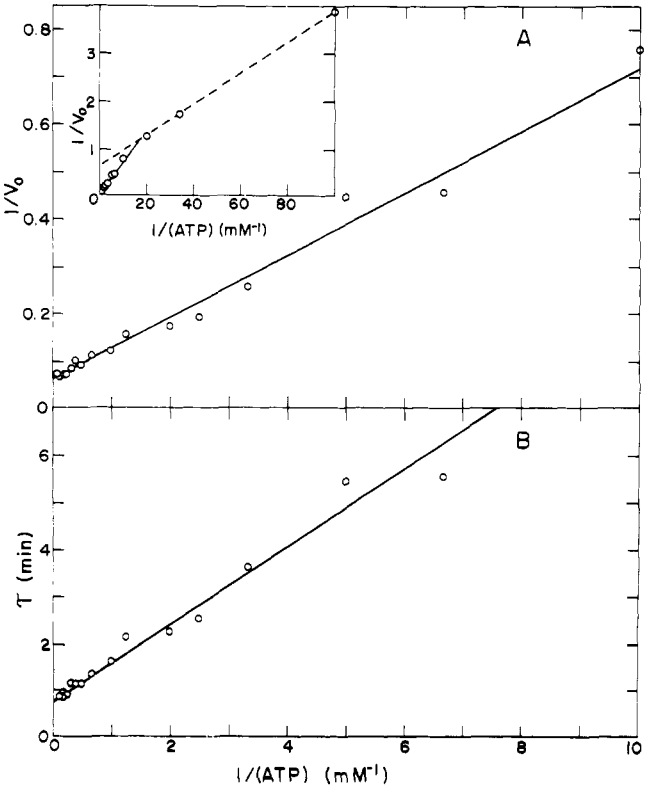

FIGURE 4: (A) Plots of the reciprocal initial velocity of ATP hydrolysis, $1/V_0$ (relative units), and (B) the reciprocal rate of inactivation, τ, as a function of 1/(ATP). The V_0 and $1/\tau$ were determined from the "coupled" assay as described in the text. All assays contained 100 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, and 10 μM to 10 mM Na_2ATP, pH 7.4, 25 °C.

Mg^{2+} was 55 ± 5%.

In order to compare the role of ATP in inactivation and in turnover, V_0 and $1/\tau$ were measured as a function of ATP at

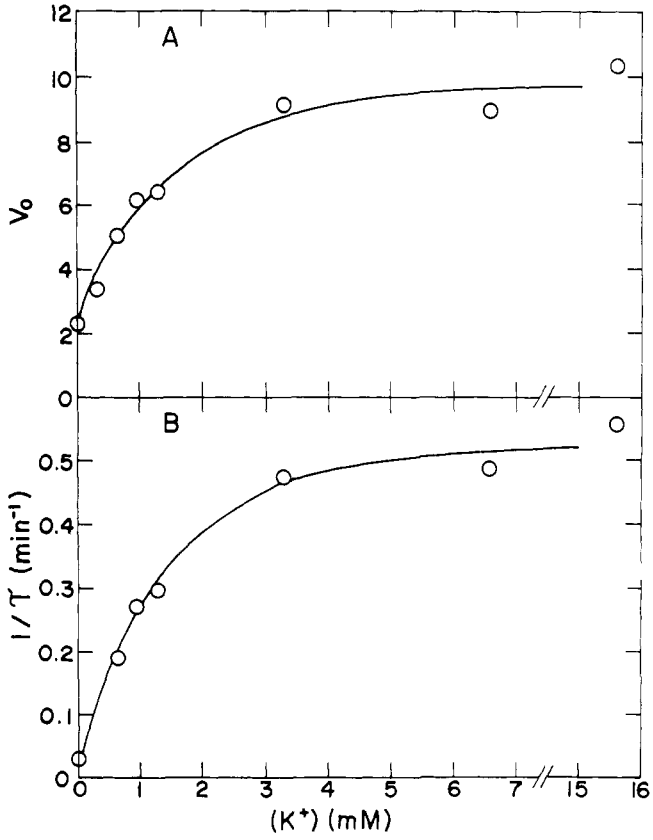

FIGURE 5: (A) The initial velocity of ATP hydrolysis, V_0, and (B) the rate of inactivation, $1/\tau$, as a function of (K^+). The pH stat assay was used with the conditions: 100 mM NaCl, 10 mM $MgCl_2$, 2.5 mM Na_2ATP, varying KCl concentrations, pH 7.4, 25 °C. The V_0 and $1/\tau$ were determined as described in the text.

saturating $MgCl_2$ concentrations (20 mM). (Since the $MgCl_2$ concentration is saturating, nearly all the ATP is present as the Mg-ATP complex in solution.) Plots of $1/V_0$ and τ vs. 1/(ATP) are presented in Figures 4A and 4B. Both V_0 and $1/\tau$ behave as normal saturation curves in the range of 0.1 to 10 mM ATP; however, at lower concentrations, a high affinity ATP site is observed (Figure 4A, inset). The data in Figure 4A inset may be interpreted as two independent ATP turnover sites with relative turnover rates of 0.1 and 0.9, and K_M values of 50 μM and 1.1 mM, respectively. Alternatively, the data may be explained by negative cooperativity in substrate binding with the second site accelerating turnover, or by a tight catalytic site and a weaker allosteric site. The apparent dissociation constant for ATP affecting $1/\tau$ is 1.1 mM and the maximum value of $1/\tau$ is 1.31 min^{-1} (Figure 4B). This suggests that the low affinity ATP site (K_M = 1.1 mM) is involved in inactivation. Measurements of $1/\tau$ at low ATP concentrations ($1/\tau$ < 0.1 min^{-1}; ATP < 0.1 mM) were not accurate enough to determine if the high affinity ATP site had any effect on $1/\tau$.

The affinities for K^+ affecting V_0 and $1/\tau$ are compared in Figure 5 where concentrations of ATP, Mg^{2+}, and Na^+ are maintained at nearly saturating levels. Both effects of K^+ were 50% maximal at about 1.4 mM KCl, suggesting that the same K^+ sites are involved in turnover and inactivation. The effects of NaCl on $1/\tau$ and V_0 at nearly saturating levels of $MgCl_2$, ATP, and KCl are presented in Figure 6. In the presence of high concentrations of free Mg^{2+}, NaCl caused a 50% stimulation of V_0 at 10 mM but had only a slight effect on the rate of inactivation.

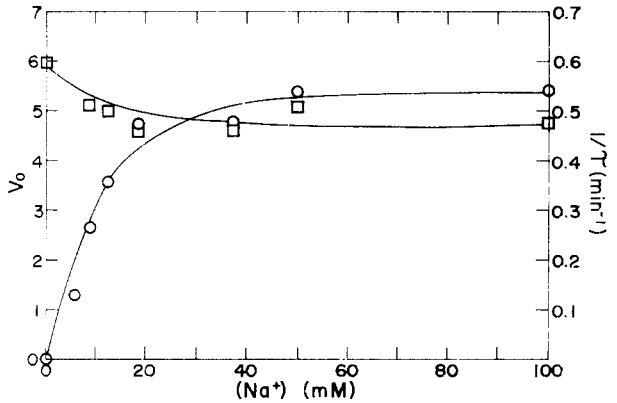

FIGURE 6: The initial velocity of ATP hydrolysis, V_0 (○), and the rate of inactivation, $1/\tau$ (□), as a function of (Na^+). The V_0 and $1/\tau$ were determined as described in the text using the "coupled" assay with 20 mM KCl, 20 mM Tris-Cl, 20 mM $MgCl_2$, 5 mM Tris-ATP, varying NaCl concentrations, pH 7.4, 25 °C. The $1/\tau$ in the absence of NaCl was determined by adding the enzyme to the above assay lacking NaCl and then completing the assay with 100 mM NaCl after a measured incubation time (10 s to 10 min). The natural log of the initial velocity measured immediately after NaCl addition was then plotted vs. the incubation time, and $1/\tau$ was calculated from the slope.

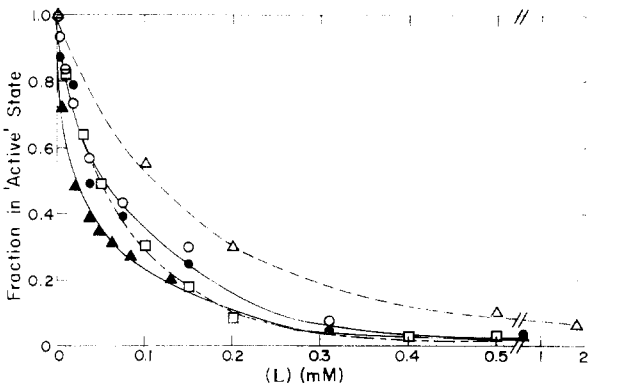

FIGURE 7: The fraction of enzyme in the "active" state at equilibrium as a function of ATP or free Mg^{2+}, where (L) is the concentration of ligand varied. The conditions were (△) 100 mM NaCl, 30 mM KCl, 5 mM Na_2ATP, and 0–6.7 mM $MgCl_2$, 25 μg/ml enzyme, 16 min, for the variation of free Mg; (□) 100 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 0–0.5 mM Na_2ATP, 10 mM Hepes-triethylamine, 10 μg/ml enzyme ("coupled" assay), 15 min; (○) 90- and (●) 180-min incubation in 20 mM KCl, 20 mM $MgCl_2$, 0–0.62 mM Tris-ATP, 10 mM Hepes-triethylamine, 50 μg/ml enzyme. (▲) Incubation (30 min) in 20 mM KCl, 5 mM Tris-ATP, 0–6 mM $MgCl_2$, 10 mM Hepes-triethylamine, and 50 μg/ml enzyme. All assays were at pH 7.4 and 25 °C. The fraction of enzyme in the active state was determined as described in the text.

In view of the reversibility of the inactivation process, the inactivation rates, $1/\tau$, are actually relaxation rates to an equilibrium position. Only a single relaxation could be observed in the time scale (minutes) and under the conditions studied. The plots of $1/\tau$ vs. either free Mg^{2+}, ATP, or K^+ (with the other two at saturating concentrations: Figures 3B, 4B, and 5B) are hyperbolic. The simplest kinetic model which explains these results is a rapid ligand binding followed by a slow conformational change as illustrated in eq 1.

$$E + L \overset{K_{1(L)}}{\rightleftharpoons} EL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'L \qquad (1)$$

In this model, E is the active form of the enzyme, E′ is the inactive form, L is the ligand (free Mg^{2+}, Mg-ATP, or K^+), $K_{1(L)}$ is a rapid dissociation constant, and k_2 and k_{-2} are slow rate constants for the conformational change. The other two ligands are assumed to be saturating the enzyme. If (L) is much greater than (E), then the slower of the two relaxation times is given by eq 2.

$$1/\tau = \frac{k_2}{(1 + K_{1(L)}/(L))} + k_{-2} \qquad (2)$$

A hyperbolic relationship is predicted for $1/\tau$ vs. (L) with the ordinate intercept equal to k_{-2} and the saturating value of $1/\tau$ equal to $k_2 + k_{-2}$.

This model may be expanded to include both Mg^{2+} and Mg-ATP at saturating K^+ concentration by assuming that concomitant binding of Mg^{2+} and Mg-ATP to independent sites is necessary for the slow conformational change to take place. The slow relaxation rate may be expressed by eq 3

$$1/\tau = \frac{k_2}{(1 + K_{1(Mg)}/(Mg))(1 + K_{1(ATP)}/(ATP))} + k_{-2} \qquad (3)$$

where the symbols are the same as in eq 2. The data are consistent with this model using the constants $K_{1(Mg)} = 2.5$ mM (Figure 3B) and $K_{1(ATP)} = 1.1$ mM (Figure 4B). In Figure 3B, the upper limit for k_{-2} is 0.07 min^{-1}, where $(Mg^{2+}) \ll K_{1(Mg)}$. The value of $k_2 = 1.3$ min^{-1} may be approximated from the extrapolated value of $1/\tau$ in Figure 5B since $k_2 \gg k_{-2}$ and

TABLE II: The Concentration of Ligand Causing a 50% Maximal Effect in the Initial ATPase Velocity ($K_{V_0(L)}$), the Initial Rate of Inactivation ($K_{1(L)}$), and the Equilibrium Fraction of Enzyme in the Active State ($K_{0.5(L)}$).

Ligand	$K_{V_0(L)}$ (mM)[a]	$K_{1(L)}$ (mM)[a]	$K_{0.5(L)}$ (mM)[a]	Comment[b]
Mg-ATP	0.05, 1.1	1.1	0.05	Figures 4 and 7 (100 mM Na^+)
			0.05	Figure 7 (no Na^+)
Free Mg^{2+}	2.5	2.5	0.11	Figures 3 and 7 (100 mM Na^+)
			0.02	Figure 7 (no Na^+)
K^+	1.4	1.4	0.4	Figure 5[c] (100 mM Na^+)

[a] The uncertainties in these constants are approximately 20%. [b] The ligands not varied (i.e., Mg-ATP, free Mg^{2+}, or K^+) were maintained at near-saturating concentrations. The Na^+ concentration is given in parentheses. All constants were determined at pH 7.4, 25 °C. [c] The $K_{0.5(K^+)}$ was determined by the procedure used in Figure 7 with the conditions described in Figure 5 (data not shown).

Mg^{2+} and K^+ are close to saturating their respective sites. Although the data in Figure 5B suggest a similar model for the effect of K^+ on $1/\tau$ at constant Mg^{2+} and ATP, the complex interaction between ATP binding and K^+ binding (Hegyvary and Post, 1971; Norby and Jensen, 1971) precludes incorporation of K^+ into this independent site model. The K^+ site is discussed more extensively below.

Effects of Ligands on Equilibrium between Active and Inactive Forms. The concentrations of ligands controlling the equilibrium ratio of active to inactive enzyme provide important information about the mechanism of enzyme inactivation. The fraction of enzyme active at equilibrium may be obtained by measuring initial and final hydrolytic rates from the pH stat technique or "coupled" assay and dividing the final by the initial hydrolytic rate at each ligand concentration. Figure 7 shows the effects of free Mg^{2+} and ATP on the fraction of

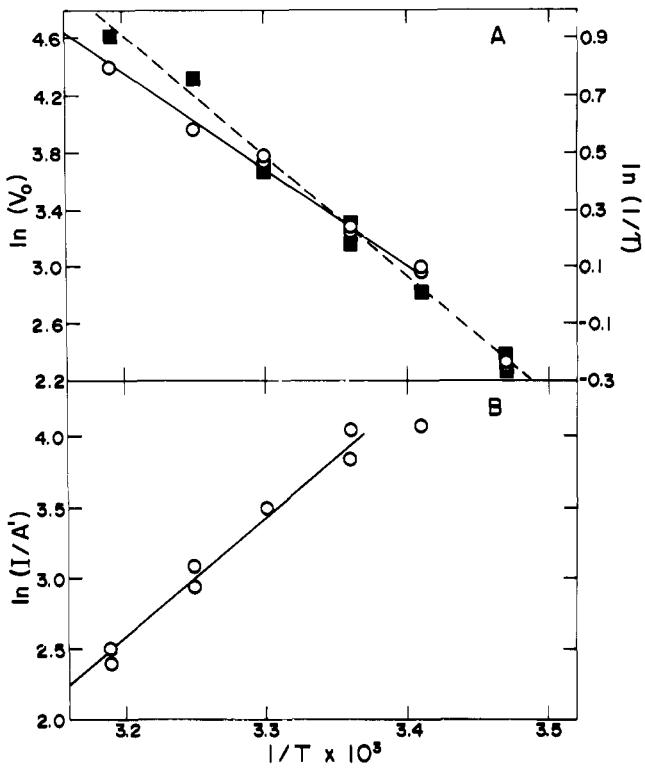

FIGURE 8: (A) Arrhenius plots of the initial ATP hydrolysis V_0 (relative units) (○), the rate of loss of activity, $1/\tau$ (■), and (B) the ratio of "inactive" to "active" enzyme (I/A'). The "coupled" assay was used with 100 mM NaCl, 20 mM KCl, 20 mM $MgCl_2$, 5 mM Na_2ATP, 10 μg/ml enzyme, 10 mM Hepes-triethylamine, pH 7.4, 15-40 °C. The V_0 and $1/\tau$ were measured as described in the text, and (I/A') was determined by dividing the equilibrium steady-state ATPase velocity (~15-min incubation), V_{eq}, by $V_0 - V_{eq}$ at each temperature, T.

enzyme in the active state at equilibrium. Either $MgCl_2$ or ATP was varied and all other ligands (Na^+, K^+, and ATP or Mg^{2+}) were maintained at saturating concentrations. In order to obtain the affinities for free Mg^{2+} and ATP in the absence of Na^+, enzyme was incubated in the presence of varying concentrations of free Mg^{2+} or ATP and in the absence of Na^+ but in the presence of other ligands required for inactivation. The fraction of enzyme in the active state was determined by a 10 to 1 dilution of the enzyme into the coupled assay system as described in Figure 2. A variation in incubation time established that equilibrium had been reached after approximately 15 min in all experiments. Values of the affinities at equilibrium together with affinities affecting initial velocities are listed in Table II. A comparison of the affinity for the ligand in the absence of inactivation ($t = 0$) with the affinity at equilibrium indicates a 20-fold increase in the affinities for free Mg^{2+} and ATP but a 3.5-fold increase in the affinity for K^+ as the system equilibrates. The significance of the change in affinity is discussed below.

Figures 8A and 8B are Arrhenius plots of the initial ATPase activity, V_0, the relaxation rate, $1/\tau$, and the equilibrium ratio of inactive to active enzyme, (I)/(A'), at near-saturating concentrations of all ligands in the temperature range 15-40 °C. Under these conditions, (I)/(A') = $k_2/k_{-2} = K_2$ and $1/\tau = k_2 + k_{-2} \cong k_2$ since ATP, Mg^{2+}, and K^+ are saturating and (I)/(A') is greater than 10. The activation energy for ATP turnover is 13.6 kcal/mol and the activation energy for k_2 is 8.2 kcal/mol as measured from the lines in Figure 8A. The change in enthalpy upon inactivation is −17 kcal/mol and the change in entropy is −50 cal mol^{-1} deg^{-1} as measured from

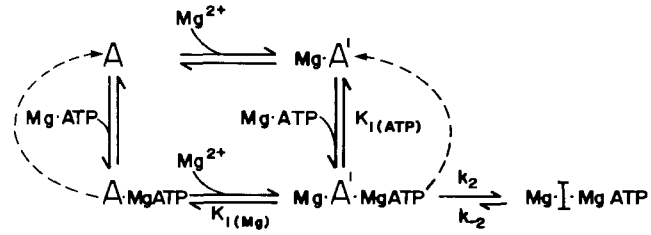

FIGURE 9: A model describing the effects of Mg^{2+} and Mg-ATP on the (Na-K)ATPase activity. The relative turnover numbers for the three enzyme states A, A', and I are 1, 0.45, and 0, respectively. The symbols are explained in the text.

the line in Figure 8B. From these data, $K_2 = 50 \pm 30$ at 25 °C and 16 ± 5 at 37 °C. At 25 °C, $k_2 = 1.31$ min^{-1} (Figure 4B) and $k_{-2} = k_2/K_2 = 0.03$ min^{-1}.

A Model for Enzyme Inactivation. The roles of free Mg^{2+} and Mg-ATP in the inactivation process at saturating K^+ concentrations (20 mM) are summarized in Figure 9. The symbols are the same as in eq 1, except the enzyme is represented by the symbols A, A', and I, indicating states with relative ATP turnover rates of 1.0, 0.45, and 0 respectively. The Mg-ATP site is the low affinity site in Figure 4A. The free Mg^{2+} binds independently of Mg-ATP and rapidly converts the enzyme from A to A' causing a 55% decrease in specific activity (see Figure 3A). The dashed arrow indicates the ATP turnover cycle that takes place in the presence of NaCl. The high affinity ATP site and the sequence of Na^+ and K^+ binding and product release are omitted for simplicity. All steps are rapid compared with k_2 and k_{-2}.

The model predicts that the equilibrium dissociation constant for free Mg^{2+} in the presence of saturating ATP, Na^+, and K^+ is $K_{I(Mg)}/K_2 = 2.5$ mM/50 = 0.05 mM. This value compares with the concentration of Mg^{2+} causing 50% inactivation at equilibrium in Figure 7 ($K_{0.5(Mg)} = 0.11$ mM).[2] The equilibrium dissociation constant for ATP in the presence of saturating Mg^{2+} and K^+ is predicted to be $K_{I(ATP)}/K_2 = 1.1$ mM/50 = 0.022 mM which is comparable to the $K_{0.5(ATP)}$ determined in Figure 7 ($K_{0.5(ATP)} = 0.05$ mM).[3] Adding 100 mM NaCl to the solution has no significant effect on either $K_{0.5(ATP)}$ or k_2 (Figures 7 and 6), suggesting that neither $K_{I(ATP)}$ nor K_2 is affected by increasing the turnover rate under these conditions. The model stresses the allosteric effect of the weak ATP site but does not eliminate it as a possible ATP hydrolysis site.

Discussion

Purified dog kidney (Na-K)ATPase undergoes a slow interconversion between active and inactive forms. Conversion from active to inactive forms requires the presence of KCl, Mg-ATP, and free Mg^{2+} but is independent of the enzyme turnover and only affected by Na^+ ions at very low free Mg^{2+} concentrations (Figure 7 and Table I). Activities which do not require all three ligands such as (Na)ATPase or PNPase occur without progressive inactivation. The effects of these ligands on ATP hydrolysis may be understood in terms of a rapid binding step determining initial rates of ATP hydrolysis followed by a slower conformational change to a form with no catalytic activity. Because of the vastly different rates of

[2] Using the assigned relative turnover rates for A, A', and I, $K_{0.5(Mg)} = K_{I(Mg)}/(K_2 + 0.05) \cong K_{I(Mg)}/K_2$.

[3] From the binding scheme in Figure 11, $K_{0.5(ATP)} = K_{I(ATP)}/(K_2 - 1) \cong K_{I(ATP)}/K_2$.

binding and inactivation, it is possible to obtain the rate constants associated with this interconversion.

The model we have developed for the interconversion between active and inactive enzyme is consistent with kinetics of enzyme turnover and inactivation. It must be noted, however, that, under the ionic conditions employed in Table I (high Na^+, low free Mg^{2+}, K^+, and ATP), reactivation occurs through a pathway not presented in Figure 9 since the observed rate of reactivation ($t_{1/2} < 1$ min) is far greater than k_{-2} (0.03 min^{-1}). It is possible that, under these conditions, NaCl or EDTA removes or displaces ligands from the inactive state prior to the "conformational" change, permitting a rapid reactivation of the enzyme. The data are not inconsistent with the existence of an inactive state lacking Mg^{2+}, K^+, Mg-ATP, or all three, but require that the equilibrium position favor the active state in the absence of any one of the ligands. This state does not appear in our transient kinetics since ligand binding precedes the conformational change.

Free Mg^{2+} has long been known to have an inhibitory effect on the (Na-K)ATPase (Skou, 1960; Hexum et al., 1970) but the effects of free Mg^{2+} have not been resolved into slow and fast components. The binding of free Mg^{2+} ions to the (Na-K)ATPase has been inferred from effects on the transphorylation of ADP (Fahn et al., 1966), effects on ouabain binding (Schwartz, 1968; Albers, 1968; Hansen and Skou, 1973), changes in the affinity and cooperativity for Na^+ ions (Robinson, 1972), and from the loss in ion sensitivity of the dephosphorylation (Post et al., 1975).

Recently a number of authors have proposed "flip-flop" models for the (Na-K)ATPase (Schon et al., 1974; Glynn and Karlish, 1975a,b; Froelich et al., 1976) and the (Ca)ATPase (Froelich and Taylor, 1976). According to these models, ATP acts as an allosteric effector accelerating product release prior to acting as a substrate for phosphorylation. The low and high affinity ATP sites observed in Figure 4A are in agreement with results of Froelich et al. (1976) and Glynn and Karlish (1976). The relationship between $1/\tau$ and V_0 in Figure 4 suggests that binding ATP to the low affinity site induces a rapid conformational change accelerating both turnover and inactivation at high Mg^{2+} concentrations.

The inactivation of the (Na-K)ATPase appears to differ with the constant rates of ATP hydrolysis seen by others (Barnett, 1970; Kyte, 1972; Robinson, 1972; Skou, 1974; Josephson et al., 1974) but cannot be attributed to product accumulation, an unusual instability of the enzyme under our assay conditions or the method of enzyme preparation. ADP fails to accumulate in the coupled assay system, while inorganic phosphate fails to inhibit the (Na-K)ATPase at the concentrations produced by ATP hydrolysis (Hexum et al., 1970; Garay and Garrahan, 1975). Phosphatidylserine, bovine serum albumin, and 2-mercaptoethanol have been employed in some assays for (Na-K)ATPase (Siegel and Albers, 1967; Kyte, 1971) but failed to alter the rate of inactivation when present at concentrations used by others (Cantley and Josephson, unpublished observations). Step IV microsomes from dog kidney prepared by the method of Kyte (1971) inactivate in a fashion identical with enzyme prepared by the method of Jorgenson, although different detergents and chaotropic agents are employed for each preparation (Josephson and Cantley, unpublished observations). The hysteretic nature of the enzyme depicted in Figure 2 may account for some of the discrepancy in the literature concerning specific activity and linearity of assays.

From the in vitro experiments presented here, it appears that a substantial fraction of the dog kidney (Na-K)ATPase may exist in an inactive form in vivo. The concentration of free Mg^{2+} in normal plasma is approximately 0.6 mM and appears to vary considerably in man (Walser, 1973). The intracellular free Mg^{2+} is probably similar to that in plasma, while the intracellular concentration of ATP is about 1 mM. The K^+ concentration in dog plasma remains relatively constant at about 4 mM (Baylis and O'Connor, 1976). Although an estimate of the fraction of active enzyme under physiological conditions is hampered by the inability to determine ligand concentration in the vicinity of the enzyme as well as by the lack of knowledge concerning the orientation of the free Mg^{2+} sites, the concentrations of K^+, ATP, and free Mg^{2+} in the cytosol and plasma appear to be sufficient to produce a substantial inactivation of the enzyme. Variations in the concentrations of free Mg^{2+} and ATP may be capable of altering the fraction of active enzyme in vivo. The equilibrium between active and inactive states in vivo might also be influenced by covalent modifications, hormones, or lipid composition.

Acknowledgments

We thank Professor Guido Guidotti in whose laboratories these experiments were carried out. We are also grateful to Dr. John Fagan and Professor Efraim Racker for making us aware of the potassium dependence of this inactivation and providing us with a rough draft of their manuscript.

References

Albers, R. W., Koval, G. J., and Siegel, G. J. (1968), *Mol. Pharmacol. 4,* 324.
Barnett, R. E. (1970), *Biochemistry 9,* 4644.
Barnett, R. E., and Palazzotto, J. (1974), *Ann. N.Y. Acad. Sci. 242,* 69.
Baylis, C., and O'Connor, W. J. (1976), *Q. J. Exp. Physiol. 61,* 145.
Burton, K. (1958), *Biochem. J. 71,* 388.
Fagan, J., and Racker, E. (1976), *Biochemistry* (in press).
Fahn, S., Koval, G. J., and Albers, R. W. (1966), *J. Biol. Chem. 241,* 1882.
Frieden, C. (1970), *J. Biol. Chem. 245,* 5788.
Froelich, J. P., Albers, R. W., Koval, G. J., Goebel, R., and Berman, M. (1976), *J. Biol. Chem. 251,* 2186.
Garay, R. P., and Garrahan, P. J. (1975), *J. Physiol. (London) 249,* 51.
Glynn, I. M., and Karlish, S. J. D. (1975a), *Energy Transform. Biol. Syst., Ciba Found. Symp. 31 (new ser.),* 205–220.
Glynn, I. M., and Karlish, S. J. D. (1975b), *Annu. Rev. Physiol. 58,* 48.
Glynn, I. M., and Karlish, S. J. D. (1976), *J. Physiol. 256,* 465.
Green, I., and Mommaerts, W. (1953), *J. Biol. Chem. 202,* 541.
Hansen, O., and Skou, J. C. (1973), *Biochim. Biophys. Acta 311,* 51.
Hegyvary, C., and Post, R. L. (1971), *J. Biol. Chem. 246,* 5234.
Hexum, T., Samson, F. E., and Himes, R. M. (1970), *Biochim. Biophys. Acta 212,* 322.
Jorgensen, P. L. (1974), *Biochim. Biophys. Acta 356,* 36.
Josephson, L., Mangold, J., and Simon, S. R. (1974), *Anal. Biochem. 60,* 312.
Kanazawa, T., Saito, M., and Tonomura, Y. (1970), *J. Biochem. (Tokyo) 67,* 693.
Kyte, J. (1971), *J. Biol. Chem. 246,* 4157.
Kyte, J. (1972), *J. Biol. Chem. 247,* 7634.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Norby, J. G., and Jensen, J. (1971), *Biochim. Biophys. Acta 233,* 104.
Post, R. L., Toda, G., and Rogers, F. N. (1975), *J. Biol. Chem. 250,* 691.
Robinson, J. D. (1972), *Biochim. Biophys. Acta 266,* 97.
Schon, R., Dittrich, F., and Repke, K. R. H. (1974), *Acta Biol. Med. Ger. 33,* K9.
Schwartz, A., Matsui, M., and Laughter, A. M. (1968), *Science 160,* 323.
Siegel, G. J., and Albers, R. W. (1967), *J. Biol. Chem. 242,* 4972.
Skou, J. C. (1960), *Biochim. Biophys. Acta 42,* 6.
Skou, J. C. (1974), *Biochim. Biophys. Acta 339,* 246.
Sullivan, W. J., and Perrin, D. D. (1964), *Biochemistry 3,* 18.
Walser, M. (1973), in Handbook of Physiology Section 8: Renal Physiology, American Physiological Society, Chapter 18, p 555.

Papain-Catalyzed Reactions at Subzero Temperatures†

Anthony L. Fink* and Kimon J. Angelides

ABSTRACT: As a first step in the investigation of papain catalysis using subzero temperatures to detect, accumulate, and characterize enzyme–substrate intermediates, we have studied some potential cryosolvents and carried out preliminary intermediate trapping experiments. The effects of subzero temperatures and aqueous dimethyl sulfoxide solutions on the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester have been investigated in detail. At 0 °C, the value of k_{cat} decreases with increasing dimethyl sulfoxide concentration, decreasing in proportion to the decreased water concentration; however, the value of K_m increases exponentially. The effect on K_m can be accounted for by a combination of both dielectric and competitive inhibition effects. The Arrhenius plot for the deacylation reaction in 7.65 M (60% v/v) dimethyl sulfoxide is linear over the temperature range 0 to −45 °C and extrapolates to a calculated value of k_{cat} at 25 °C in excellent agreement with that obtained in the absence of organic solvent. The pH–rate profile is not substantially perturbed by the presence of 7.65 M dimethyl sulfoxide. At −45 °C and below, turnover occurs extremely slowly, and is essentially negligible, although acylation is still quite rapid. Consequently, the acyl enzyme, N^{α}-carbobenzoxy-L-lysyl-papain, can be readily accumulated and trapped at temperatures below −50 °C. At these low temperatures, under conditions of excess substrate, the amount of *p*-nitrophenol liberated in the acylation reaction is equivalent to the active-site normality of the enzyme, indicating a 1:1 stoichiometry in formation of the acyl enzyme. The effect of dimethyl sulfoxide up to 7.65 M, on the intrinsic ultraviolet, fluorescence, and circular dichroic properties of the enzyme shows no evidence of any solvent-induced structural changes. All experimental observations are consistent with the conclusion that 7.65 M dimethyl sulfoxide and subzero temperatures have no deleterious effects on papain-catalyzed reactions. A related series of experiments indicate that aqueous ethanol cryosolvents up to 13.7 M (80% v/v) are also suitable. Preliminary experiments at subzero temperatures using N^{α}-carbobenzoxy-L-lysine methyl ester suggest the existence of three enzyme–substrate intermediates which can be detected and accumulated.

The rapidity of enzyme-catalyzed reactions and the concomitant short life times of intermediates have been a major problem in studying the dynamic processes which occur during catalysis. However, knowledge of the existence and structure of intermediates on the reaction pathway is necessary before specific details of the mechanism may be successfully resolved. Much of our current understanding of enzyme mechanisms has been obtained through the use of kinetically nonspecific substrates and substrate analogues. However, the fact that intermediates from such pseudo-substrates can be isolated does not necessarily indicate that similar intermediates are on the productive pathway for good substrates. Consequently, a method using specific substrates which permits the detection and characterization of intermediates would be very valuable. We have been developing such a method utilizing subzero temperatures and fluid solvent systems (Fink, 1973a,b, 1976a,b; Fink and Wildi, 1974; Fink et al., 1976). The potential of such an approach was first clearly shown by Douzou's studies on peroxidase (Douzou et al., 1970). The procedure not only allows the accumulation of kinetic and thermodynamic information, which may otherwise be very difficult to obtain, but also permits the trapped intermediates to be studied by a variety of chemical and physical techniques to provide details of their structure.

The methodology is predicated on the following basis. A drop of 100 °C will decrease the rate of a typical step in an enzyme-catalyzed reaction by a factor of 10^5 to 10^8. Thus, the rates of individual steps in the overall enzyme-catalyzed reaction will be decreased to such an extent that, for some, the reaction will be negligible at such low temperatures. If the free energies and enthalpies of activation are such that at the very low temperatures the faster reactions precede the slower ones, it should be possible to accumulate each intermediate successively. The potential of this cryoenzymological approach for detecting and accumulating intermediates in enzyme-catalyzed reactions has been demonstrated in recent studies with a number of enzymes, e.g., peroxidase (Douzou et al., 1970), chymotrypsin (Fink and Wildi, 1974; Fink, 1976a; Fink

† From the Division of Natural Sciences, Thimann Laboratories, The University of California, Santa Cruz, California 95064. *Received June 10, 1976.* This work was supported by grants from the National Science Foundation.