Kirk, G. L., Gruner, S. M., & Stein, D. L. (1984) *Biochemistry 23*, 1093–1101.
Knight, D. E., Hallam, T. J., & Scrutton, M. C. (1982) *Nature (London) 296*, 256–257.
Kraft, A. S., & Anderson, W. B. (1983) *Nature (London) 301*, 621–623.
Larsson, K. (1983) *Nature (London) 304*, 664–665.
Lecuyer, H., & Dervichian, D. (1969) *J. Mol. Biol. 45*, 39–57.
Lis, L. J., McAlister, M., Fuller, N., Rand, R. P., & Parsegian, V. A. (1982a) *Biophys. J. 37*, 657–665.
Lis, L. J., McAlister, M., Fuller, N., Rand, R. P., & Parsegian, V. A. (1982b) *Biophys. J. 37*, 667–672.
Longley, W., & McIntosh, J. (1983) *Nature (London) 303*, 612–614.
Loosley-Millman, M. E. (1980) Ph.D. Thesis, Guelph University, Guelph, Canada.
Loosley-Millman, M. E., Rand, R. P., & Parsegian, V. A. (1982) *Biophys. J. 40*, 221–232.
Luzzati, V. (1962) *J. Cell Biol. 12*, 207–219.
Luzzati, V. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 71–123, Academic, New York.
Michell, R. H. (1975) *Biochim. Biophys. Acta 415*, 81–147.
Moolenaar, W. H., Tertoolen, L. G. J., & De Laat, S. W. (1984) *Nature (London) 312*, 371–374.
Nishizuka, Y. (1983) *Philos. Trans. R. Soc. London, B 302*, 101–112.
Nishizuka, Y. (1984) *Nature (London) 308*, 693–698.
Ohki, K., Sekiya, T., Yamauchi, T., & Nozawa, Y. (1982) *Biochim. Biophys. Acta 693*, 341–350.
Parsegian, V. A. (1977) *Soc. Neurosci. Symp. 2*, 161–171.
Parsegian, V. A., Fuller, N., & Rand, R. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2750–2754.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Rand, R. P., & Parsegian, V. A. (1984) *Can. J. Biochem. Cell Biol. 62*, 752–759.
Rand, R. P., Reese, T. S., & Miller, R. G. (1981) *Nature (London) 392*, 237–238.
Rink, T. J., Sanchez, A., & Hallam, T. J. (1983) *Nature (London) 258*, 317–319.
Siegel, D. (1984) *Biophys. J. 45*, 399–420.
Stockton, G. W., & Smith, I. C. P. (1976) *Chem. Phys. Lipids 17*, 251–263.
Verkleij, A. J., Mombers, C., Gerritsen, W. J., Leunissen-Bijvelt, J., & Cullis, P. R. (1979a) *Biochim. Biophys. Acta 555*, 358–361.
Verkleij, A. J., Mombers, C., Leunissen-Bijvelt, J., & Ververgaert, P. (1979b) *Nature (London) 279*, 162–163.
Wakelam, M. J. O. (1983) *Biochem. J. 214*, 77–82.
Wakelam, M. J. O. (1985) *Biochem. J. 228*, 1–12.
Whitaker, M., & Aitchison, M. (1985) *FEBS Lett. 182*, 119–124.

# Thermoinactivation and Aggregation of $\alpha\beta$ Units in Soluble and Membrane-Bound (Na,K)-ATPase†

Peter Leth Jørgensen* and Jens Peter Andersen
*Institute of Physiology, Aarhus University, 8000 Aarhus C, Denmark*
*Received July 10, 1985; Revised Manuscript Received December 5, 1985*

ABSTRACT: Stability and conformational transitions of soluble and fully active $\alpha\beta$ units of (Na,K)-ATPase in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$) are examined. Sedimentation equilibrium centrifugation gave a molecular weight of 143 000 for the $\alpha\beta$ unit eluting from TSK 3000 SW gel chromatography columns. Fluorescence analysis and phosphorylation experiments show that $E_1$–$E_2$ transitions between both dephospho and phospho forms of soluble (Na,K)-ATPase are similar to those previously observed in the membrane-bound state. The two conformations can also be identified by their different susceptibilities to irreversible temperature-dependent inactivation. $E_1$ forms of both soluble and membrane-bound (Na,K)-ATPase are more thermolabile than $E_2$ forms. Gel chromatography on TSK 3000 SW and 4000 SW columns shows that thermal inactivation of soluble (Na,K)-ATPase at 40 °C is accompanied by aggregation of $\alpha\beta$ units to $(\alpha\beta)_2$ units and higher oligomers. The aggregates are stable in $C_{12}E_8$ but dissolve in sodium dodecyl sulfate. Similar aggregation accompanies inactivation of membrane-bound (Na,K)-ATPase at 55–60 °C. These data suggest that inactivation both in the soluble and in the membrane-bound state involves exposure of hydrophobic residues to solvent. The instability of the soluble $E_1$ form may be related to inadequate length of the dodecyl alkyl chain of $C_{12}E_8$ for stabilization of hydrophobic protein domains that normally associate with alkyl chains of phospholipids in the membrane. Interaction between $\alpha\beta$ units does not seem to be required for the $E_1$–$E_2$ conformational change, but irreversible aggregation appears to be a consequence of denaturation of (Na,K)-ATPase in both soluble and membranous states.

Soluble and fully active $\alpha\beta$ units can be prepared from purified membrane-bound (Na,K)-ATPase from outer renal medulla in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] (Brotherus et al., 1981, 1983; Craig, 1982). Each soluble $\alpha\beta$ unit binds one molecule of ATP without evidence for cooperative interaction (Jensen & Ottolenghi, 1983), and the soluble enzyme reconstitutes to fully active Na,K pumps in

† This work was supported by the Danish Medical Research Council and Novo's Foundation.

[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; $E_1$ and $E_2$, major conformational states of (Na,K)-ATPase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography.

0006-2960/86/0425-2889$01.50/0 

phospholipid vesicles (Brotherus et al., 1983). However, direct evidence for competence of the $\alpha\beta$ unit as the (Na,K)-transport system has not been obtained. Transport across phase boundaries cannot be measured in detergent solution, and the molecular size cannot be determined in phospholipid vesicles. The $\alpha\beta$ unit is the minimum asymmetric unit in two-dimensional crystals of pure membrane-bound (Na,K)-ATPase from the outer medulla of mammalian kidney (Hebert et al., 1982). Although they are transient and more scarce, dimeric crystals consisting of $(\alpha\beta)_2$-unit cells can also be prepared (Hebert et al., 1982), and it remains uncertain whether the $\alpha\beta$ unit can catalyze active cation transport or if association to $(\alpha\beta)_2$ units is required.

Interpretation of experiments with soluble (Na,K)-ATPase has been hampered by the relative instability and tendency for aggregation of soluble (Na,K)-ATPase. It is not known whether this instability is due to loss of protein–protein associations or if it reflects the replacement of phospholipid with detergent molecules. The purpose of this study has been to examine the stability and conformational transitions of preparations consisting of soluble and fully active $\alpha\beta$ units. Particular emphasis was placed on determination of thermodynamic constants for heat inactivation of the soluble (Na,K)-ATPase and the dependence of the inactivation rate constant on the conformational state of the protein. The aggregation state and molecular weight of the soluble protein particles were determined by HPLC gel chromatography on TSK columns and analytical ultracentrifugation.

## Experimental Procedures

(Na,K)-ATPase was purified in membrane-bound form from pig kidney outer medulla by incubation of crude membrane protein (1.5–2 mg/mL) with 0.65–0.71 mg of Na-DodSO$_4$/mL in the presence of 3 mM $Na_3ATP$/2 mM EDTA/25 mM imidazole, pH 7.5. After 30 min at 20 °C, 75 mL of this medium was injected into Ti-14 Beckman or Kontron TZT 48.650 zonal rotors loaded with a 15–40% (w/v) nonlinear sucrose gradient and a cushion of 45% (w/v) sucrose in 25 mM imidazole/1 mM EDTA, pH 7.5 (Jørgensen, 1974). Centrifugation was for 120 min at 48 000 rpm. Fractions of 16 mL containing the purified (Na,K)-ATPase were diluted to 35 mL with 25 mM imidazole/1 mM EDTA, pH 7.5, and centrifuged at 40 000 rpm overnight (16–17 h) to collect the enzyme in the pellet. Prior to solubilization, the membrane-bound (Na,K)-ATPase was sedimented at 100 000 rpm for 10 min in the Beckman airfuge and resuspended in 20 mM KCl or 20 mM NaCl/20 mM Tes/2 mM dithiothreitol/2 mM Tris–EDTA, pH 7.5, at 1.3–2.6 mg of protein/mL. After homogenization, this suspension was mixed with equal volumes of $C_{12}E_8$ (Nikko Chemicals, Co., Tokyo, Japan) in water to final concentrations of 0.65–1.3 mg of protein/mL, 1.16–4.8 mg/mL $C_{12}E_8$/10 mM NaCl, or KCl/10 mM Tes/1 mM dithiothreitol/1 mM Tris–EDTA, pH 7.5, as previously reported in detail (Brotherus et al., 1981, 1983). After incubation for 30 min at 20 °C, the insoluble residue was removed by centrifugation for 10 min at 100 000 rpm in a Beckman airfuge.

The sedimentation velocity at 20 °C was examined in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and temperature control unit. Calculation of sedimentation coefficients was based on scans showing protein concentration (monitored as absorbance at 280 nm) as a function of radial distance and of the first derivative (concentration gradient) calculated from these scans. In a few experiments with fluorescein-labeled enzyme, scans were also obtained at 496 nm.

The molecular weight of $(Na^+,K^+)$-ATPase protein eluting from the TSK 3000 SW gel filtration column (Figure 8) was determined by sedimentation equilibrium studies (Tanford et al., 1974). Scans taken 16 h after initiation of sedimentation at 12 000 rpm were used for calculation. To obtain the molecular weight, correction was made for carbohydrate (0.15 mg/mg of protein) and detergent (0.70 mg/mg of protein) bound to the protein by using previously described procedures (Brotherus et al., 1983).

Labeling with fluorescein 5-isothiocyanate was performed as previously described (Karlish, 1980; Hegyvary & Jørgensen, 1981). With respect to solubilization in $C_{12}E_8$, the behavior of fluorescein-labeled enzyme was identical with that of membrane-bound (Na,K)-ATPase (cf. Figure 3). Fluorescence measurements were made in a Perkin-Elmer MPF 44 A spectrofluorometer in a thermostated continuously stirred cuvette. Ligands were added from Hamilton syringes equipped with push-button dispensers. Tryptophan fluorescence was recorded at 328 nm with an excitation wavelength of 295 nm using 10-nm slits on both monochromators (Karlish, 1980; Hegyvary & Jørgensen, 1981).

For phosphorylation, (Na,K)-ATPase was solubilized in 15 mM Tes/150 mM NaCl/1.5 mM EDTA/1 mM dithiothreitol to 0.8 mg of protein/mL. Aliquots containing 100–160 $\mu$g were incubated at 2 °C with 30 $\mu$M [$\gamma$-$^{32}$P]ATP (purified Tris salt from Amersham, 0.3 $\mu$Ci/mL) in 3 mL of 150 mM NaCl/3 mM $MgCl_2$/30 mM Tris-HCl, pH 7.5. The reaction was stopped with 3 mL of 8% ice-cold perchloric acid/1 mM pyrophosphate. The protein was separated by centrifugation for 60 min at 18 000 rpm in the Sorwall centrifuge and washed 3 times with 4% perchloric acid/0.5 mM pyrophosphate before counting.

For inactivation studies, 150-$\mu$L aliquots of native or soluble (Na,K)-ATPase preparations were incubated at 0.3–0.5 mg of protein/mL in carefully thermostated water baths at temperatures in the range 28–60 °C. After 2–30 min, 35 $\mu$L was taken out for assay at 20 °C and supplemented with 10 $\mu$L of 650 mM NaCl/100 mM KCl and 5 $\mu$L of 100 mM ATP/100 mM $MgCl_2$ to final concentrations of 130 mM NaCl/20 mM KCl/10 mM ATP/10 mM $MgCl_2$. After 30–60 s at 20 °C, the reaction was stopped with 1 mL of ice-cold 0.5 M HCl containing 30 mg of ascorbic acid/5 mg of ammonium heptamolybdate/10 mg of NaDodSO$_4$. The tubes were transferred to an ice bath. For color development, 1.5 mL containing 30 mg of sodium metaarsenite/30 mg of sodium citrate/30 $\mu$L of acetic acid was added. The tubes were heated for 10 min at 37 °C, and the absorbance was read at 820 nm as before (Brotherus et al., 1983).

Spectrophotometric assay of soluble and membrane-bound (Na,K)-ATPase was performed in a Beckman Acta recording photometer. Reaction was started by adding (Na,K)-ATPase to 3 mL of 20 mM Tes, pH 7.5, in thermostated, stirred cuvettes containing 140 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, and ATP varying between 2 and 2000 $\mu$M. Phosphorylation of ADP from phosphoenolpyruvate (1 mM) was catalyzed by pyruvate kinase (0.03 mg/mL) and reduction of pyruvate to lactate by lactate dehydrogenase (0.03 mg/mL) coupled with oxidation of NADH (0.18 mM) that was monitored at 340 nm. $C_{12}E_8$ (0.1 mg/mL) was present in experiments with soluble (Na,K)-ATPase. The detergent did not interfere with the activity of the enzymes of the coupled assay (Møller et al., 1980).

Molecular sieve HPLC was performed with a TSK 3000 SW (7.5 × 600 mm) Toyo Soda gel filtration column with a TSK SW guard column (Hayashi et al., 1983). They were

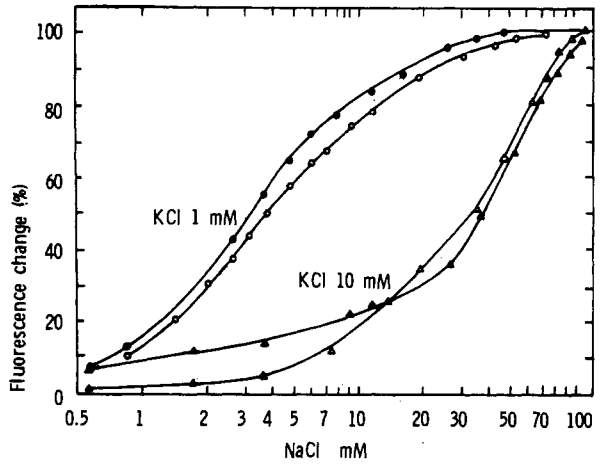


FIGURE 1: Changes in fluorescence from fluorescein bound to soluble (●, ▲) or membrane-bound (○, △) (Na,K)-ATPase. Labeling with fluorescein 5-isothiocyanate as described under Experimental Procedures. 100 μL of soluble (Na,K)-ATPase (0.25 mg of protein/mL) was added to 2.5 mL of 1 mM KCl/10 mM Tes/0.1 mM EDTA, pH 7.5, and fluorescence was measured with excitation at 496 nm, emission at 520 nm, and slit widths of 5 nm on both monochromators. NaCl (5 M) was added from Hamilton syringes mounted on push-bottom dispensers.

operated at flow rates of 0.5 mL/min using a Waters 6000A solvent delivery system. Compositions of elution media are given in the figure legends. For resolution of higher oligomers, two columns were operated in series, first a TSK 4000 SW and then a TSK 3000 SW column, both 7.5 × 600 mm.

## RESULTS

*$E_1$–$E_2$ Transitions in Soluble αβ Units.* To monitor changes in fluorescence intensity, the soluble (Na,K)-ATPase was prepared in KCl in order to avoid inactivation of the $E_1$ form. The soluble (Na,K)-ATPase was suspended in cuvettes containing 1–2 mM KCl and $C_{12}E_8$ in concentrations higher than the critical micelle concentration. Titration with NaCl of the changes in emission from fluorescein covalently attached to the protein is shown in Figure 1. The amplitude and direction of these changes in intensity were similar to those previously observed for the membrane-bound enzyme (Karlish & Yates, 1978; Jørgensen & Karlish, 1980). The $K_{1/2}$ for NaCl for the increase in intensity of fluorescence from fluorescein was 3–4 mM NaCl at 1 mM KCl and 36–40 mM NaCl at 10 mM KCl. Previously, we published experiments demonstrating (Jørgensen, 1983) that changes in tryptophan fluorescence were identical for soluble and membrane-bound (Na,K)-ATPase. The large quenching of fluorescence from fluorescein bound to the α subunit after addition of vanadate or of phosphate and ouabain could also be demonstrated in soluble (Na,K)-ATPase.

*Phosphorylation and Dephosphorylation.* The phosphorylation experiment in Figure 2 was performed to examine if soluble (Na,K)-ATPase can undergo a transition between $E_1P$ and $E_2P$ conformations. $E_1P$, the ADP-sensitive fraction of phosphoenzyme under steady-state conditions, was determined by extrapolation of the dephosphorylation curve after ADP addition at zero time as described in detail before (Jørgensen et al., 1978). For soluble (Na,K)-ATPase, the intercept of the curve was equal to 1.0 at 150 mM NaCl, and $E_1P$ was not demonstrable. At 300 mM NaCl, the intercept was equal to 0.8, thus demonstrating that ADP-sensitive $E_1P$ formed about 20% of total phosphoenzyme in the soluble state. For (Na,K)-ATPase in the membrane-bound state, the intercepts at 150 and 300 mM NaCl, were equal to 0.82 and 0.6, corresponding to 18% and 40% $E_1P$, respectively. Thus, the amount

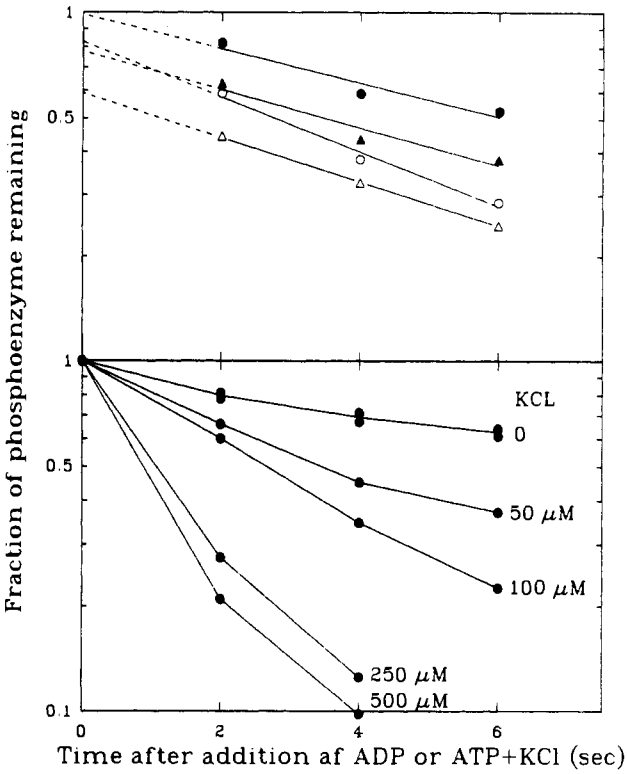


FIGURE 2: ADP-dependent and K-dependent dephosphorylation of soluble (Na,K)-ATPase. (Upper panel) ADP-dependent dephosphorylation of soluble (Na,K)-ATPase (▲, ●) or membrane-bound enzyme (△, ○) at 150 mM NaCl (●, ○) or 300 mM NaCl (▲, △). After phosphorylation for 6 s, 2.5 mM ADP was added, and the amount of phosphoenzyme was determined after 2, 4, or 6 s as described under Experimental Procedures. (Lower panel) Effect of $K^+$ on the rate of dephosphorylation of soluble (Na,K)-ATPase. After phosphorylation for 6 s, 1 mM ATP was added without or with $K^+$ in the concentration range 50–500 μM, and the amount of phosphoenzyme was determined after 2, 4, or 6 s. Zero-time values were determined by adding perchloric acid before ADP or ATP plus $K^+$.

of ADP-sensitive $E_1P$ as a fraction of total phosphoenzyme was smaller for soluble than for membrane-bound enzyme. K-dependent dephosphorylation of soluble (Na,K)-ATPase is demonstrated in the lower part of Figure 2. The soluble phosphoenzyme was more sensitive to $K^+$ than the membrane-bound enzyme. Addition of 100 μM KCl increased the rate constant for dephosphorylation of soluble enzyme 2.8-fold as compared to a 1.8-fold increase in the rate constant for dephosphorylation for membrane-bound (Na,K)-ATPase in previous data (Jørgensen et al., 1978). These data show that the phospho form of soluble (Na,K)-ATPase may exist in both $E_1P$ and $E_2P$ forms. The data furthermore suggest that the equilibrium between the phospho forms is poised in the direction of $E_2$ forms in the soluble enzyme as compared to the membrane-bound enzyme.

*Sedimentation Velocity Studies.* Figure 3 shows sedimentation velocity analysis of the soluble (Na,K)-ATPase both without modification and after covalent labeling with fluorescein isothiocyanate. Analytical ultracentrifugation was performed at relatively high protein concentrations, 0.6–0.7 mg of protein/mL. Analysis of the first derivative (concentration gradient) of the sedimentation profiles showed a high degree of homogeneity with more than 80% of the material sedimenting as a single boundary with $s_{20,w} = 6.9 \pm 0.2$ S. The remainder consisted of faster sedimenting material, well separated from the main component. The value of 6.9 S is consistent with an αβ-protomer structure (Brotherus et al., 1983). This value is slightly lower than the previously reported average sedimentation coefficients (7.2–7.45 S). Fluores-

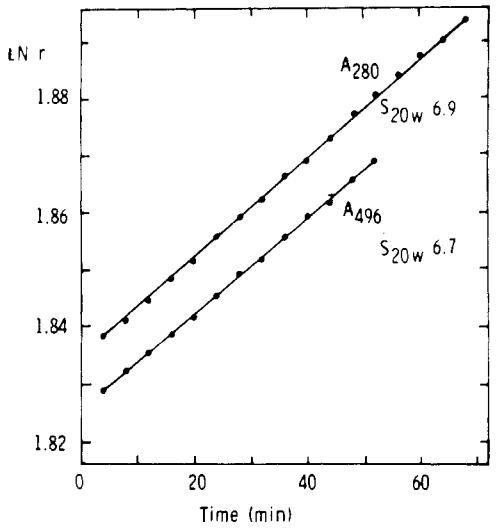


FIGURE 3: Sedimentation velocity analysis of soluble unlabeled (Na,K)-ATPase or fluorescein-labeled (Na,K)-ATPase. Procedure as described under Experimental Procedures. Sedimentation was registered at 280 or 496 nm which is the absorption maximum for fluorescein bound to protein.

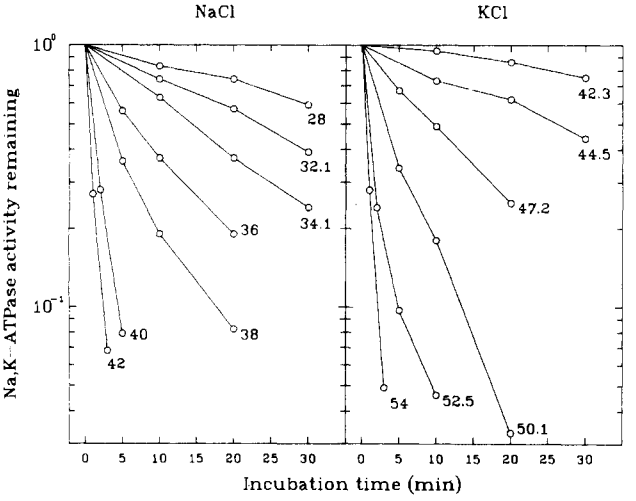


FIGURE 4: Temperature dependence of inactivation of soluble (Na,K)-ATPase in NaCl or KCl as a function of incubation time. Membrane-bound (Na,K)-ATPase was suspended at 0.5 mg of protein/mL in Tes (30 mM)/EDTA (3 mM)/dithiothreitol (2 mM), pH 7.5, and 20 mM NaCl (left panel) or 20 mM KCl (right panel). 800 $\mu$L of this mixture was mixed with 800 $\mu$L of $C_{12}E_8$ (1.2 mg/mL) and incubated for 30 min at 20 °C. After centrifugation for 10 min at 100 000 rpm in the Beckman airfuge, 200-$\mu$L aliquots were incubated in thermostated water baths at temperatures in the range 28–54 °C. After 2–30 min, 35 $\mu$L was taken out for assay at 20 °C as described under Experimental Procedures.

cein-labeled (Na,K)-ATPase used for demonstration of conformational transitions also sedimented as a nearly homogeneous preparation with a sedimentation coefficient of 6.8 ± 0.2 S for the main component, whether scanning was done at the absorption maximum for fluorescein, 496 nm, or at 280 nm. The sedimentation analysis thus demonstrated that also at the higher protein concentrations used in this work the soluble (Na,K)-ATPase from kidney consists predominantly of $\alpha\beta$ units.

*Thermolability of $E_1$ Forms of Soluble (Na,K)-ATPase.* Figure 4 shows semilogarithmic plots of (Na,K)-ATPase activity measured at various time intervals after incubation of soluble (Na,K)-ATPase in either NaCl or KCl medium in suitable ranges of temperatures. The soluble enzyme is irreversibly inactivated during the incubation period. The inactivation appears to be a monoexponential process with rate

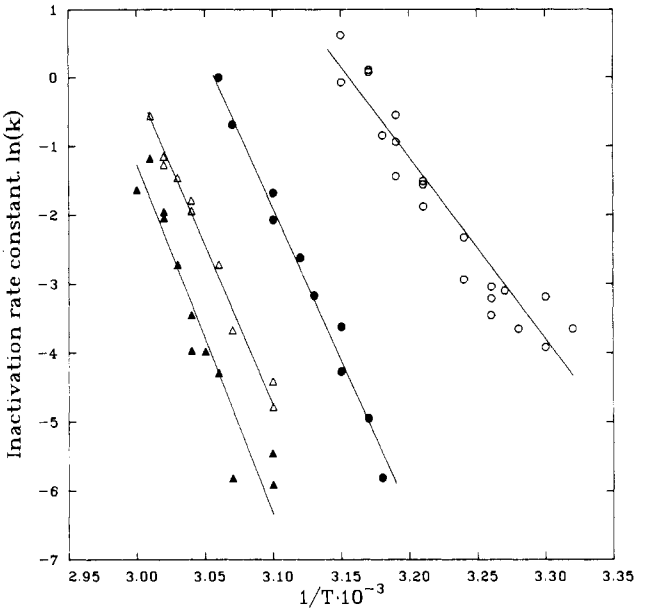


FIGURE 5: Arrhenius plots of inactivation rate constants (ln $k$) vs. temperature ($1/T$, $T$ in degrees kelvin). The rate constants were calculated from inactivation curves as in Figure 4 for membrane-bound (Na,K)-ATPase in KCl (▲) or NaCl (△) and for soluble (Na,K)-ATPase in KCl (●) or NaCl (○). Thermodynamic parameters calculated from the data appear in Table I.

Table I: Thermodynamic Data of Inactivation of (Na,K)-ATPase

| state and conformation | activation energy, $E_a$ (kcal/mol) | enthalpy of activation, $\Delta H^{\ddagger}$ (kcal/mol) | entropy of activation, $\Delta S^{\ddagger}$ [kcal/(mol·K)] | activation free energy, $\Delta G^{\circ\,\ddagger}$ (kcal/mol) |
|---|---|---|---|---|
| membrane, KCl | 100.7 | 100.1 | 0.231 | 28.6 |
| membrane, NaCl | 92.0 | 91.4 | 0.207 | 27.3 |
| soluble, KCl | 87.8 | 87.2 | 0.200 | 25.3 |
| soluble, NaCl | 52.3 | 51.7 | 0.106 | 18.1 |

constants that increase steeply with increasing temperature. It is seen that the enzyme is much more labile in NaCl than in KCl. As demonstrated by Fischer (1983), the $E_1$Na form of membrane-bound (Na,K)-ATPase was also more thermolabile than the $E_2$K form, but this difference was small when compared with the pronounced difference in thermolability of the soluble forms.

Arrhenius plots of the rate constants measured at different temperatures for both membrane-bound and soluble enzyme in the presence of NaCl or KCl are linear as shown in Figure 5. The slopes of the lines and the ordinate intercepts for the membrane-bound (Na,K)-ATPase in NaCl or KCl and for the soluble (Na,K)-ATPase in KCl differ little, whereas the line for the rate constants of soluble (Na,K)-ATPase in NaCl is displaced to the right with significantly smaller values for both slope and intercept. Having obtained the activation energy ($E_z$) and the frequency factor (A) from the Arrhenius plots (slope equal to $-E_a/R$, ordinate intercept equal to ln $A$), it is possible to calculate the enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation from the equations:

$$E_a = RT + \Delta H^{\ddagger}$$

and

$$A = (RT/Nh)e^{(\Delta S^{\ddagger}+R)/R}$$

The free energy of activation is calculated as

$$\Delta G^{\circ\,\ddagger} = -RT \ln (AhN/RT) + E_a$$

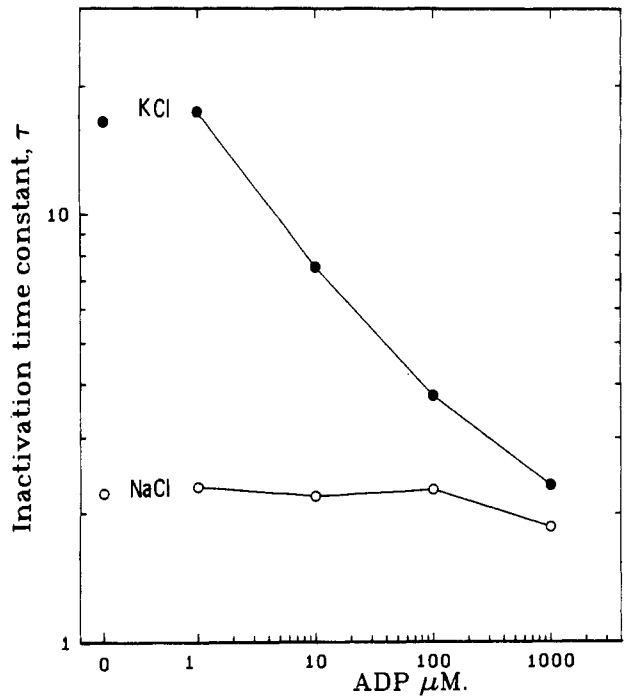


FIGURE 6: Effect of ADP on inactivation of soluble (Na,K)-ATPase in NaCl (○) or KCl (●) at 40 °C. Procedure as in Figure 4 with addition of ADP–Tris or Tris-HCl to constant ionic strength. The time constant ($\tau$) was calculated from linear inactivation curves as the time required to reach $1/e$ of the original (Na,K)-ATPase activity.

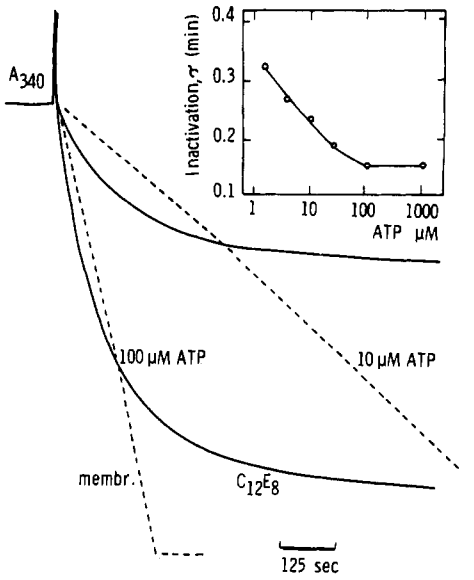


FIGURE 7: Effect of ATP on the rate of inactivation of soluble (Na,K)-ATPase. Spectrophotometric assay of soluble and membrane-bound (Na,K)-ATPase was performed at 38 °C in a Beckman Acta recording photometer as described under Experimental Procedures. Reaction was started by adding soluble (—) or membrane-bound (- - -) (Na,K)-ATPase to the cuvettes. The inactivation time constant was calculated as the time required to reach $1/e$ of the initial velocity from the slopes of activity curves for soluble (Na,K)-ATPase vs. time.

The results are shown in Table I. It is seen that both activation enthalpies and activation entropies are larger in the presence of *K* than in the presence of Na, irrespective of whether the enzyme is membrane bound or soluble. The difference between K and Na forms is most pronounced for the soluble (Na,K)-ATPase. In the presence of Na, solubilization lowers $\Delta G^{\circ\ddagger}$ approximately 9 kcal/mol, in agreement with the labilization induced by solubilization in this condition.

The experiment in Figure 6 was done to examine if the thermolability depends on occupancy of ligand binding sites by $Na^+$ or $K^+$ or if it is related to the conformational state of the protein. The data show that ADP with $K_{1/2}$ = 8–10 μM increased the rate of thermoinactivation in KCl medium. Transition from $E_2K$ to $E_1$ with formation of the ternary complex ADP–$E_1K$ increased the rate of inactivation to the level observed for the $E_1Na$ form. Binding of the nucleotide did not protect against inactivation in NaCl medium.

These observations suggested that the inactivation of soluble (Na,K)-ATPase during hydrolysis of ATP would depend on the conformational equilibrium. To test this, inactivation rates were examined during turnover at different ATP concentrations. At 130 mM $Na^+$ and 20 mM $K^+$, a large fraction of the membrane-bound enzyme accumulates in the $E_2K$ form at low ATP concentration, due to potassium–nucleotide antagonism, while at high ATP concentration a larger fraction is present as $E_1$ forms in the steady state. In the case of the soluble monomeric enzyme, the rate of thermoinactivation increased when the ATP concentration was increased in the range from 2 to 100 μM (Figure 7). In conjunction with the data showing higher thermolability of $E_1$ forms, this result indicates that during turnover of the soluble enzyme, the $E_1$–$E_2$ equilibrium depends on the ATP concentration, qualitatively in the same manner as the equilibrium in membrane-bound enzyme.

The initial velocities were higher for soluble than for membrane-bound (Na,K)-ATPase. Determination of initial velocities at 2–200 μM ATP showed that the half-maximum concentration for ATP was 33 μM for soluble (Na,K)-ATPase as compared to 107 μM for membrane-bound enzyme [cf. Huang et al. (1985)]. The reason for the higher apparent affinity of soluble (Na,K)-ATPase for ATP is not clear.

*HPLC Gel Chromatography.* HPLC chromatography on TSK gel filtration columns has been shown to provide efficient methods for separation of monomer and oligomer forms of soluble (Na,K)-ATPase (Hayashi et al., 1983). Figure 8 shows that the large peak eluting at 28–29 min after charging soluble (Na,K)-ATPase on a TSK 3000 SW column has a molecular weight of 143 000 as determined by sedimentation equilibrium analysis in the analytical ultracentrifuge. This result is in agreement with our previous analysis (Brotherus et al., 1983). It shows that the peak eluting at 28–29 min consists of $\alpha\beta$ units.

Using analytical ultracentrifugation, we previously demonstrated aggregation of soluble $\alpha\beta$ units of (Na,K)-ATPase and monomeric Ca-ATPase to higher oligomers upon storage (Brotherus et al., 1981). Chromatography on TSK 3000 SW columns shows that soluble $\alpha\beta$ units of (Na,K)-ATPase aggregate to $(\alpha\beta)_2$ units and higher oligomers when stored at 20 °C. Similarly, Figure 9 shows that Ca-ATPase from sarcoplasmic reticulum with $M_r$ 115 000 undergoes aggregation to dimers and higher oligomers.

Data in Figures 4 and 5 demonstrate the widely different rates of thermal inactivation of soluble (Na,K)-ATPase in NaCl and KCl medium. In the experiment in Figure 10, it was examined if this inactivation is accompanied by aggregation of the soluble $\alpha\beta$ units. It is seen that in parallel with the faster inactivation of soluble (Na,K)-ATPase in the $E_1$ form aggregation is also faster in NaCl than in KCl medium. In both media, the amount of soluble $\alpha\beta$ units expressed as a fraction of total protein was reduced at a slower rate than the rate of inactivation of (Na,K)-ATPase. This suggests that aggregation is secondary to denaturation of the protein. These aggregates are stable in $C_{12}E_8$, but they dissolve in $NaDodSO_4$ plus mercaptoethanol. Thus, after thermal inactivation of soluble (Na,K)-ATPase, the appearance of $\alpha$ and $\beta$ subunits

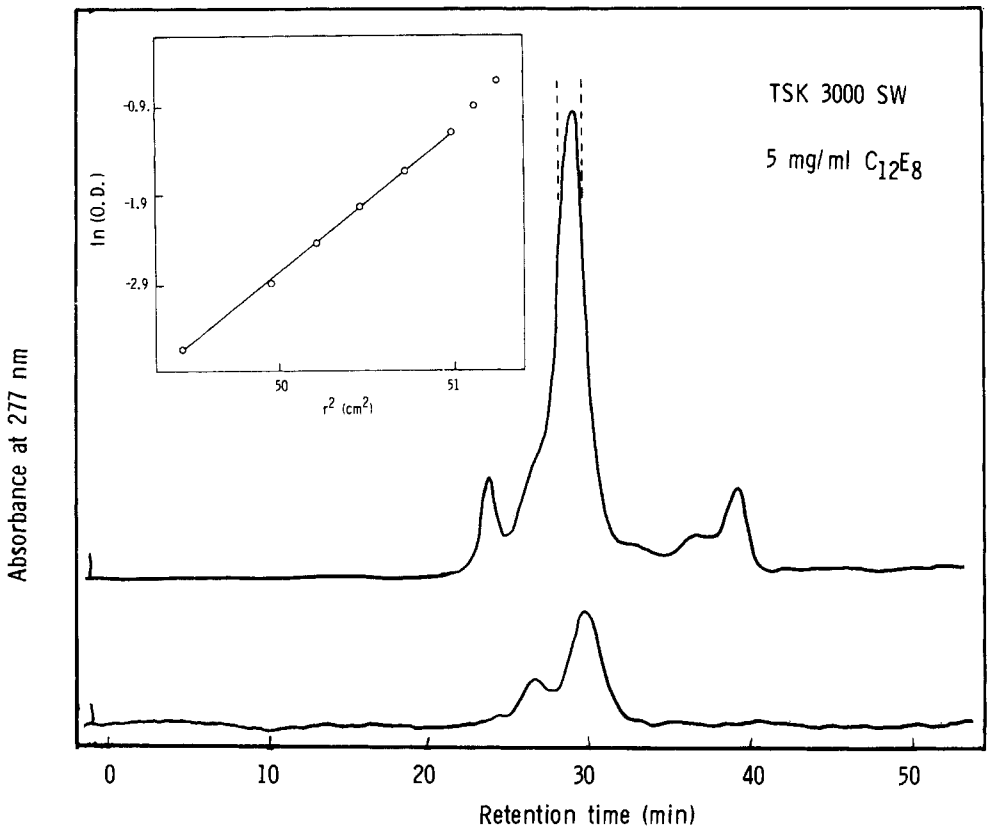


FIGURE 8: Analytical ultracentrifugation of the protein peak eluted from a 7.5 × 600 mm TSK-GEL G 3000 SW column equilibrated with 5 mg/mL $C_{12}E_8$/150 mM potassium acetate/25 mM Tris–acetate, pH 7.0, and operated at 0.5 mL/min. For preparation of sample, (Na,K)-ATPase (5 mg of protein/mL) was solubilized in 12.5 mg/mL $C_{12}E_8$/150 mM potassium acetate/25 mM Tris–acetate, pH 7.5. After centrifugation in the airfuge, an aliquot of 100 μL was injected into the column. Fractions containing the peak of protein eluting at 28–29 min were divided in two aliquots. One was used as a sample for sedimentation equilibrium centrifugation in a Beckman analytical ultracentrifuge as described under Experimental Procedures. The other was stored at 20 °C and rechromatographed after 16 h to produce the lower tracing. The inset shows the sedimentation equilibrium data. Molecular weight was calculated from the linear part of the plot.

after electrophoresis in $NaDodSO_4$ was identical with that of native (Na,K)-ATPase (not shown).

These data led us to examine if inactivation of (Na,K)-ATPase in the membrane-bound state also is accompanied by aggregation. Membrane-bound (Na,K)-ATPase was incubated at 57 °C and subsequently solubilized in $C_{12}E_8$ and injected into TSK 4000–3000 SW columns. The data in Figure 11 show that disappearance of $\alpha\beta$ units is faster in NaCl than in KCl medium, in agreement with the faster rate of inactivation of membranous (Na,K)-ATPase in NaCl medium. Also in the membrane, thermoinactivation of (Na,K)-ATPase is accompanied by aggregation of $\alpha\beta$ units to oligomers that are insoluble in $C_{12}E_8$. The aggregates did dissolve in $NaDodSO_4$ plus mercaptoethanol, and the gel electrophoresis pattern remained similar to that of native (Na,K)-ATPase. Some aggregates remain undissolved in the absence of mercaptoehtanol (not shown), suggesting that formation of S–S bridges may in part explain the aggregation. Otherwise, the nature of the association between $\alpha\beta$ units in these aggregates remains to be examined.

## Discussion

The data show that soluble (Na,K)-ATPase in the monomer state as $\alpha\beta$ units can undergo transitions between $E_1$ and $E_2$ forms of the $\alpha$ subunit that are similar to if not identical with the conformational transitions in membrane-bound (Na,-K)-ATPase. In the analytical ultracentrifuge, the soluble (Na,K)-ATPase in $C_{12}E_8$ has an $s_{20,w}$ of 6.8–6.9 S even at relatively high protein concentrations. A molecular weight of 143 000 for the $\alpha\beta$ unit is obtained by sedimentation equilibrium analysis after high-resolution gel chromatography. Sedimentation velocities are the same in media containing NaCl or KCl, and they are unaffected by addition of the ligands ($Mg^{2+}$, vanadate, or phosphate) that are known to stabilize $E_2P$ or $E_2$–vanadate forms of the protein (Brotherus et al., 1983). The gel chromatography data also confirm that the soluble (Na,K)-ATPase used in the present study consists predominantly (>80%) of protomeric $\alpha\beta$ units. In the high-resolution gel filtration system, the distribution between monomer and oligomer units is also independent of whether the enzyme is in the $E_1Na$ or the $E_2K$ conformation. Our data therefore allow the conclusion that dephospho forms of soluble $\alpha\beta$ units can undergo $E_1$–$E_2$ transitions. Transitions between $E_1Na$ and $E_2K$ forms of the $\alpha$ subunit have been shown to be related to cation binding and translocation in a passive exchange mode (Karlish & Stein, 1982). In the absence of ligands other than cations, the $\alpha\beta$ unit may therefore be competent as a passive (Na,K)-transport system, and the $\alpha$–$\alpha$-subunit interaction does not seem to be an obligatory element in the $E_1$–$E_2$ conformational transition.

The decision whether the $\alpha\beta$ unit can catalyze ATP-driven active (Na,K)-transport requires demonstration that the protein remains monomeric during ATP hydrolysis. The specific activity of soluble (Na,K)-ATPase containing more than 80% $\alpha\beta$ units is the same as for membrane-bound enzyme (Brotherus et al., 1983). This very high activity can only be attributed to oligomer forms (<20%) if they have about 5-fold higher specific activities than the membrane-bound (Na,-K)-ATPase.

Each soluble $\alpha\beta$ unit in $C_{12}E_8$ binds one molecule of ATP (Jensen & Ottolenghi, 1983). The two phosphoenzyme forms, $E_1P$ and $E_2P$, can be demonstrated in the soluble preparation,

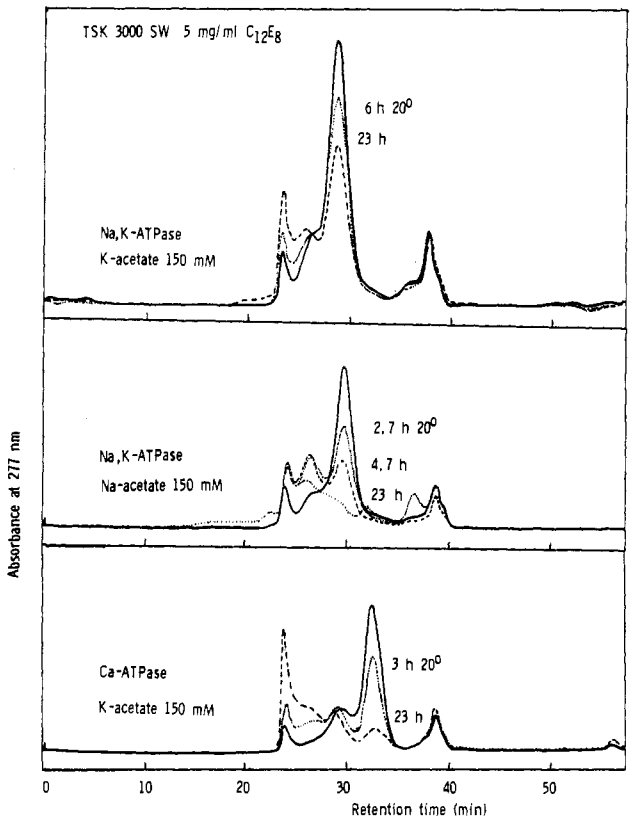


FIGURE 9: Time-dependent aggregation of soluble (Na,K)-ATPase and Ca-ATPase. (Na,K)-ATPase (2 mg of protein/mL) was solubilized in 5 mg/mL $C_{12}E_8$ in 150 mM potassium acetate or sodium acetate/25 mM Tris–acetate, pH 7.5, and incubated at 20 °C. Ca-ATPase from sarcoplasmic reticulum was solubilized as described above in potassium acetate. After the indicated time, aliquots of 100 μL were injected into a TSK 3000 SW column equilibrated with 5 mg/mL $C_{12}E_8$/150 mM potassium acetate/25 mM Tris–acetate, pH 7.0, and operated at 0.5 mL/min.

and the turnover-dependent inactivation experiment (Figure 7) shows that the (Na,K)-ATPase reaction involves $E_1$–$E_2$ transitions. We have shown that the soluble monomeric $\alpha\beta$ unit can assume the dephospho forms, $E_1$Na and $E_2$K, and the phosphorylated $E_2$P form. These are three of the four major conformations that the protein must pass to carry out the transport reaction in the $E_1$–$E_2$ reaction cycle of the Na,K pump. The only reasonable possibility for attributing the (Na,K)-ATPase activity to oligomer forms of the protein is that ATP binding or formation of the $E_1$P form induces oligomerization of $\alpha\beta$ units. The possibility for dimerization-dependent activity has been considered for soluble Ca-ATPase from sarcoplasmic reticulum. Martin (1983) has calculated that this would require bimolecular diffusion rate constants beyond the currently accepted limits for diffusion-limited protein–protein interactions.

Evidence that the monomer Ca-ATPase possesses ATPase activity has been obtained by the active enzyme centrifugation (Martin, 1984). High-resolution chromatography of Ca-ATPase shows that binding of vanadate or ATP as well as phosphoenzyme turnover shifts the equilibrium toward the monomer forms (Andersen & Vilsen, 1985). Neither of these experiments shows directly whether ATP hydrolysis by the monomer is coupled to transport, but it has been shown that $E_1$–$E_2$ transitions are associated with Ca release from soluble monomeric Ca-ATPase (Andersen et al., 1985).

Conformational transitions have previously been demonstrated in renal (Na,K)-ATPase after solubilization in Lubrol WX or Brij 58 (Steinberg & George, 1982), but the aggregation state of the enzyme in detergent solution was not examined. The soluble (Na,K)-ATPase from shark rectal gland also undergoes transitions between $E_1$ and $E_2$ forms and occludes $Rb^+$ ions (Esmann, 1985), but this preparation consists predominantly of $(\alpha\beta)_2$ units (Esman & Skou, 1984).

The thermoinactivation experiments demonstrate that the $E_1$ and $E_2$ forms can be distinguished in the soluble and membrane-bound states by differences in the susceptibility of (Na,K)-ATPase to irreversible inactivation. The inactivation process is strongly temperature dependent in all conditions, but inactivation proceeded faster in the presence of ligands stabilizing the $E_1$ form of the protein than in the presence of ligands inducing the $E_2$ state. The enthalpies and entropies of activation of the $E_2$K form are much larger than for the $E_1$Na form of the soluble protein. Similar but much smaller differences are observed for membranous (Na,K)-ATPase, in agreement with Fischer (1983). The difference in stability between the $E_1$Na form and the $E_2$K form is not due to simple protection of ligand binding sites, since nucleotide binding promotes inactivation in medium containing K. We therefore conclude tht the lower enthalpy and entropy of activation of the $E_1$ form relative to the $E_2$ form must depend on the protein structure, per se, or on the interaction of protein with lipid and water.

The finding that aggregation of $\alpha\beta$ units to higher oligomers accompanies thermal inactivation in both soluble and membranous (Na,K)-ATPase suggests that the steps of the denaturation process are the same in the two states. It is conceivable that thermal inactivation of (Na,K)-ATPase activity involves an increasing exposure of hydrophobic residues to polar solvent with resulting destabilization of protein structure (Tanford, 1970). The exposure of hydrophobic residues may be accompanied by an increased tendency for aggregation due to hydrophobic association between $\alpha\beta$ units. This would explain the faster inactivation of the soluble (Na,K)-ATPase since the hydrophobic residues are better shielded from solvent in the membrane than after solubilization. Inadequate covering of the hydrophobic residues by the relatively short alkyl chains of $C_{12}E_8$ may increase unfavorable interactions between protein and solvent, thus accounting for the extraordinary low free energy of activation in the soluble $E_1$ form. The difference in the free energy of activation between soluble and membranous enzymes is much larger for $E_1$ forms than for $E_2$ forms. This suggests that $E_1$–$E_2$ transitions involve parts of the protein that are normally engaged in lipid–protein associations. In agreement with this interpretation, preferential labeling of the $E_2$ form of membranous (Na,K)-ATPase from within the lipid bilayer with the hydrophobic [$^{125}$I]iodonaphthyl azide (Karlish et al., 1977) suggests that the $E_1$–$E_2$ conformational transition involves a decrease in protein–lipid interactions.

This situation is comparable to that observed for sarcoplasmic reticulum Ca-ATPase (Andersen et al., 1982; Martin et al., 1984). In the soluble monomeric state, this protein is also much less stable in one of the two major conformations than in the other. However, in this case, the $E_2$ form in the absence of $Ca^{2+}$ is the less stable. $Ca^{2+}$ and nucleotides stabilize the $E_1$ form and protect against inactivation. This "opposite behavior" relative to (Na,K)-ATPase is paralleled by opposite directions of the changes in intensities of fluorescence from intrinsic tryptophan fluorescence or fluorescein in relation to the $E_1$–$E_2$ transition (Andersen et al., 1982). Thus, Ca-ATPase behaves as if it has more hydrophobic residues exposed to solvent in the $E_2$ state than in the $E_1$ state. The irreversible inactivation of the $E_2$ conformation

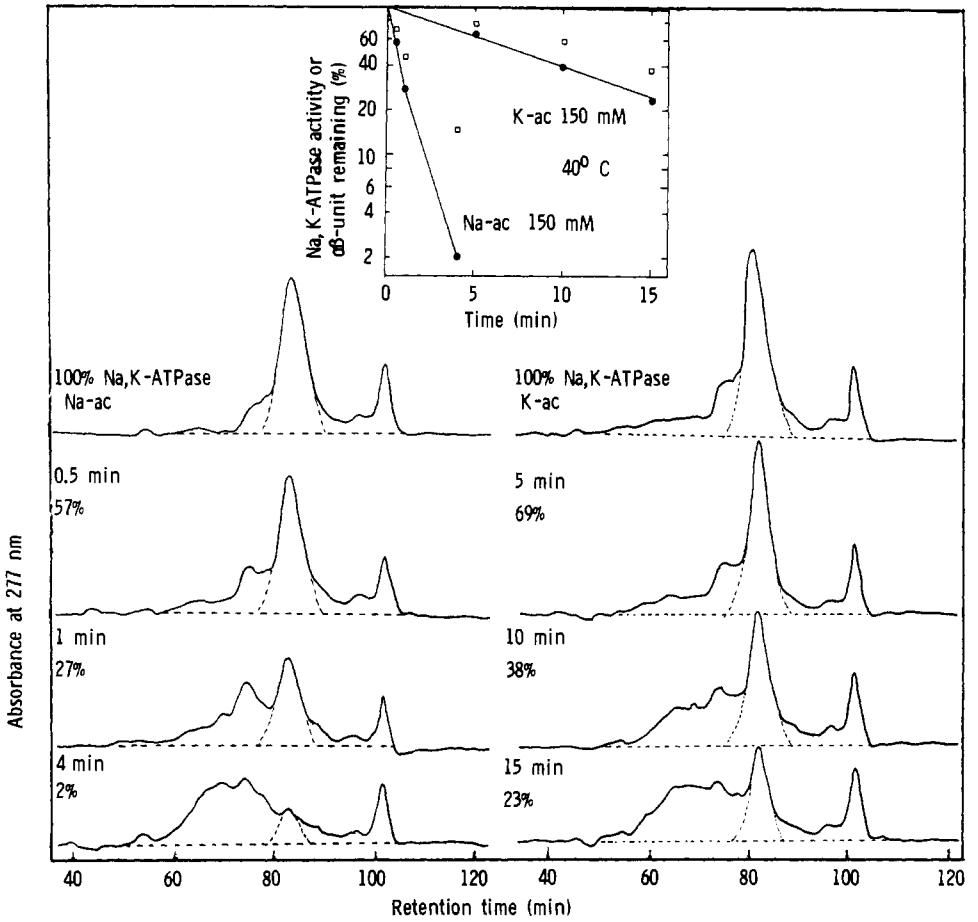


FIGURE 10: Inactivation and aggregation of soluble (Na,K)-ATPase in $Na^+$- or $K^+$-containing media. (Na,K)-ATPase was solubilized as in Figure 9 and incubated at 40 °C. At the indicated times, aliquots of 100 μL were taken out for assay of (Na,K)-ATPase, and aliquots of 100 μL were injected into TSK 4000 SW and TSK 3000 SW columns operated in series at 0.5 mL/min and equilibrated with 2 mg/mL $C_{12}E_8$/150 mM sodium acetate or potassium acetate/25 mM Tris–acetate, pH 7.0. The inset shows the decay of (Na,K)-ATPase activity (●) and of the amount of αβ units as a fraction of total protein (□).

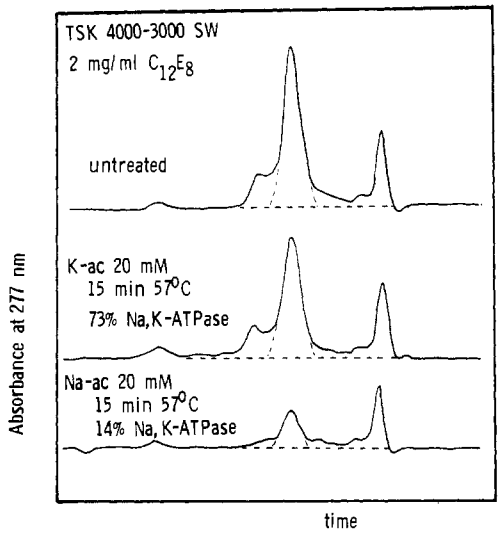


FIGURE 11: Inactivation and aggregation of membrane-bound (Na,K)-ATPase. The enzyme was suspended at 1 mg of protein/mL in 20 mM potassium acetate or sodium acetate/25 mM Tris–acetate, pH 7.5. After incubation for 15 min at 57 °C, (Na,K)-ATPase was sedimented at 100 000 rpm for 10 min in the Beckman airfuge and resuspended at 2 mg of protein/mL in 5 mg/mL $C_{12}E_8$/150 mM potassium acetate/25 mM Tris–acetate, pH 7.5. Aliquots of 100 μL were injected into the TSK 4000 and 3000 SW columns used for the experiment in Figure 10.

of Ca-ATPase has been shown to involve a decrease in α-helix content as monitored by circular dichroism and an increased exposure of sulfhydryl groups to solvent (Andersen et al., 1980).

## ACKNOWLEDGMENTS

We thank Lene Jacobsen for excellent technical assistance.

**Registry No.** ADP, 58-64-0; ATP, 56-65-5; ATPase, 9000-83-3; $C_{12}E_8$, 101226-62-4; K, 7440-09-7; Na, 7440-23-5.

## REFERENCES

Andersen, J. P., & Vilsen, B. (1985) *FEBS Lett. 189*, 13–17.
Andersen, J. P., Le Maire, M., & Møller, J. V. (1980) *Biochim. Biophys. Acta 603*, 84–100.
Andersen, J. P., Møller, J. V., & Jørgensen, P. L. (1982) *J. Biol. Chem. 257*, 8300–8307.
Andersen, J. P., Jørgensen, P. L., & Møller, J. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4573–4577.
Brotherus, J. R., Møller, J. V., & Jørgensen, P. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 146–154.
Brotherus, J. R., Jacobsen, L., & Jørgensen, P. L. (1983) *Biochim. Biophys. Acta 731*, 290–303.
Craig, W. S. (1982) *Biochemistry 21*, 5707–5717.
Esmann, M. (1985) *Biochim. Biophys. Acta 815*, 196–202.
Esmann, M., & Skou, J. C. (1984) *Biochim. Biophys. Acta 787*, 71–80.
Fischer, T. H. (1983) *Biochem. J. 211*, 771–774.
Hayashi, Y., Takagi, T., Maezawa, S., & Matsui, H. (1983) *Biochim. Biophys. Acta 748*, 153–167.

Hebert, H., Jørgensen, P. L., Skriver, E., & Maunsbach, A. B. (1982) *Biochim. Biophys. Acta 689*, 571–574.
Hegyvary, C., & Jørgensen, P. L. (1981) *J. Biol. Chem. 256*, 6296–6303.
Huang, W. H., Kakar, S. S., & Ascari, A. (1985) *J. Biol. Chem. 260*, 7356–7361.
Jensen, J., & Ottolenghi, P. (1983) *Biochim. Biophys. Acta 731*, 282–289.
Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Jørgensen, P. L. (1983) in *Structure and Function of Membrane Proteins* (Palmieri et al., Eds.) pp 245–254, Elsevier, Amsterdam.
Jørgensen, P. L., & Karlish, S. J. D. (1980) *Biochim. Biophys. Acta 597*, 305–317.
Jørgensen, P. L., Klodos, I., & Petersen, J. (1978) *Biochim. Biophys. Acta 507*, 8–16.
Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111–136.
Karlish, S. J. D., & Yates, D. W. (1978) *Biochim. Biophys. Acta 527*, 115–130.
Karlish, S. J. D., & Stein, W. D. (1982) *J. Physiol. (London) 328*, 295–316.
Karlish, S. J. D., Jørgensen, P. L., & Gitler, C. (1977) *Nature (London) 269*, 715–717.
Martin, D. W. (1983) *Biochemistry 22*, 2276–2282.
Martin, D. W., Tanford, C., & Reynolds, J. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6623–6626.
Møller, J. V., Lind, K. E., & Andersen, J. P. (1980) *J. Biol. Chem. 255*, 1912–1920.
Steinberg, M., & George, J. J. D. (1982) *Arch. Biochem. Biophys. 219*, 359–365.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2369–2376.

# Triplet–Singlet Energy Transfer in the Complex of Auramine O with Horse Liver Alcohol Dehydrogenase†

Jeffry G. Weers‡ and August H. Maki*
*Department of Chemistry, University of California, Davis, California 95616*
*Received September 27, 1985; Revised Manuscript Received December 17, 1985*

ABSTRACT: Triplet–singlet energy transfer has been studied in the complex formed between auramine O (AO) and horse liver alcohol dehydrogenase with optically detected magnetic resonance (ODMR) spectroscopy. The results show that Trp-15 and Tyr residues transfer triplet energy mainly by a trivial process, whereas Trp-314 transfers triplet energy by a Förster process with two observed lifetimes at 77 K of 170 and 50 ms. The different Förster energy-transfer lifetimes are ascribed either to quenching of the two Trp-314 residues of the dimer by a single asymmetrically bound AO or to two distinct conformations of the enzyme–dye complex with differing separations and/or orientations of donor and acceptor. Individual spin sublevel transfer rate constants are reported for the major decay component with the 170-ms Trp triplet-state lifetime; these are found to be highly selective with $k_x^{tr} \gg k_y^{tr}$ and $k_z^{tr}$.

The development of chemical modification techniques for proteins has led to important information regarding the structure, function, and dynamics of many proteins (Means & Feeney, 1971). In particular, introduction of fluorophores into protein structures gives the experimentalist a direct probe for obtaining this information (Cantor & Schimmel, 1980; Chen & Edelhoch, 1975). The binding of dyes to globular proteins has received considerable attention [Glazer (1970) and refereces cited therein]. Many dyes have the interesting property of being virtually nonfluorescent in aqueous solution but become highly fluorescent when bound to proteins (Edelman & McClure, 1968; Turner & Brand, 1968; Chen, 1977). In addition to the dramatic change in their radiative quantum yields in various environments, the dye fluorescence maximum exhibits large shifts. Also, denaturation of the protein or the binding of small molecules such as cofactors or substrates can lead to differences in dye properties (Velick, 1961; Weber & Daniel, 1966; Heitz & Brand, 1971). Glazer (1970) has reviewed a number of dye–protein complexes and has shown that the strong binding of dyes to simple globular proteins generally takes place in areas overlapping the binding sites for substrates, coenzymes, or prosthetic groups, in preference to other regions of the protein structure. Thus, dyes are useful probes for investigating protein structure and function near enzyme active sites.

An enzyme–dye complex that has received considerable attention is that formed between the cationic dye auramine O (AO)[1] and the enzyme horse liver alcohol dehydrogenase (HLAD) (Conrad et al., 1970; Sigman & Glazer, 1972; Chen, 1977; Heitz & Brand, 1971). AO has a fluorescence quantum yield of $4 \times 10^{-5}$ in aqueous solution, which increases over 1000-fold to $5.5 \times 10^{-2}$ when it is bound to HLAD (Chen, 1977). By monitoring changes in the AO binding and fluorescence properties upon addition of certain active site directed compounds, Heitz and Brand (1971) were able to identify the location of the dye binding site. The dye was found to bind directly adjacent to the active site in a region overlapping the cyclohexanol binding site but not the ethanol binding site.

† This research was supported by a grant from the National Science Foundation.
‡ Present address: Clorox Technical Center, Pleasanton, CA 94566.

[1] Abbreviations: ODMR, optical detection of triplet-state magnetic resonance; AO, auramine O; HLAD, horse liver alcohol dehydrogenase; MIDP(L), microwave-induced delayed phosphorescence (luminescence); Trp, tryptophan; Tyr, tyrosine.

0006-2960/86/0425-2897$01.50/0 