Biochimica et Biophysica Acta, 693 (1982) 34–44
Elsevier Biomedical Press

BBA 71452

CHARACTERIZATION OF PARTIALLY PURIFIED ($Na^+ + K^+$)-ATPase FROM PORCINE LENS

PARIMAL C. SEN and DOUGLAS R. PFEIFFER

Hormel Institute, University of Minnesota, Austin, MN 55912 (U.S.A.)

(Received April 27th, 1982)

Key words: ($Na^+ + K^+$)-ATPase; Protein purification; Membrane fluidity; Cataract; (Porcine lens)

The partial purification of ($Na^+ + K^+$)-ATPase from pig lens has been achieved by treatment with deoxycholate followed by density gradient centrifugation. The specific activity of the final preparation, ranging from 300 to 500 nmol/h per mg protein, is increased approx. 100-fold compared to the homogenate. A parallel increase in *p*-nitrophenylphosphatase activity is also observed. Sodium dodecyl sulfate (SDS) gel electrophoresis reveals six major protein bands, one of which is the 93 kDa α subunit of ($Na^+ + K^+$)-ATPase which can be phosphorylated by reaction with [γ-^{32}P]ATP. A second band contains a glycoprotein which displays an apparent molecular weight of 51000 and thus appears to be the β subunit of the enzyme. The enzyme is sensitive to ouabain with the I_{50} for ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase inhibition being 1.2 and 1.3 μM, respectively. Several agents which inhibit ($Na^+ + K^+$)-ATPase from other tissues such as oligomycin, Ca^{2+}, vanadate, *N*-ethylmaleimide, *p*-chloromercuribenzenesulfonic acid (PCMBS) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) also inhibit the lens enzyme. Monovalent cations other than K^+ are partially effective in activating the ($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase activities. The K^+ congeners were relatively more effective in supporting ($Na^+ + K^+$)-ATPase compared to *p*-nitrophenylphosphatase activity. Other kinetic properties of the lens enzyme are also comparable to those of the enzyme from other tissues. Utilizing the partially purified membrane bound enzyme, discontinuities in Arrhenius plots of ($Na^+ + K^+$)-ATPase activity, *p*-nitrophenylphosphatase activity and fluoresence polarization of the fluidity probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), are observed near the physiological temperature of lens. The possible significance of these observations for the mechanism of cataract formation are discussed.

Introduction

The ($Na^+ + K^+$)-ATPase (ATP-phosphohydrolase, EC 3.6.1.3) has been shown to be responsible for the energy-dependent transport of Na^+ and K^+ across cell membranes (see Refs. 1–3 for reviews). The enzyme has been purified from a number of sources [4–8] and the properties have been widely studied. Purification and characterization of ($Na^+ + K^+$)-ATPase from lens is of considerable interest because of the important role played by this enzyme in the inward transport of K^+ and outward transport of Na^+ in fiber cells (see Refs. 9 and 10 for reviews). An abnormal distribution of Na^+ and K^+ in this tissue can lead to the loss of osmotic regulation and subsequent cataract formation [11]. Loss of ($Na^+ + K^+$)-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMBS, *p*-chloromercuribenzenesulfonic acid; SDS, sodium dodecyl sulfate.

0005-2736/82/0000–0000/$02.75

ATPase activity has been reported to occur during cataract formation in the galactose-fed rat [12], the triparanol-fed rat [13], in certain animal hereditary cataracts [14], and in human senile cataracts [15]. It has thus been proposed that a failure of this enzyme may be a primary defect leading to the development of several types of cataracts [11,14].

Despite the apparent central role of $(Na^+ + K^+)$-ATPase in the maintenance of lens transparency, purification and characterization of the enzyme from this tissue has not been previously reported. One reason for this may be that the enzyme activity is very low in lens. In addition, the methods available for the purification of $(Na^+ + K^+)$-ATPase from other tissue [4–8] are not directly applicable to lens because fiber cell plasma membranes, which contain the enzyme, have unusual lipid properties [13,16] and also contain large amounts of other tightly bound intrinsic proteins [17]. In the present communication, we describe the partial purification of $(Na^+ + K^+)$-ATPase from pig lens by a detergent extraction method. The enzyme is about 6% pure compared to total protein. Subunit phosphorylation, ouabain binding capacity, and the kinetic parameters for substrates, cofactors and inhibitors of the partially purified enzyme have also been investigated.

Materials

Adenosine 5′-triphosphate (Tris salt), *p*-nitrophenyl phosphate (Tris salt), other nucleotides, Trizma base, ethylenediaminetetraacetic acid (EDTA), imidazole-HCl, histidine-HCl, ouabain octahydrate, bovine serum albumin (crystalline lyophilized, fraction V), glycylglycine-HCl and sodium deoxycholte, were purchased from the Sigma Chemical Co. Ultrapure NaCl and KCl were obtained from Alfa Products. All reagents used for gel electrophoresis were from BioRad Laboratories; [^{3}H]ouabain and [γ-^{32}P]ATP were obtained from New England Nuclear. The radiochemical purity of the [^{3}H]ouabain was determined by thin-layer chromatography on Silica gel G, developed with chloroform/methanol/water (65 : 30 : 5, v/v) and found to be greater than 98%. The remaining reagents utilized were from the Fisher Chemical Co. (A.C.S. grade).

Methods

Collection of lenses and isolation of the fiber cell membrane fraction

Fresh pig eyes were obtained from a local slaughterhouse and brought to the laboratory on ice. Subsequent procedures were carried out at 0–4°C. The lenses were obtained, and the capsules plus the adhering epithelial cell layer were removed by dissection. Four to five lenses were homogenized in a Dounce apparatus (20 strokes) with 10 vol. of 50 mM Tris-HCl, pH 7.5, containing 5 mM sodium-EDTA (homogenate). Approx. 0.5 ml of the homogenate was retained and the remainder centrifuged at $13000 \times g$ for 30 min. The resultant pellet was rehomogenized (10 strokes) with the same volume of buffer, centrifuged as described above, and the two supernatants pooled (combined supernatants). The second pellet was resuspended in 25 mM imidazole-Tris, pH 7.0, containing 250 mM sucrose and 2 mM EDTA-Tris. The pellet (washed membrane suspension), pooled supernatant and the original homogenate fractions were assayed for protein and $(Na^+ + K^+)$-ATPase activity. Protein determinations were conducted by the method of Lowry et al. [18] using bovine serum albumin as standard.

Assay of $(Na^+ + K^+)$-ATPase and p-nitrophenylphosphatase activities

$(Na^+ + K^+)$-ATPase was assayed according to the method of Robinson [19]. The standard medium contained in a volume of 0.5 ml, 30 mM histidine-HCl/Tris (pH 7.5), 3 mM $MgCl_2$, 20 mM KCl, 130 mM NaCl and 3 mM ATP-Tris. Reaction was initiated by the addition of 150–250 μg of protein in the case of homogenate fractions, or 50–75 μg in the case of partially purified fractions. When present, ouabain was utilized at a 1 mM concentration. After a 30 min incubation at 37°C, hydrolysis was terminated by the addition of 0.1 ml of 30% ice-cold trichloroacetic acid. The liberated phosphate was measured spectrophotometrically following the method of Sanui [20] using reduced volumes to increase the sensitivity. $(Na^+ + K^+)$-ATPase activity was taken to be the ouabain sensitive component of the total ATPase activity. *p*-Nitrophenylphosphatase activity was measured as described by Robinson [19]. The as-

say mixture contained in a volume of 0.5 ml, 30 mM histidine-HCl/Tris (pH 7.5), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate/Tris, 100–150 μg of protein in the case of the partially purified enzyme or 200–300 μg of protein in the case of homogenate fractions. When present, the concentration of KCl was 10 mM. After a 30 min incubation at 37°C, the reaction was terminated by the addition of 0.5 ml of 1.5 M NaOH and the liberated *p*-nitrophenol determined as described by Sen and Ray [21]. *p*-Nitrophenylphosphatase activity was taken to be the K^+-dependent component of the total *p*-nitrophenyl phosphate hydrolytic activity.

Both hydrolytic activities were linear with respect to protein concentration over a range of 50–300μg per assay and linear with respect to time for at least 60 min at 37°C.

Enzyme purification

Partial purification of ($Na^+ + K^+$)-ATPase was carried out by modifying the method of Jørgensen and Skou [22]. The membrane suspension was adjusted to a protein concentration of 1.2 mg/ml in 250 mM sucrose, 25 mM imidazole-Tris (pH 7.0), 2 mM EDTA-Tris and then incubated for 30 min at 21–23°C with an equal volume of this medium which also contained sodium deoxycholate at 1.2 mg/ml. The resultant suspension was layered over an equal volume of 40% (w/v) sucrose and centrifuged at 50000 × *g* for 1 h in a Beckman SW 27 rotor at 0–4°C. The supernatant was discarded and the pellet was resuspended in 250 mM sucrose, 25 mM imidazole-Tris (pH 7.5), and 2 mM EDTA-Tris (storage buffer). The optimum pH for extraction with deoxycholate was 7.0 as in the procedure of Jørgensen and Skou [22]. The partially purified enzyme thus obtained (final preparation) was membrane-bound. It was stored in a frozen state, below −20°C, and utilized within one week of preparation. The enzyme did not lose activity during this period.

Polyacrylamide gel electrophoresis

Gel electrophoresis was carried out in 7.5% acrylamide containing 1% SDS according to the method of Fairbanks et al. [23]. 25–50 μg of protein was applied to each gel and electrophoresis was performed at 80–100 V with a current of 5–7 mA/tube. The time required to complete a run was normally 2.5–3.0 h. Fixing, staining with Coomassie blue and destaining were carried out as described by Fairbanks et al. [23]. The protein content of individual bands was estimated from densitometric scans obtained at 550 nm in a Beckman DU 8 spectrophotometer equipped with a peak area integrator. Molecular weights were graphically estimated from standard plots of the elecrophoretic mobility of other proteins, which ranged from 10000 to 96000.

Quantitation of ouabain binding

[^{3}H]Ouabain binding was measured as described by Van Winkle et al. [24]. The medium contained in a volume of 1.0 ml, 30 mM histidine-Tris (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA-Tris, 10 μM [^{3}H]ouabain plus either 2 mM Tris-ATP plus 50 mM NaCl or 2 mM Tris-inorganic phosphate. 250–500 μg of membrane-associated protein were utilized per determination. After a 10 min incubation at 37°C, the reaction mixture was filtered through 0.45 μm Gelman membrane filters and washed four times with 1 ml portions of ice-cold water. The filters were dried at 60°C and the associated radioactivity was determined by liquid scintillation counting in a Beckman LS 7500 scintillation spectrophotometer. The data were corrected for the fraction of ouabain bound directly to the filters by preparing and filtering additional samples from which the enzyme fraction was omitted.

Quantitation of ATP-dependent enzyme phosphorylation

The partially purified ($Na^+ + K^+$)-ATPase was labeled with [γ-^{32}P]ATP (10^7–10^8 cpm/μmol) according to the method of Post et al. [25]. The reaction mixture contained in a total volume of 0.5 ml, 10 mM imidazole-glycylglycine (pH 7.5), 0.1 mM $MgCl_2$, 20 mM NaCl, 0.1 mM [γ-^{32}P]ATP and 250–500 μg of membrane-associated protein. After an incubation period of 30 s at 21–23°C, the reaction was terminated by the addition of 0.5 ml of ice-cold 10% $HClO_4$ containing 2 mM unlabeled ATP and 2 mM unlabeled inorganic phosphate. The resultant mixture was filtered through Gelman 0.45 μm membrane filters which were subsequently washed with 30 ml of cold 5% $HClO_4$ containing 1 mM unlabeled ATP and 1 mM un-

labeled inorganic phosphate, and finally with 5 ml of cold water. Filters were dried at 60°C and the associated radioactivity determined as described above. To observe K^+-dependent dephosphorylation of the enzyme, the labeled preparation was treated with 20 mM KCl for 60 s prior to the addition of $HClO_4$.

For SDS gel electrophoresis of the phosphorylated enzyme, the reaction was carried out and terminated as described above, after which the suspension was centrifuged for 10 min at 15 000 × *g* in an Eppendorf microcentrifuge. The pellet was resuspended in 1 ml of the stopping solution, centrifuged again for 10 min, and the final pellet was dispersed and gel electrophoresis carried out as described above. The gels were sliced in 2 mm thick sections which were dissolved in hydrogen peroxide by heating at 80°C overnight. The associated radioactivity was then determined by scintillation counting.

Kinetic parameters

$K_{0.5}$ and K_m apparent values were estimated from double reciprocal plots of enzyme activity vs. substrate or cofactor concentration. Assays were carried out as described above except for varying the concentration of the medium component in question. To initially determine the optimal concentrations of Na^+ and K^+, the concentrations of both ions were altered simultaneously, such that the ionic strength of the reaction media remained constant. Temperature and pH effects on activity were determined by varying these parameters under assay conditions which were otherwise optimal.

I_{50} values for the inhibition of $(Na^+ + K^+)$-ATPase and *p*-nitrophenylphosphatase activity by ouabain were estimated from plots of percent inhibition vs. ouabain concentration. The media contained optimal concentrations of Mg^{2+}, Na^+ and ATP or Mg^{2+} and *p*-nitrophenyl phosphate for the ATPase and phosphatase reactions, respectively. Media containing the enzyme preparation and ouabain was preincubated for 5 min at 37°C after which the reactions were initiated by the addition of K^+. Released P_i and *p*-nitrophenol were measured as described above.

Fluorescence polarization measurements on membrane-associated DPH

Membrane fractions were suspended in the storage buffer to a protein concentration of 1 mg/ml. DPH, a probe for the fluidity state of membrane hydrocarbon regions [26,27], was added at 85 ng/mg protein in negligible volumes of double distilled ethanol and fluorescence polarization was determined by the double-ended ratiometric technique [28]. An SLM 8000 DS spectrofluorometer operated in the proton counting mode was employed with excitation of the fluorophore at 360 nm. Temperature was maintained with circulating bath equipped with an external flow circuit.

Results

Prepartion of lens $(Na^+ + K^+)$-ATPase

The results of the enzyme purification procedure are summarized in Table I. The method is similar to that for the isolation of this enzyme

TABLE I

SUMMARY OF LENS $(Na^+ + K^+)$-ATPase PURIFICATION

Fractions are defined in Methods. Values are presented on a per lens basis and were obtained from three preparations on which each parameter was determined in triplicate. The ranges presented are standard deviations of the data from the three preparations. The triplicate determinations were reduced to a mean value before calculating the standard deviations.

Fraction	Total protein (mg)	Total activity (μmol/h)	Specific activity (nmol/mg/h)	-Fold purification
Homogenate	178 ± 5.1	0.58 ± 0.041	3.3 ± 0.7	-
Washed membrane suspension	11.2 ± 1.6	1.53 ± 0.083	137 ± 10.5	42
Combined supernatants	161 ± 15.1	0	0	-
Final preparation	4.3 ± 0.8	1.41 ± 0.069	331 ± 19.1	101

from rabbit kidney as described by Jørgensen and Skou [22], although several modifications are necessary to obtain optimal results with the lens enzyme. As when isolating the enzyme from other tissues, the protein and deoxycholate concentrations, as well as the temperature, duration and pH of the incubation with deoxycholate, are particularly important. The specific activity of the resultant preparation is 100-fold higher than the initial homogenate and about 92% of the original activity is recovered (Table I). 75–80% of the ATPase activity in the final preparation is ouabain sensitive. A parallel increase in *p*-nitrophenylphosphatase activity was also observed; however, this activity is only 8–10% of the $(Na^+ + K^+)$-ATPase activity (not shown). The enzyme remains membrane-bound and is stable for at least one week when stored below −20°C.

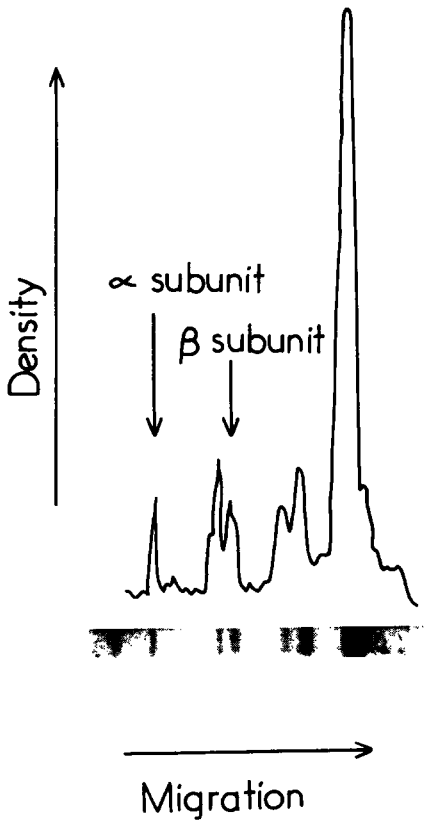

Fig. 1. SDS gel electrophoretogram of partially purified $(Na^+ + K^+)$-ATPase from pig lens. Electrophoresis was carried out and the densitometric scan was obtained as described in Methods.

A typical SDS gel electrophoresis pattern and densitometric scan of the partially purified enzyme is shown in Fig. 1. Six major protein bands, present in the preparation, are revealed by the electrophoretic technique. Assuming that the lens enzyme is composed of α and β subunits with molecular weights of approx. 100000 and 50000, respectively, as in other tissues [1–3], then the bands identified in the densitometric scan (Fig. 1) should represent those two subunits in this preparation. Supporting these tentative identifications are the findings that the apparent α band (mol. wt. 93000) is phosphorylated by ATP under conditions expected to result in formation of the phosphoenzyme intermediate (see below), while the apparent β band (mol. wt. 51000) is stained by periodate Schiff's reagent (data not shown), indicating that it is a glycoprotein as is the β subunit of the enzyme in othr tissues [4–8]. The 93 kDa component of the preparation contains $4.2 \pm 0.5\%$ of the total protein (average of five determinations). The percent of total protein in the 51 kDa component is more difficult to estimate since this band is not completely resolved from other components of similar molecular weight. If this component is present at a 1 : 1 mole ratio compared to the α subunit, then $(Na^+ + K^+)$-ATPase constitutes approx. 6% of the total protein in the preparation.

Enzymatic properties of lens $(Na^+ + K^+)$-ATPase

Table II shows that the partially purified enzyme is phosphorylated by ATP and binds the specific inhibitor ouabain. Using the apparent extent of phophorylation in the presence of K^+ and the apparent extent of labeled ouabain binding in the presence of excess unlabeled ouabain to correct for the nonspecific components of the two reactions, the degree of enzyme labeling by both procedures is seen to be equivalent within experimental error and to equal approx. 200 pmol/mg total protein. Using a value of 4.2 percent (see above) as the amount of total protein which is α subunit, the extent of phosphorylation and ouabain binding corresponds to 0.44 mol/mol of α subunit, whereas the expected ratio is near 1.0 [29–31]. Possible explanations for this discrepancy are considered in the Discussion.

As expected [2,25], high concentrations of ouabain largely prevent formation of the phos-

TABLE II

FORMATION OF THE PHOSPHORYLATED INTERMEDIATE AND DEMONSTRATION OF OUABAIN BINDING BY $(Na^+ + K^+)$-ATPASE FROM PIG LENS

The quantitations of enzyme phosphorylation by [γ-^{32}P]ATP and [^{3}H]ouabain binding were carried out as described in Methods. Where indicated, Na^+ or K^+ were present at a concentration of 20 mM. Values presented are the mean ± the standard deviation of six determinations obtained as triplicate assays on two different preparations.

Parameter	Extent of reaction (pmol/mg protein)
Phosphoenzyme formation	
Presence of Na^+	232 ± 32
Presence of Na^+ plus 1 mM ouabain	65 ± 21
Presence of K^+	29 ± 9.2
Ouabain binding	
Presence of Mg^{2+} and P_i	198 ± 25
Presence of Mg^{2+}, Na^+ and ATP	219 ± 47
Presence of excess unlabeled ouabain, Mg^{2+} and P_i	21 ± 11.8

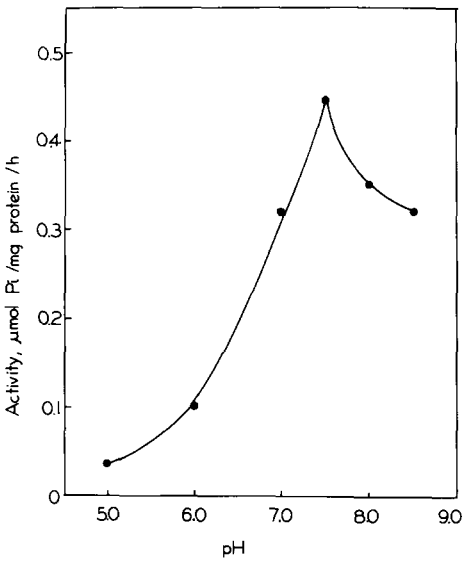

Fig. 2. The effect of medium pH on the activity of $(Na^+ + K^+)$-ATPase from pig lens. $(Na^+ + K^+)$-ATPase assays were conducted as described in Methods except that pH was adjusted to the values shown by addition of appropriate amounts of Tris. The activity values presented are the mean of four determinations obtained as duplicate assays on two different preparations.

phoenzyme. This inhibitor binds to the enzyme to the same extent either in the presence of Mg^{2+} plus inorganic phosphate or in the presence of Mg^{2+}, Na^+ and ATP. Treatment of the phosphorylated preparation with 20 mM KCl released 90% of the radioactive phosphate within 60 s. When the phosphorylated preparation was subjected to electrophoresis, a comparable amount of the labeled phosphate was found to be associated with the 93 kDa component identified as the α subunit in Fig. 1 (data not shown).

Under the assay conditions employed in this study, the enzyme shows a sharp pH optima located at 7.5 (Fig. 2), above or below which it is less active. The specific activity of the enzyme under optimal conditions was not affected by substituting imidazole for the histidine buffer, whereas the use of Tris at pH 7.5 reduced the activity by 65% (data not shown).

The effects of characteristic inhibitors of $(Na^+ + K^+)$-ATPase obtained from other tissues on the activities of the lens enzyme are shown in Table III. The $(Na^+ + K^+)$-ATPase activity is completely inhibited by all of the agents when they are employed at levels which produce a comparable effect on the enzyme from other sources. Complete inhibition of *p*-nitrophenylphosphatase activity is also observed except in the cases of Ca^{2+} and oligomycin. The latter agent blocks the transition of the $E_1 \sim P$ to the E_2-P form of the enzyme and, hence, is characteristically without effect on *p*-nitrophenylphosphatase activity [1–3]. However, in this preparation, we consistently observed a 35–45% inhibition of the *p*-nitrophenyl phosphatase activity by this well known antibiotic. Inhibition by the sulfhydryl reactive agents, PCMBS, *N*-ethylmaleimide, and DTNB, indicates that the lens enzyme is sulfhydryl-dependent as is the $(Na^+ + K^+)$-ATPase in other tissues [1–3]. Comparing the relative hydrolytic rates for different nucleotides showed that ATP hydrolysis is the dominant activity of the preparation. A 20% component of this activity is not sensitive to ouabain. Thus, it appears that other ATPase(s) are also present in measurable amounts. The preparation also has measurable activities for the hydrolysis of GTP (9% compared to ATP) and ADP (5% compared to ATP) but not for CTP, UTP or AMP when rates are compared at nucleotide concentra-

TABLE III

THE EFFECT OF SELECTED INHIBITORS OF ($Na^+ + K^+$)-ATPase and *p*-NITROPHENYLPHOSPHATASE ACTIVITIES OF THE PARTIALLY PURIFIED LENS ENZYME

The effects of ouabain, oligomycin, Ca^{2+}, VO_4^- and PCMBS were determined by comparing the activity of the enzyme in the presence and absence of the inhibitor. For the agents listed above, assays were performed as described in Methods with the inhibitor in question added immediately after the enzyme preparation. In the case of *N*-ethylmaleimide (NEM), the enzyme (1.0 mg protein/ml) was incubated with the inhibitor (5 mM) in medium containing 0.25 M sucrose, 10 mM imidazole-Tris (pH 7.0), for 30 min at 37°C. The mixture was subsequently diluted 5-fold with the same medium (*N*-ethylmaleimide excluded) which contained in addition 1 mM dithiothreitol. The enzyme-membrane fraction was recovered by centrifugation at 100000 × *g* for 1 h, the pellet resuspended in storage buffer, and assayed for protein and enzyme activities as described in Methods. In the case of DTNB, the enzyme was prereacted with the inhibitor in an equivalent manner except that the media consisted of 0.25 M sucrose, 50 mM Tris, pH 7.5, with DTNB present at 1 mM. The values presented were determined from triplicate assays performed on two preparations, except in the case of oligomycin, where four preparations were tested.

Inhibitor (concentration)	($Na^+ + K^+$)-ATPase activity (% of control)	*p*-Nitrophenylphosphatase activity (% of control)
None	100	100
Ouabain (1 mM)	0	0
Oligomycin (10 μg/ml)	0	57 ± 10
$CaCl_2$ (1 mM)	0	15
NH_4VO_3 (50 μM)	0	0
PCMBS (0.25 mM)	0	0
NEM (prereacted)	0	0
DTNB (prereacted)	0	0

tions of 3 mM. Hydrolysis of GTP is ouabain sensitive, whereas hydrolysis of ADP is not. ADP hydrolysis is also unaffected by the myokinase inhibitor P^1, P^5-di(adenosine-5′)pentaphosphate (data not shown). Thus, the hydrolysis of GTP represents an activity of the ($Na^+ + K^+$)-ATPase while another contaminating activity is apparently responsible for ADP hydrolysis.

Table IV shows the effect of replacing K^+ by equimolar concentrations of ammonium and other alkali metal cations on the hydrolytic activities of the lens enzyme. In the case of ($Na^+ + K^+$)-ATPase activity, NH_4^+ and Rb^+ are effective substitutes for K^+, whereas substituting Cs^+ or Li^+ for K^+ decreases the activity. $K_{0.5}$ for activation of the enzyme by Li^+ is substantially higher than for K^+ [32], which may explain the diminished activity seen with that cation under the present conditions. In the case of *p*-nitrophenylphosphatase activity, no cation tested is as effective as K^+.

TABLE IV

ACTIVATION OF ($Na^+ + K^+$)-ATPase AND *p*-NITROPHENYLPHOSPHATASE WHEN POTASSIUM IS SUBSTITUTED BY EQUIMOLAR AMOUNTS OF OTHER MONOVALENT CATIONS

($Na^+ + K^+$)-ATPase and *p*-nitrophenylphosphatase activities were measured in the standard assay media except that potassium was replaced by an equimolar concentration of the cation indicated. Values presented are the mean of six determinations obtained as triplicate assays on two different preparations.

Cation	($Na^+ + K^+$)-ATPase activity (% of control)	*p*-Nitrophenylphosphatase activity (% of control)
K^+	100	100
NH_4^+	114	42
Rb^+	95	71
Cs^+	63	41
Li^+	43	13.3

Fig. 3 shows the activation of the partially purified ($Na^+ + K^+$)-ATPase by different concentration ratios of Na^+ and K^+. Under the conditions employed, the ionic strength of the assay media is kept constant, thereby avoiding distortions of the enzyme's apparent affinities for these cations by changes in this parameter. Characteristic of this enzyme from other sources [1–3], a broad dependence of activity on the concentration ratio of these cations is observed. High concentration ratios with respect to either cation are inhibitory, presumably due to reciprocal competition at the respective activator sites [33]. As expected, the preparation does not contain K^+-ATPase activity. The apparent absence of Na^+-ATPase activity may indicate that optimal conditions for this reaction are significantly different than those employed in Fig. 3 or that the activity is too low to be detected in the present preparation.

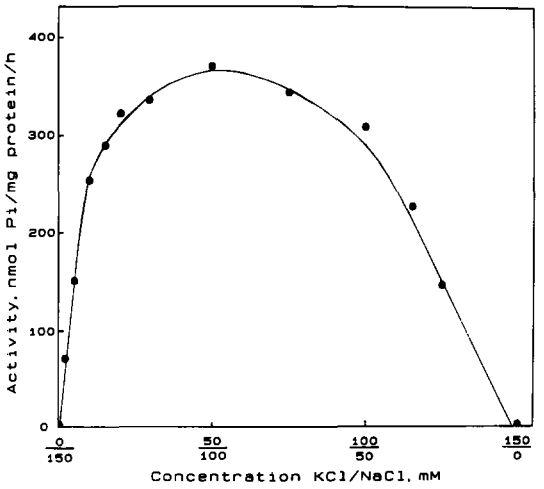

Fig. 3. Effect of varied K^+/Na^+ ratios on the activity of $(Na^+ + K^+)$-ATPase from pig lens. $(Na^+ + K^+)$-ATPase assays were conducted as described in Methods except that the concentrations of KCl and NaCl were altered as indicated. Ultrapure samples of KCl and NaCl were utilized in this experiment to assure that the calculated K^+/Na^+ ratios, near the high and low extremes of this parameter, were not in error due to the cross contamination of these cations which often exist in lower purity grades of these reagents. The activity values presented are the mean of four determinations obtained as duplicate assays on two different preparations.

Kinetic constants for substrates, cofactors and the inhibitor ouabain are shown in Table V. The values found with this preparation are comparable to those reported previously for the highly purified enzyme [1–3]. The enzyme is strongly inhibited by ouabain as revealed by the low I_{50} values for inhibition of both hydrolytic activites. The values observed are in the same range reported for the membrane-bound enzyme from other sources [6,34–36].

TABLE V

KINETIC PARAMETERS FOR THE $(Na^+ + K^+)$-ATPase AND *p*-NITROPHENYLPHOSPHATASE REACTION OF THE PARTIALLY PURIFIED LENS ENZYME

The constants were determined as described in Methods. Individual points used to construct the double reciprocal plots were the mean of four values obtained as duplicate determinations on two preparations.

Constant	$(Na^+ + K^+)$-ATPase activity	*p*-Nitrophenylphosphatase activity
K_m ATP (mM)	0.77	–
K_m pNPP (mM)	–	1.11
$K_{0.5}$ Mg^{2+} (mM)	0.4	0.50
$K_{0.5}$ Na^+ (mM)	25.0	–
$K_{0.5}$ K^+ (mM)	2.2	1.70
I_{50} ouabain (μM)	1.2	1.30

The effect of membrane fluidity on the activities of lens $(Na^+ + K^+)$-ATPase

The lens is unusual compared to many other tissues in that the fiber cell membranes contain high proportions of cholesterol and esterified fatty acids which are highly saturated [13,16]. These properties would be expected to impart a relatively high liquid to gel phase transition temperature to the membrane. This in turn could produce an unusual temperature dependency of the associated $(Na^+ + K^+)$-ATPase, since activity of this enzyme is markedly affected by the fluidity state of its membrane enviroment [1,3]. Fig. 4 shows Arrhenius plots of the $(Na^+ + K^+)$-ATPase and *p*-nitrophenylphosphatase activitities of the lens preparation. Breaks in the plots for both activities are seen near 37°C. That these temperature char-

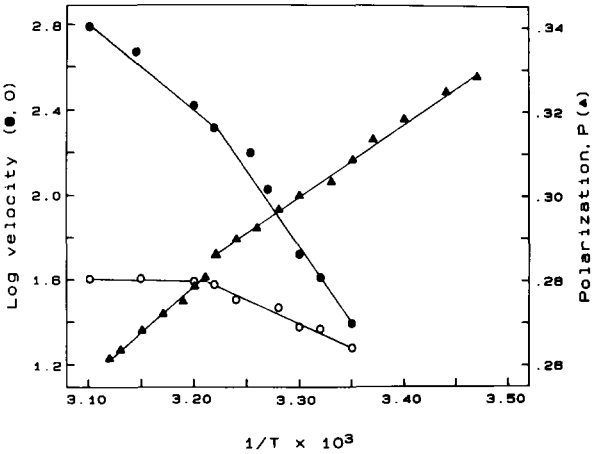

Fig. 4. Comparison of temperature effects on the activities of pig lens $(Na^+ + K^+)$-ATPase and fluorescence polarization of diphenylhexatriene (DPH) bound to the preparation. Activity assays and fluorescence polarization measurements were conducted as described in Methods at the temperatures indicated in the figure. For the activity assays, the pH of the incubation media was adjusted at the temperature to be employed. ●, $(Na^+ + K^+)$-ATPase activity. ○, *p*-nitrophenylphosphatase activity. ▲, fluorescence polarization of DPH bound to the enzyme preparation.

acteristics reflect the fluidity properties of the membrane hydrocarbon interior is supported by the fact that the Arrhenius plot of fluorescence polarization of DPH displays a discontinuity and a change in slope which are located at approximately the same temperature.

Discussion

A number of studies have appeared in which the level of $(Na^+ + K^+)$-ATPase activity in lens has been quantitated [13–15,37,38]; however, purification of the enzyme from this tissue has not been previously attempted. In this study a partially purified preparation has been obtained by use of the deoxycholate extraction technique, optimized for application to lens. The specific activity of the final preparation, 331 ± 19 nmol/h per mg protein, is increased about 100-fold compared to the homogenate and is 11-fold higher than a preparation from chicken lens obtained as a sedimented membrane fraction [39].

The six major protein bands present in the preparation range from 20 000 to 93 000 in molecular weight. The major contaminant displays a molecular weight of approx. 26 000 and constitutes approx. 70% of total protein. This protein is likely to be the so-called membrane intrinsic protein component of lens membranes which is known to be tightly associated, and is believed to constitute a component of the abundant gap junctions between fiber cells [40,41]. The highest molecular weight component (approx. 93 000) contains a component which is phosphorylated by the γ phosphate of ATP, dephosphorylated in response to K^+, and thus appears to include the α subunit of the $(Na^+ + K^+)$-ATPase. A protein of similar molecular weight and susceptibility to phosphorylation has recenlty been found to be associated with chicken lens membranes [39]. In that report, the apparent phosphorylated subunit could be resolved into two components with slightly different electrophoretic mobilities. We found no evidence for such heterogeneity in this preparation. The 51 kDa protein band of this preparation was the only component stained by periodate Schiff's reagent, indicating that it is the only glycoprotien present in significant quantities and thus is likely to include the β subunit of the $(Na^+ + K^+)$-ATPase.

The present data allow an estimation of the molecular activity of the lens $(Na^+ + K^+)$-ATPase. The amount of protein in the 93 kDa component of the preparation constitutes 4.2% of the total. The phosphorylation and ouabain binding data presented in Table II show that 44% of this band can be considered to arise from an enzymatically active α subunit, which corresponds to 1.85% of total protein. Assuming that the α subunit represents 70% of the protein content in the intact enzyme and that the mole ratio of the α to β subunits in the final preparation is 1 : 1, then 2.64% of the total protein would represent active enzyme. Utilizing the specific activity of 331 nmol/h per mg protein for the final preparation, it can be calculated that the specific activity of the pure, active enzyme would be 1.25 μmol/h per mg protein. This value corresponds to a turnover number in the region of 50/min as compared to turnover numbers of 10 000/min which have been reported for preparations from kidney [42]. This difference could indicate that inhibitory components remain in the relatively impure lens preparation, that the fiber cell plasma membranes provide a substantially less than optimal lipid environment for the enzyme or that significant protein structure differences exist between the enzymes from these two sources. Further work will be necessary to differentiate between these possibilities.

Similar to erythrocytes, lens fiber cells arise by a terminal differentiation during which subcellular organelles and the capacity to maintain significant protein turnover is lost. However, unlike erythrocytes which have a limited lifetime, once fiber cells are formed they persist in the lens throughout the remaining lifetime of the animal. Thus, critical metabolism such as $(Na^+ + K^+)$-ATPase activity is maintained by 'old enzyme molecules'. In this context it is interesting that the substrate and cation specificities, kinetic properties and inhibitor sensitivities of lens $(Na^+ + K^+)$-ATPase are similar to those of the enzyme from tissues in which protein turnover can occur. The total population of $(Na^+ + K^+)$-ATPase molecules present in the lens may include a substantial proportion which have become inactivated during the life of the animal. This is suggested by the fact that the level of phosphorylation and ouabain binding constitutes slightly less than 50% of what would be

expected if all protein in the 93 kDa band consisted of active α subunits. Alternative explanations such as the partial denaturation of enzyme or a preferential loss of β subunits during isolation seems improbable since 92% of the initial activity is recovered by the isolation procedure. It is possible, of course, that other peptides are present in substantial quantities in the 93 kDa band, however, the sharp and relatively symmetrical appearance of this component in the electrophoretogram argues against this.

$(Na^+ + K^+)$-ATPase normally displays high affinity (K_m 0.3–1.0 μM) and low affinity (K_m 0.1–0.3 mM) binding of ATP. Binding at the low affinity site markedly stimulates the $(Na^+ + K^+)$-ATPase reaction [43–45] and it is probably the affinity at this site which the ATP K_m value reported here (0.77 mM) represents. This value is comparable to the one obtained by Sen et al. for the highly purified dog kidney enzyme [46]; however, the presence of other proteins in the preparation may be influencing the apparent K_m for ATP. The slightly elevated $K_{0.5}$ for Na^+ may also relate to the presence of other components. More detailed kinetics of the lens enzyme must await the availability of a more highly purified preparation. The findings that oligomycin consistently produces a 35–45% inhibition of *p*-nitrophenylphosphatase activity and that the enzyme has some hydrolytic activity for GTP but not CTP are unexpected and interesting. However, further work will be necessary before the significance of these findings are clear.

The existence of a membrane fluidity dependent break in the Arrhenius plot of $(Na^+ + K^+)$-ATPase activity near the physiological temperature is an unusual and potentially significant property of the lens enzyme. While the activity is known to be dependent on the fluidity state of associated lipids [47–49], the transition temperatures of other preparations are normally located near 20°C [50], well below the range physiologically accessible. However, in lens it would appear that relatively small changes in temperature or relatively minor changes in the lipid composition of fiber cell membranes could markedly alter the activation energy for the $(Na^+ + K^+)$-ATPase reaction, thereby affecting the ability of lens to maintain normal osmotic regulation, metabolite transport activities and, hence, transparency. From the data in Fig. 4 it can be calculated that the activation energy for the $(Na^+ + K^+)$-ATPase reaction is increased from 17.2 to 34.7 kcal/mol upon decreasing the temperature below the transition point at 37°C. Significant lipid alterations occur during aging [51], preceding senile cataract formation, and during the induction of experimental cataracts by triparanol feeding [13,16]. The effects of these alterations on $(Na^+ + K^+)$-ATPase activity may be marked and a central aspect of the cataractogenic mechanisms.

The existence of an apparent fluidity-dependent break in the Arrhenius plot of *p*-nitrophenylphosphatase activity is also an unexpected finding. Phospholipids are generally not essential for this activity (however, see Ref. 52) and the activation energy of the phosphatase reaction is normally not affected by the fluidity state of associated lipids [53,54]. This finding, together with the sensitivity of *p*-nitrophenylphosphatase activity to oligomycin, suggests that the phosphatase reaction of the lens enzyme does not proceed in a manner entirely equivalent to this reaction in the enzyme from other tissues.

Acknowledgements

This investigation was supported in part by U.S. Public Health Service Grants EY 02307 and EY 02613; by Research Grant HL 08214 from the Program Projects Branch, Extramural Programs, National Heart, Lung and Blood Institute; and by the Hormel Foundtion. The authors thank Mr. Randy Krebsbach for expert technical assistance.

References

1 Skou, J.C. (1975) Q. Rev. Biophys. 7, 401–434
2 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176
3 Stekhoven, F.S. and Bonting, S.L. (1981) Physiol. Rev. 61, 1–76
4 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
5 Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973) J. Biol. Chem. 248, 2593–2605
6 Dixon, J.F. and Hokin, L.E. (1974) Arch. Biochem. Biophys. 163, 749–758
7 Hopkins, B.E., Wagner, H., Jr. and Smith, T.W. (1976) J. Biol. Chem. 251, 4365–4371

8 Uesugi, S., Gulak, N.C., Dixon, J.F., Hexum, T.D., Dahl, J.L., Perdue, J.F. and Hokin, L.E. (1971) J. Biol. Chem. 246, 531–543

9 Duncan, G. (1973) in Comparative Physiology of Lens Membranes in the Eye (Darison, H., ed.), Vol. 5, pp. 99–116, Academic Press, London

10 Rae, J.L. (1979) in Current Topics in Eye Research (Zadunaisky, J.H. and Darison, H., eds.), Vol. 1, pp. 37–90, Academic Press, New York

11 Kinoshita, J.H. (1974) Invest. Ophthalmol. 13, 713–724

12 Fournier, D.J. and Patterson, J.W. (1971) Proc. Soc. Exp. Biol. Med. 137, 826–832

13 Mizuno, G.R., Chapman, C.J., Chipault, J.R. and Pfeiffer, D.R. (1981) Biochim. Biophys. Acta 644, 1–12

14 Iwata, A. and Kinoshita, J.H. (1971) Invest. Ophthalmol. 10, 504–512

15 Gupta, J.D. and Harley, J.D. (1975) Exp. Eye Res. 20, 207–209

16 Mizuno, G., Ellison, E., Chipault, J.R. and Harris, J.E. (1974) Ophthalmol. Res. 6, 206–215

17 Takemoto, L.J., Hansen, J.S. and Horwitz, J. (1981) Comp. Biochem. Physiol. 68B, 101–106

18 Lowry, O.H., Rosebrough, N.J., Farr, N.J. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

19 Robinson, J.D. (1969) Biochemistry 6, 3250–3258

20 Sanui, H. (1974) Anal. Biochem. 60, 489–504

21 Sen, P.C. and Ray, T.K. (1979) Arch. Biochem. Biophys. 198, 548–555

22 Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366–380

23 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617

24 Van Winkle, W.B., Allen, J.C. and Schwartz, A. (1972) Arch. Biochem. Biophys. 151, 85–92

25 Post, R.L., Sen, A.K. and Rosenthal, A.S. (1965) J. Biol. Chem. 240, 1437–1445

26 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657

27 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394

28 Jameson, D.M., Weber, G., Spencer, R.O. and Mitchell, G. (1978) Rev. Sci. Instrum. 49, 510–514

29 Peters, W.H.M., Swarts, H.G.P., DePont, J.J.H.H.M., Schuurmans Stekhoven, F.M.A.H. and Bonting, S.L. (1981) Nature 290, 338–339

30 Skou, J.C. and Esmann, M. (1981) Biochim. Biophys. Acta 647, 232–240

31 Huang, W., Askari, A. (1981) Biochim. Biophys. Acta 645, 54–58

32 Robinson, J.D. (1975) Biochim. Biphys. Acta 384, 250–264

33 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 234–245

34 Robinson, J.D. (1980) Biochim. Pharmacol. 29, 1995–2000

35 Braughler, J.M. and Corder, C.N. (1977) Biochim. Biophys. Acta 481, 313–327

36 Gache, C., Rossi, B. and Lazdunski, M. (1976) Eur. J. Biochem. 65, 293–306

37 Bonting, S.L., Caravaggio, L.L. and Hawkins, N.M. (1963) Arch. Biochem. Biophys. 101, 47–55

38 Unakar, N.J. and Tsui, J.Y. (1980) Invest. Ophthalmol. Vis. Sci. 19, 630–641

39 Takemoto, L.J., Hansen, J.S. and Hokin, L.E. (1981) Biochem. Biophys. Res. Commun. 100, 58–64

40 Broekhuyse, R.M., Kuhlmann, E.D. and Stols, A.H. (1976) Exp. Eye Res. 23, 365–372

41 Roy, D. and Specter, A. (1978) Exp. Eye Res. 26, 429–443

42 Jørgensen, P.L. (1980) Physiol. Rev. 60, 864–917

43 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006–1019

44 Froehlich, J.P., Alberts, R.W., Koval, G.J., Gobel, R. and Berman, M. (1966) J. Biol. Chem. 251, 2186–2188

45 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. 256, 465–496

46 Sen, P.C., Kapakos, J.G. and Steinberg, M. (1981) Arch. Biochem. Biophys. 211, 652–661

47 Grisham, C.M. and Barnett, R.E. (1973) Biochemistry 12, 2635–2637

48 Kimelberg, H.K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071–1080

49 Walker, J.A. and Wheeler, K.P. (1975) Biochim. Biophys Acta 394, 135–144

50 Charnock, J.S. and Bashford, C.L. (1975) Mol. Pharmacol. 11, 766–774

51 Broekhuyse, R.M. (1969) Biochim. Biophys. Acta 187, 354–365

52 Ottolenghi, P. (1979) Eur. J. Biochem. 99, 113–131

53 Barnett, R.E. and Palazzotto, J. (1974) Ann. N.Y. Acad. Sci. 242, 69–76

54 Walker, J.A. and Wheeler, K.P. (1975) Biochem. J. 151, 439–442