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MINIMAL FUNCTIONAL UNIT FOR TRANSPORT AND ENZYME ACTIVITIES OF $(Na^+ + K^+)$-ATPase AS DETERMINED BY RADIATION INACTIVATION

STEVEN J.D. KARLISH [a] and ELLIS S. KEMPNER [b]

[a] *Biochemistry Department, The Weizmann Institute of Science, Rehovot 76100 (Israel) and* [b] *Laboratory of Physical Biology, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)*

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Frozen aqueous suspensions of partially purified membrane-bound renal $(Na^+ + K^+)$-ATPase have been irradiated at $-135°C$ with high-energy electrons. $(Na^+ + K^+)$-ATPase and K^+-phosphatase activities are inactivated exponentially with apparent target sizes of 184 ± 4 kDa and 125 ± 3 kDa, respectively. These values are significantly lower then found previously from irradiation of lyophilized membranes. After reconstitution of irradiated $(Na^+ + K^+)$-ATPase into phospholipid vesicles the following transport functions have been measured and target sizes calculated from the exponential inactivation curves: ATP-dependent Na^+-K^+ exchange, 201 ± 4 kDa; (ATP + P_i)-activated Rb^+-Rb^+ exchange, 206 ± 7 kDa and ATP-independent Rb^+-Rb^+ exchange, 117 ± 4 kDa. The apparent size of the α-chain, judged by disappearance of Coomassie stain on SDS-gels, lies between 115 and 141 kDa. That for the β-glycoprotein, though clearly smaller, could not be estimated. We draw the following conclusions: (1) The simplest interpretation of the results is that the minimal functional unit for $(Na^+ + K^+)$-ATPase is $\alpha\beta$. (2) The inactivation target size for $(Na^+ + K^+)$-dependent ATP hydrolysis is the same as for ATP-dependent pumping of Na^+ and K^+. (3) The target sizes, for K^+-phosphatase (125 kDa) and ATP-independent Rb^+-Rb^+ exchange (117 kDa) are indistinguishable from that of the α-chain itself, suggesting that cation binding sites and transport pathways, and the *p*-nitrophenyl phosphate binding site are located exclusively on the α-chain. (4) ATP-dependent activities appear to depend on the integrity of an $\alpha\beta$ complex.

Introduction

The cell membrane $(Na^+ + K^+)$-ATPase or Na^+/K^+ pump consists of two major polypeptides, an α catalytic chain and a β-glycoprotein. The α-chain is involved in the chemical reactions leading to ATP hydrolysis and the conformational changes associated with cation transport. The function of the β-chain is not known. There is strong evidence for a 1:1 ratio of $\alpha:\beta$ in the membrane, but the state of sub-unit aggregation, $\alpha\beta$, $(\alpha\beta)_2$ etc. and the role if any of subunit interactions, are controversial issues [1–3]. A substantial body of recent work provides evidence for the notion that $\alpha\beta$ is the minimal functional unit of $(Na^+ + K^+)$-ATPase, particularly for enzyme solubilized in the non-ionic detergent $C_{12}E_8$ in a fully active state [4–9].

Radiation inactivation by high energy electrons, γ rays etc. together with classical target theory, provides a useful, perhaps unique, technique for

Abbreviations: $(Na^+ + K^+)$-ATPase, sodium and potassium ion transport adenosine triphosphatase (EC 3.6.1.3); SDS, sodium dodecyl sulphate; $C_{12}E_8$, dodecyloctaethyleneglycol monoether.

estimating the molecular size of the minimal functional units of membrane bound proteins [10,11]. In many studies of both soluble and membrane bound proteins in situ samples have been lyophilized before irradiation and are rehydrated prior to assay of the protein's function. Removal of water from the target protein avoids possible complications of radical formation and secondary inactivation of the protein, but it also removes the natural environment of the protein. As described by Kempner and Schlegel [11], equally satisfactory results can be obtained by irradiating frozen aqueous samples at temperatures low enough to effectively preclude radical diffusion (e.g. $-135\,^{\circ}C$).

Following the original work of Kepner and Macey [10] a number of studies of inactivation of $(Na^+ + K^+)$-ATPase and partial reactions such K^+-activated phosphatase have been reported, utilizing lyophilized membranes as targets [12–15]. The apparent target size for $(Na^+ + K^+)$-ATPase and smaller size for inactivating K^+-phosphatase, found in work to date (see Table II and the Discussion) has been seen [14,15] as implying a dimeric structure of the intact Na^+/K^+ pump and a minimal functional unit of $(\alpha\beta)_2$. This, however, appears to conflict with the other recent evidence suggesting a functional unit of $\alpha\beta$. Very recently Ottolenghi and Ellory [15] have reported a detailed study of radiation inactivation of $(Na^+ + K^+)$-ATPase using lyophilized preparations of pig brain or partially purified kidney enzyme. The results obtained were complex in that not all activities tested were inactivated along a single exponential, as required for a single-sized target by classical target theory. The authors have proposed that the enzyme is an $(\alpha\beta)_2$ dimer showing multiple and independent radiation-sensitive domains in both α-chains and radiation energy transfer between all four α- and β-chains in the protein. The assumptions on which their model is based are of course in sharp contradiction with the conclusions based on experience with most other proteins studied by the radiation technique in either the lyophilized or frozen wet state [10,11]. One object of the present study was to reinvestigate the problem by irradiating deep-frozen samples of pig-kidney $(Na^+ + K^+)$-ATPase, that is membranes containing the protein in an aqueous environment.

One might accept that the minimal functional unit for $(Na^+ + K^+)$-ATPase activity is $\alpha\beta$, but argue that the functional unit for active Na^+ and K^+ transport could be different, e.g. $(\alpha\beta)_2$. We have described a simple and efficient procedure for reconstituting the pig kidney $(Na^+ + K^+)$-ATPase into soybean phospholipid vesicles and used these vesicles for detailed investigation of the transport capabilities of the reconstituted pumps [16]. A further objective of our work was therefore to attempt to reconstitute the $(Na^+ + K^+)$-ATPase from the irradiated frozen membranes, and use the vesicles to estimate apparent target sizes for inactivating normal ATP-dependent Na^+ and K^+ pumping and also partial transport reactions. Recently we reported in detail properties of (ATP + P_i)-activated Rb^+-Rb^+ exchange which is analogous to the classical K^+-K^+ exchange model known in red cells, and those of a newly discovered class of slow passive fluxes, including Rb^+-Rb^+ exchange, which occur in the complete absence of ligands other than Rb itself [17–19]. The apparent target sizes of these fluxes were of particular interest because it was thought that they might be different from that of the active Na^+ and K^+ pumping.

Methods

Partially purified $(Na^+ + K^+)$-ATPase was obtained from pig kidney red outer medulla by the procedures described by Jørgensen [20]. Specific activities were in the range 800–1100 μmol P_i released per h per mg protein. The membranes were stored frozen at $-20\,^{\circ}C$ at a protein concentration of 1–2 mg/ml in a medium containing: 250 mM sucrose, 25 mM histidine (pH 7.5) and 1 mM EDTA (Tris). An enzyme preparation with an activity of 1806 μmol released per h per mg protein was used for the experiments of Figs. 3 and 4. This was a gift from R.L. Post of Vanderbilt University.

Irradiation procedures. Samples of enzyme suspended in 300 to 500 μl of storage medium were frozen at $-78\,^{\circ}C$ in 2-ml glass ampules. Vials were sealed with an oxygen-gas flame while keeping the sample frozen. Samples were maintained at $-80\,^{\circ}C$ prior to and subsequent to radiation exposure. A 13 MeV beam of electrons produced by

a linear accelerator at the Armed Forces Radiobiology Research Institute (Bethesda, MD) was spread with a water-scatterer to provide a uniform beam of 10 MeV electrons over a 225 cm^2 area (dose variation less than 10%). Radiation exposure was determined with thermoluminescent dosimeters which were periodically checked with radiochromic dye films and ionization chambers.

During irradiation, samples were maintained at −135 °C by a stream of cold nitrogen gas. Temperature was monitored continuously during exposure and varied less than 2 °C.

Enzyme assays. Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was measured in duplicate over 10 min at 37 °C by an assay described originally for use with Ca^{2+}-ATPase [21]. The standard reaction mixture of volume 200 μl, consisted of: 130 mM NaCl, 20 mM KCl, 25 mM histidine (pH 7.5), 3 mM $MgCl_2$ and 3 mM ATP + [γ-^{32}P]ATP, without or with 1 mM ouabain. K^+-Phosphatase was measured essentially as described by Robinson [22], using the following reaction conditions (total volume, 1 ml): 3 mM *p*-nitrophenyl phosphate (Tris salt), 3 mM MgCl, 20 mM KCl, 25 mM Tris-HCl (pH 7.8), 5–10 μg of protein was added to tubes in triplicate, and the tubes incubated for 20 min at 37 °C. A preliminary experiment showed that ouabain-insensitive phosphatase was only about 0.1% of the total activity, and tubes containing ouabain were therefore omitted. Absolute K^+-phosphatase activity, calibrated with standard nitrophenol solutions was between 90 and 150 μmol nitrophenol released per h per mg protein.

Reconstitution and transport assays. Prior to reconstitution, all control and irradiated samples were dialyzed against 500 volumes of a solution containing 25 mM imidazole (pH 7.5) and 1 mM EDTA (Tris). After dialysis the absorbances at 280 nm of small aliquots of membranes suspended in 2 ml of water were recorded, as a relative measure of their protein concentrations, and any small differences between samples which had occurred during dialysis (usually less than 10%) were corrected for by appropriate dilutions to equalize the absorbance of all samples. (Prior to dialysis the absorbance at 280 nm of all samples was the same as in the sample before irradiation). Reconstitution was done as described in detail in Refs. 16 and 17 and briefly as follows. ($Na^+ + K^+$)-ATPase was mixed with recrystallised cholate (5.5 mg per mg protein) and the partially dissolved membrane suspension was added to sonicated soybean phospholipid (40 mg per mg protein) suspended in a solution containing 25 mM imidazole (pH 7.5, 1 mM EDTA and RbCl such that the final concentration was 150 mM. For most experiments the volume of vesicle suspension was 250 μl, and contained 0.15 mg protein, 0.75 mg of cholate and 6 mg of the azolectin. The protein/cholate/lipid mixtures were frozen in liquid nitrogen, thawed at room temperature, and sonicated briefly using a Laboratory Supplies Circular Sonicator Bath. The samples were then centrifuged briefly on columns of Sephadex G-50 washed with 150 mM Tris-HCl (pH 7.0) in order to replace the exterior RbCl with Tris-HCl (cf. Ref. 16). Small variations in volumes of vesicle suspension eluted from the columns were compensated for by adding 150 mM Tris-HCl solution.

For transport assays, see Refs. 16–19, 40 μl of vesicle were incubated at 20 °C for 2–4 min in duplicate with 40 μl of an appropriate reaction mixture containing ^{22}Na or ^{86}Rb (see below). After incubation the suspension was removed to columns of Dowex 50W-X8 and the vesicles were eluted with 1.5 ml of ice-cold 250 mM sucrose solution, into counting vials (see Ref. 16). ^{86}Rb was estimated by Cerenkov radiation and ^{22}Na by scintillation counting.

The reaction mixtures for the three types of fluxes measured, were such that the final concentrations of the constituents were as follows:

(a) ATP-dependent Na^+-Rb^+ exchange: 10 mM NaCl (+ ^{22}Na), 3 mM $MgCl_2$, 130 mM Tris-HCl (pH 7.0) without or with 3 mM ATP(Tris).

(b) (ATP + P_i)-activated Rb^+-Rb^+ exchange: 5 mM RbCl (+ ^{86}Rb), 3 mM $MgCl_2$, 20 mM phosphate (Tris) (pH 7.0), 25 mM Tris-HCl (pH 7.0), without or with 5 mM ATP (Tris).

(c) ATP-independent Rb^+-Rb^+ exchange: 0.5 mM RbCl (+ ^{86}Rb), 150 mM Tris-HCl (pH 7.0), 75 mM or RbCl (+ ^{86}Rb), 75 mM Tris-HCl (pH 7.0).

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed on 7.5% polyacrylamide running gel slabs, essentially as described by Laemmli [23]. Samples of 60 μl volume containing 5–40 μg of protein (Lowry) dissolved in 2% SDS

were applied to each slot of the gel. After running overnight at 2.5 V and approx. 2 A, the gels were fixed for 2 h in methanol (50% v/v)/acetic acid (10% v/v) and were then heavily stained overnight with Coomassie blue R, 0.01% (w/v) in the fixing solution. For destaining, gels were soaked for 2 h in methanol (5% v/v)/acetic acid (7.5% v/v) and were then soaked with shaking in 7.5% acetic acid solution for 5–6 days with frequent changes of the destaining solution.

Destained gels were photographed and the α-band was removed and the Coomassie stain estimated as follows. The α-chain of all samples (cf. Fig. 3) was cut out in equal rectangular areas of gel. The individual pieces of gel were cut up, suspended in 1 ml of 5% SDS solution, and were throughly homogenized using glass and a teflon homogenizer revolving at 1000 rpm. After standing for several hours the polyacrylamide was removed by centrifugation and the absorbance of the clear blue supernatant was read at 605 nm. A background correction was made for excess unextracted Coomassie stain, by subtracting from the absorbance extracted with the α-chain, the absorbance extracted from an equal area of gel immediately above the position of the α-chain (see also Results).

Analysis of data. Biological activity (A) remaining in samples exposed to various doses (D) of ionizing radiation was plotted as an inactivation curve, $\ln A/A_0$ vs. D, where A_0 is the activity in the unirradiated control. For single exponential decay of activity, the inactivation curves were fitted by a (constrained) least-squares analysis, and the D_{37} was estimated from the inverse of the slope of the line. The standard deviation of the slope was also calculated.

The target size was calculated from the relationship $M_r = 6.4 \cdot 10^5/D_{37}$ (+30 °C), in which the conversion factor was determined empirically by Kepner and Macey [10], and can also be calculated from independent physical measurements [24,25].

The D_{37} (−135 °C) was divided by a temperature correction factor of 2.8 in order to obtain the D_{37} (+30 °C) (see Ref. 26 for a systematic study of the magnitude of this temperature correction for five different soluble enzymes, and Refs. 27–30 for work with several membrane proteins in which the same factor has been found necessary to correct data obtained at −135 °C).

The accuracy of all the experimental measurement of activities falls at the low activities or high radiation doses. Rather than correct individual points with arbitrary weighting factors, all points below a certain percentage of the initial value were excluded as unreliable. On the basis of experience, these were 1% for $(Na^+ + K^+)$-ATPase and K^+-phosphatase, 2% for ATP-dependent Na^+-K^+ exchange; 3% for both modes of Rb^+-Rb^+ exchange and 10% for the α-chain. All other points were included in the analysis without weighting factors.

Materials

p-Nitrophenyl phosphate; ATP (vanadate-free); Sephadex G-50–40; Dowex 50W-X8 (50–100 msh); acrylamide and bisacrylamide were obtained from Sigma. Radioisotopes [γ-^{32}P]ATP, ^{22}Na and ^{86}Rb were purchased from Amersham Radiochemicals. All conventional chemicals were of analytical grade.

Results

Table I shows calculated apparent molecular weights for the various activities tested, both for individual experiments and as average values obtained by collecting all reliable data points.

Enzymatic activities

Fig. 1 shows representative inactivation curves for $(Na^+ + K^+)$-ATPase and K^+-phosphatase estimated for the irradiated frozen enzyme. In both cases activity fell exponentially to between 1 and 2% of the initial activity. The apparent target sizes for inactivation of $(Na^+ + K^+)$-ATPase and K^+-phosphatase in Fig. 1 were 180 kDa and 122 kDa, respectively, while the average values for all experiments by linear regression were 184 kDa and 125 kDa, respectively (Table I). By comparison with previous estimates of the target size for both $(Na^+ + K^+)$-ATPase and K^+-phosphatase our values are, with one exception, significantly lower (cf. Discussion, and Table II).

Cation transport in reconstituted vesicles

Fig. 2 shows the results of experiments to study the inactivation of ATP-dependent Na^+-K^+ ex-

TABLE I

TARGET SIZES OBTAINED BY RADIATION INACTIVATION OF RENAL ($Na^+ + K^+$)-ATPase WITH HIGH-ENERGY ELECTRONS

Activity measured	No. of expts.	Apparent molecular weight (mean ± S.D.) (kDa)	
		Individual experiments	Average for all experiments
($Na^+ + K^+$)-ATPase	4	180 ± 5, 175 ± 8 207 ± 8, 182 ± 4	184 ± 4
K^+-phosphatase	2	122 ± 2, 132 ± 7	125 ± 3
ATP-dependent Na^+-K^+ exchange	4	173 ± 5, 195 ± 7 236 ± 4, 205 ± 3	201 ± 6
(ATP + P_i)-activated Rb^+-Rb^+ exchange	2	210 ± 4, 175 ± 33	206 ± 7
ATP-independent Rb^+-Rb^+ exchange	3	112 ± 5, 117 ± 4 117 ± 4	117 ± 4
α-chain	2	> 110 ± 5, < 137 ± 8 > 124 ± 7, < 144 ± 8	> 115 ± 4 < 141 ± 5 Median value 128 ± 6

change and (ATP + P_i)-activated Rb^+-Rb^+ exchange measured on the same set of reconstituted vesicles, and ATP- and P_i-independent Rb^+-Rb^+ exchange measured on a separate set of vesicles. the activity of both the ATP-dependent Na^+-K^+ exchange and (ATP + P_i)-activated Rb^+-Rb^+ exchange fell rather convincingly along a single exponential to about 3% of the initial activity. This is in accordance with the requirements of the classical target theory, and the target sizes for the two activities are obviously the same. In this experiment (Fig. 2) the target size for ATP-dependent Na^+-K^+ exchange was 205 kDa and that for (ATP + P_i)-stimulated Rb^+-Rb^+ exchange was 210 kDa, while the average molecular weight for four different inactivation curves of ATP-dependent Na^+-K^+ exchange was 201 kDa and that for two (ATP +

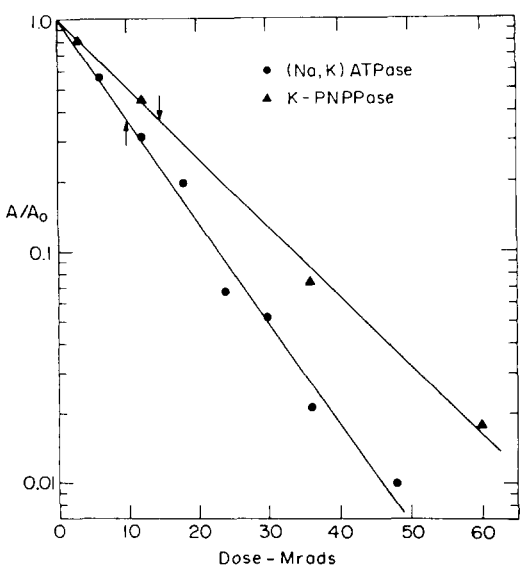

Fig. 1. Inactivation of ($Na^+ + K^+$)-ATPase (●) and K^+-phosphatase (▲) activities. The arrows in this and other figures indicate the D_{37} value. *A*, biological activity.

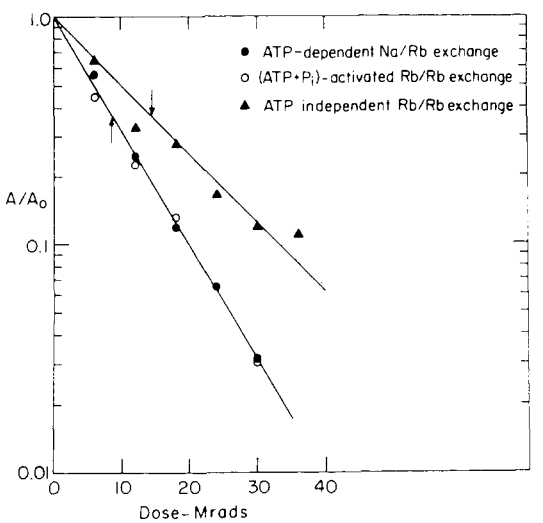

Fig. 2. Inactivation of transport functions of ($Na^+ + K^+$)-ATPase. For the purpose of demonstrating the similarity of the inactivation size of ATP-dependent Na^+-Rb^+ exchange and (ATP-P_i)-activated Rb^+-Rb^+ exchange, a single least-squares line has been drawn through the points (●,○). (See Table I for calculated target sizes of the individual flux models). *A*, biological activity.

P_i)-activated Rb^+-Rb^+ exchange experiments was 206 kDa, Table I. These values, although slightly higher, are clearly not significantly different from the target size for inactivating ($Na^+ + K^+$)-ATPase.

The range of target size estimates for four different ATP-dependent $Na^+ + K^+$ exchange experiments was significantly greater than for four ($Na^+ + M^+$)-ATPase measurements (see Table I). For pairs of different but identically prepared sets of vesicles the transport rates were found to vary by an average of 6.5% ($n = 11$). Of the various manipulations performed in reconstitution of the enzyme into vesicles, a likely source of variability is differences in effectiveness of the sonication after thawing. Another concern in transport experiments is the possibility of systematic differences in efficiency of reconstitution into vesicles of control and irradiated enzyme. Reconstitution is optimal over a particular range of concentration ratios of cholate to protein (between 4 and 8 mg cholate per mg protein), but falls quite sharply at more extreme ratios [16]. The fixed cholate concentration used for reconstitution was optimal for the control protein concentration but might not be so for the low concentrations of intact pumps remaining at high irradiation doses. A large fall in efficiency of reconstitution would cause the curve to deviate from the exponential sharply downwards at the high doses, and this can obviously be excluded. A small progressive decrease in efficiency of reconstitution would tend to make the slopes appear steeper, and it therefore seems prudent to consider the values of the target size for the ATP-dependent Na^+-K^+ exchange and other transport activities, as upper limits.

Notice that ^{22}Na and ^{86}Rb uptake into vesicles, dependent on the addition of the impermeant substrate ATP to the medium, is catalyzed by pumps oriented inside-out with respect to the normal cellular orientation. The ($ATP + P_i$) activated Rb^+-Rb^+ exchange is estimated as an ATP-dependent increment of Rb uptake, over and above that occurring in the presence only of Rb, P_i and Mg. Since in the absence of ATP, a slow pump mediated Rb^+-Rb^+ exchange can be detected [17], the target size that we are measuring here is specifically that for the stimulatory effect of ATP on Rb^+-Rb^+ exchange.

The ATP- and P_i-independent Rb^+-Rb^+ exchange was significantly less sensitive to radiation than the ($ATP + P_i$)-activated Rb^+-Rb^+ exchange, as seen in Fig. 2. The least-squares analysis for three different experiments gave a target size of 117 kDa. The apparent curvature of the semi-log plot in Fig. 2 can be attributed to random scatter since in the two other experiments this was not observed. The ATP-independent Rb^+-Rb^+ exchange has been measured as the saturable fraction of the total Rb uptake, by estimating the difference in ^{86}Rb uptake at 0.5 and 75 mM RbCl, that at the high Rb concentration representing the passive (non-saturating) leak into the vesicles. This measure of the saturable ATP-independent Rb^+-Rb^+ exchange gives the sum of fluxes through pumps oriented both inside-out and right-side out by comparison with the normal cellular orientation *.

Target size of the catalytic chain

A final set of experiments was designed to study the apparent size of catalytic α-chain as judged by disappearance of the Coomassie blue staining band on polyacrylamide gels (Fig. 3). The α-chain disappeared progressively in samples exposed to increasing doses of irradiation and was apparently broken up into a wide range of smaller polypeptides as evident from the increased staining throughout the gel, below its own position. Also there was a tendency for the α-chain to be split by irradiation at particular points, as seen for example by the appearance of the new low molecular weight band in the 18 Mrad samples (see the arrow in sample D).

Control experiments showed that the absorbance at 605 nm of dye bound to α-chain was proportional to the total protein applied to the gel, in the range 5–40 μg (see also Ref. 31). Fig. 4 shows that disappearance of the α-chain in the gel of Fig. 3 followed a reasonably good exponential down to 13% of the initial value. Virtually identi-

* Previously we have used vanadate inhibition of the ATP independent Rb fluxes to distinguish Na^+/K^+-pump mediated fluxes from passive leaks and determine the orientation of the pumps involved [17]. The use of vanadate in the presence experiments was undesirable because the target size so estimated could well be that for binding of the vanadate to the protein.

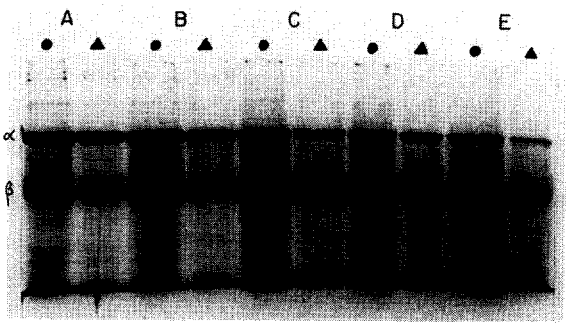

Fig. 3. SDS-gel electrophoresis of $(Na^+ + K^+)$-ATPase irradiated with high-energy electrons. ●, 35 μg protein; ▲, 17.5 μg protein. (A) Control; (B) 3 Mrad; (C) 9 Mrad; (D) 18 Mrad; (E) 24 Mrad. Absorbances at 605 nm of extracted Coomassie blue (minus background) for the 35 μg and 17.5 μg samples, respectively, were as follows: Control, 0.92 and 0.50; 3 Mrad, 0.64 and 0.36; 9Mrad 0.40 and 0.21; 18 Mrad, 0.25 and 0.13 and 24 Mrad, 0.124 and 0.069.

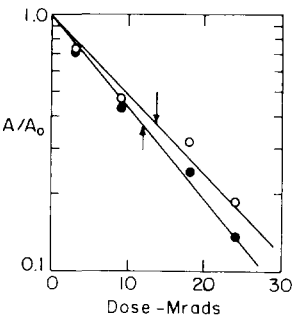

Fig. 4. Destruction of the α-chain of $(Na^+ + K^+)$-ATPase. The plotted A/A_0 value represents the average of A/A_0 values calculated at each radiation dose for the 35 μg and 175 μg samples, respectively. ●, with background correction; ○, without background correction. A, biological activity.

cal results were obtained for the 35 and 17.5 μg samples, which thus also served as independent internal controls for the linearity of Coomassie stain bound per unit mass of protein. The average target size in the experiment in Fig. 4 was 137 kDa and in another it was 144 kDa. These values probably represent upper limits for the following reason. For equal areas of gel from tracks run with decreasing amounts of control protein, or samples irradiated with increasing doses, the absorbance of the background Coomassie stain is not constant, but falls, although not in proportion to that in the α-chain. This implies that some of the dye in the gel segments adjacent to the α-chain was in reality bound to traces of protein and overestimated the true amount of unextracted stain. The effect of an excessive background correction becomes significant at high radiation doses. One can estimate a lower limit for the target size by ignoring the background stain. In Fig. 4 the lower limit was 110 kDa while in the second experiment it was 124 kDa. Therefore the best average estimate we have of the size of the α-chain by this technique would be less than 141 kDa and greater than 115 kDa (Table I) giving a median value of 128 kDa. The target size of the β-chain could not be evaluated with confidence firstly because of the poor definition of the band, and because the degradation products of the chain ran in an overlapping position, causing the extracted absorbance to actually rise somewhat for the intermediate radiation doses. Nevertheless, from visual inspection of Fig. 3, it is evident that the β-chain begins to disappear only at much higher doses than were necessary to destroy the α-chain, compare for example the intensity of the stain at α- and β-positions in samples A and E. This is, of course, the behaviour expected of the β-chain, which has a lower molecular weight than the α-chain, and demonstrates, at least qualitatively, that significant radiation energy transfer did not occur between the α- and β-chains in the membrane.

Discussion

We suggest below that the simplest interpretation of the target sizes for $(Na^+ + K^+)$-ATPase activity, ATP-dependent Na^+-K^+ exchange and $(ATP + P_i)$-dependent Rb^+-Rb^+ exchange is that these represent $\alpha\beta$, while the target sizes for K^+-phosphatase, and ATP-independent Rb^+-Rb^+ exchange are simply that of the α-chain itself. This leads to predictions concerning functional roles of α-and β-chains, which may be tested in the future by independent techniques. The conclusion in no way refutes the possibility for a dimeric structural organization within the membrane, but only that cooperation between the halves does not appear to be necessary for overall function.

Kempner and Schlegel [11] have compared the radiation target sizes of a large number of enzymes with the molecular weights estimated by more conventional techniques, and estimated that the

average discrepancy between the target sizes and accepted molecular weights is less than 14%. Given this expected degree of accuracy it is clear that the most reliable conclusions will be obtained from radiation inactivation experiments when an internal calibration of the molecular weight estimates is available by a direct measurement of the target size of a component polypeptide. In our case the target size for destroying the α-chain was estimated to be higher than 115 kDa and lower than 141 kDa giving a median value of 128 kDa.

There is still disagreement concerning the exact molecular weight of the α-chain as estimated by conventional techniques (cf. Refs. 1, 2). Analytical centrifugation of SDs-solubilized α-chains has given values of 94 or 106 kDa, respectively [32–34], in agreement with values of 97–104 kDa obtained by SDS-PAGE [31] (104 kDa for the pig kidney enzyme). A higher value of 121 kDa has has been reported, based on analytical ultracentrifugation of SDS-free α-chain [3] and values in the range 110–139 kDa based on gel filtration in SDS or guanidinium chloride solutions [35–37]. Similarly, although it is now widely agreed that the $\alpha:\beta$ stoichiometry is 1 : 1 [2], the published molecular weights for the $\alpha\beta$ complex (protein components only) range from 123 kDa (α, 94 kDa and β, 32 (34) kDa) [9], to 164 kDa (α, 121 kDa and β, 43 kDa) [3].

Given these ambiguities we avoid comparing the estimated target sizes with published molecular weight figures and the approach adopted below is to compare the target sizes for inactivating the various activities with that of our internal standard, the α-chain, and thus attempt to exclude particular models as being inconsistent with the data. This approach makes use of previous experience with many other enzymes that partial reactions can be inactivated with smaller target sizes than the overall function and the smaller molecular target size can often be identified with one or some component polypeptides in an oligomeric complex [11]. In this situation there can be little or no radiation energy transfer between neighbouring polypeptides, a condition which appears to hold in our case (see Results).

(a) The low range of target sizes

The target sizes for inactivating the α-chain (between 115 and 141 kDa), K^+-phosphatase activity (125 kDa) and ATP-independent Rb^+-Rb^+ exchange (117 kDa) are not significantly different from each other. the implication is that this target size reflects one and the same phenomenon, namely destruction of the α-chain, and all the individual steps involved in the K^+-phosphatase activity or ATP-independent Rb^+-Rb^+ exchange are dependent on the intactness only of the individual α-chains and are not affected by destruction of β-chains. From our knowledge of the mechanism the K^+-phosphatase or Rb^+-Rb^+ exchanges these individual steps must include (a) the cation binding at both cytoplasmic and extracellular surfaces of the protein, (b) conformational changes between the E_1 and E_2 forms of the protein (see Ref. 2) and (c) binding of the paranitrophenyl phosphate substrate (at what is normally a phosphate-binding site).

(b) The high range of target sizes. The minimal functional unit

The target sizes for $(Na^+ + K^+)$-ATPase (184 kDa), ATP-dependent Na^+-K^+ exchange (201 kDa) and $(ATP + P_i)$-activated Rb^+-Rb^+ exchange (206 kDa) are not significantly different from each other. The minimal functional unit is therefore the same whether measured as $(Na^+ + K^+)$-ATPase activity or the active ATP-dependent Na^+ and K^+ fluxes. The most reliable of the higher target sizes is undoubtedly that for $(Na^+ + K^+)$-ATPase, 184 kDa. A comparison of this value with that of the α-chain (Table I) shows that the $(Na^+ + K^+)$-ATPase target size is appreciably smaller than twice that of the α-chain. This finding is obviously inconsistent with models in which the minimal functional unit consists of pairs of α-chains, for example $(\alpha)_2$ or $(\alpha\beta)_2$. Interestingly in all radiation inactivation studies published to date the target size for inactivating $(Na^+ + K^+)$-ATPase has been found to be greater than for K^+-phosphatase, but the ratio of sizes $(Na^+ + K^+)$-ATPase: K^+-phosphatase has invariably been found to be smaller than 2.0, usually 1.4–1.6 (Table II). Thus the notion of an $(\alpha)_2$ or $(\alpha\beta)_2$ minimal functional unit provides no simple explanation for the observed differences between $(Na^+ + K^+)$-ATPase and K^+-phosphatase target sizes.

TABLE II

RADIATION TARGET SIZES OF $(Na^+ + K^+)$-ATPases AND K^+-PHOSPHATASES

Type of preparation	State during irradiation	Radiation source	Apparent mol. wt. (kDa)		Ref.
			$(Na^+ + K^+)$-ATPase	K^+-phosphatase	
Partially purified pig kidney $(Na^+ + K^+)$-ATPase	Frozen	13 Mev electrons	184	125	This paper
Partially purified pig kidney $(Na^+ + K^+)$-ATPase	Lyo.	20 MeV electrons	–	164	13
Purified pig kidney $(Na^+ + K^+)$-ATPase	Lyo.	16 MeV(?) electrons	261 (assuming exponential)	196	15
Guinea pig kidney microsomes	Lyo.	7.5 MeV electrons	190	140	10
Purified rabbit kidney $(Na^+ + K^+)$-ATPase	Lyo.	^{60}Co γ-rays	332	262	14
Rat liver plasma membranes	Lyo.	7.5 MeV electrons	200	–	10
Crayfish nerve	Lyo.	7.5 MeV electrons	270	–	10
Human red cell ghosts	Lyo.	7.5 MeV electrons	300	–	10
Human red cell membranes	Lyo.	20 MeV electrons	330	184	12

The most likely alternative explanation for the larger target size for inactivating $(Na^+ + K^+)$-ATPase is that it represents one α-chain plus one or possibly more β-chains, that is $\alpha\beta$ or $\alpha\beta_2$. Either of these possibilities is consistent with the observed ratio of 1.44 for the target size of $(Na^+ + K^+)$-ATPase compared to that of the α-chain or K^+-phosphatase. Irrespective of whether one accepts the higher or lower literature values for the molecular weights of α and β chain, one can calculate ratios of $(\alpha + \beta)/\alpha$ or $(\alpha + 2\beta)/\alpha$ which are close to the observed ratio of 1.44. For kidney enzyme the lower range of values in Ref. 34 α, 94 kDa and β, 32 kDa or the upper range of values in Ref. 3, α, 121 kDa and β, 43 kDa, give $(\alpha + \beta)/\alpha$ 1.34–1.36 and $(\alpha + 2\beta)/\alpha = 1.69$–1.71.

It is hard to exclude the $\alpha\beta_2$ model on the basis only of our radiation results. However, since it is now agreed that the ratio of $\alpha:\beta$ is 1:1, the hypothesis of an $\alpha\beta_2$ minimal functional unit implies that the protein is organized as an $(\alpha\beta)_2$ dimer (or higher polymer) in the membrane, and requires that a radiation hit in either β-chain destroys the $(Na^+ + K^+)$-ATPase activity of both halves, while a hit in an α-chain destroys activity only in its own half. The latter requirement seems quite unlikely. We conclude that the simplest interpretation of the results is that the minimal functional unit is $\alpha\beta$. This conclusion is consistent with other recent data suggesting also an $\alpha\beta$ minimal functional unit for $(Na^+ + K^+)$-ATPase activity [4–9].

Irradiation of lyophilized membranes

How can one account for the discrepancy between the target sizes for $(Na^+ + K^+)$-ATPase found by irradiating frozen wet or dry membranes, respectively, (Table II)? As shown in the recent study of Ottolenghi and Ellory [15] another major problem is the fact that K^+-phosphatase and Na^+-ATPase do not appear to inactivate along a single exponential in the lyophilized preparations. The model that the authors arrive at depends on the assumption that energy transfer occurs freely between all the α- and β-chains in an $(\alpha\beta)_2$ dimer, but in each α-chain there are domains which are

not destroyed or affected by absorption of radiation energy at other points in the same chain. It seems unlikely that energy transfer occurs more freely between independent chains than down the individual backbone of α-chains. Our aim here, however, is not to fault the model, 'obviously quixotic' in the authors words, for in any event the assumptions on which it is based apply only to the state of the protein when it was irradiated, i.e. dry.

The important question is whether there is inter-chain radiative energy transfer which leads to biochemical inactivation. There is evidence (Haigler, H., Woodbury, D. and Kempner, E., in preparation) that a disulphide bridge will carry radiation damage between adjacent chains of ricin in that cleavage of the S-S bridge before irradiation causes the target size to drop by half from the dimer to the monomer size. However, there is no evidence for disulphide bridges between the α- and β-chains of $(Na^+ + K^+)$-ATPase. Parkinson and Callingham [38] have shown that the apparent target size for inactivating acetylcholine esterase in lyophilized erythrocyte ghosts depends on the medium used to dry the membranes, and have proposed that in phosphate buffer, energy is transferred between adjacent chains so that a hit anywhere in the dimer inactivates both halves, while in other buffers energy transfer does not occur and the monomer target size is found.

A number of techniques provide evidence that the $(Na^+ + K^+)$-ATPase can be organized as a $(\alpha\beta)_2$ dimer in the membrane, (but not necessarily for functional interactions between the halves, cf. Ref. 2). It is conceivable that when water is removed the strength of the non-covalent interchain bonds increases, permitting energy transfer in this dry state. Obviously then a hit anywhere in a dimer would inactivate both halves even if normally in the wet state there were not significant functional interactions.

Another possibility is that rehydration after lyophilization is not fully reversibly in that destruction of half of a structural dimer precludes reactivation of the other intact half. An explanation along these lines might even explain non-exponential inactivation curves for one might produce heterogeneous populations of rehydrated enzyme molecules, some fully and others only partially reactivated.

Roles for the α- and β-chains

The α-chain is of course known to participate in specific ligand binding, the catalytic events of ATP hydrolysis and conformational changes associated with cation transport. The role of the β-chain is unknown although recent studies on the biosynthesis of the α- and β-chains have led to suggestions that the latter acts as a form of membrane receptor for the α-chain permitting its correct alignment and insertion into the membrane [39].

The striking difference in the functions inactivated with high or low target sizes, respectively, is that the former depend on the presence of ATP (Table I). If our interpretation of the minimal functional unit, $\alpha\beta$, is correct the implication is that ATP-dependent functions are sensitive to the intactness of β-chains as well as the α-chains to which the ATP binds. The α- and β-chain contacts in the membrane may constrain or stabilize the catalytic region of the α-chain so that destruction of the β-chain leads to alteration of the catalytic domain of the α-chain and disruption of ATP binding and/or ATP dependent effects.

Conversely, ATP-independent functions, appear to require only an intact α-chain (Table I). Thus we predict that cation binding and transport pathways are located exclusively on the α-chain. This is of course quite inconsistent with the notion that cation transport pathways lie within aqueous channels located at intra-membrane subunit interfaces [40]. A major challenge for the future will be to devise means to test the prediction that α-chains in the membrane can sustain ATP-independent functions, in the absence of their normal association with the β-chains.

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