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Heterogeneity of pig kidney Na,K-ATPase as indicated by ADP- and ouabain-binding stoichiometry

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A centrifugation method has been used for determination of [^{14}C]ADP and [^{3}H]ouabain binding to Na,K-ATPase from pig kidney with high specific activity. In the presence of K^+, the fit of the [^{14}C]ADP binding data to a two-site model gives a component with high affinity which accounts for 12 ± 2% of the total sites. The figure is significantly different from 50%, i.e., two components of equal size cannot be assumed. This contrasts with a ratio between the sites of 1 : 1 obtained by the rate dialysis technique. The discrepancy may be due to the fact that the centrifugation method enables bound ADP to be determined at lower concentrations of free ligand. [^{3}H]Ouabain binding in the absence of Na^+ is compatible with a straight line in a Scatchard plot if the isotope is purified shortly before use. An unspecific binding of ouabain can be neglected if the concentration of free ouabain is not too high. In the presence of Na^+, the isotherms become upward concave. An analysis of the binding data gives a 19 : 81% division, although equilibrium is not quite attained. This is a maximum value because the lack in equilibrium will be most pronounced at the small values of free ouabain. Thus the ADP-binding studies are supported. The finding here is in some agreement with the semiquantitative immunoassay showing that pig kidney enzyme contains the isoenzymes α_1, α_2 and α_3 in a proportion of 84 : 12 : 4, respectively. Determination of ADP- and ouabain-binding site stoichiometry favours a theory with one substrate site per $(\alpha\beta)_2$.

Introduction

The first equilibrium studies on the reaction of Na,K-ATPase with its substrate, ATP, were reported independently by two laboratories in 1971 [1,2]. It is the purpose of this report to present new observations on ADP binding to Na,K-ATPase as obtained by centrifugation. This technique permits measurement of ADP at lower concentrations than previously possible (e.g., by the rate dialysis technique [3]). Furthermore, the data suggest that a reappraisal of the results obtained with rate dialysis is necessary. New observations on ouabain binding are also reported.

In the presence of potassium the binding isotherms for ADP to microsomal Na,K-ATPase from pig kidney outer medulla maximally activated with SDS are non-linear curves in Scatchard plots, which is indicative of site heterogeneity [4–8]. This phenomenon complicates the evaluation of the binding isotherms. There are a number of models that can explain upward-curved Scatchard plots. Among them are the following: (A) two or more populations of independent sites with different affinities for the ligand: (1) inhomogenous enzyme preparation, partial denaturation, and (2) pre-existing different sites on the molecule, (B) homotropic negative cooperativity between two or more sites on the enzyme; and (C) heterotropically induced differences in the affinity of the ligand for two or more otherwise identical sites on the enzyme. The nature of the experiments and the accuracy of the data do not often justify the resolution of curved Scatchard plots into more than two straight-lined components [9]. In the case of models (A2), (B) and (C) these lines should have identical intercepts on the axis representing bound ligand, whereas in the case of model (A1), the intercepts, which corresponds to the site concentrations, need not be equal.

The rate dialysis method and the centrifugation method for determination of bound ligand use different incubation times. With the rate dialysis method binding may be determined within 8 min, while in the latter method 90 min is used. Furthermore, the centrifugation method requires lower specific radioactivity of

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Abbreviations: Na^+/K^+-ATPase, Na^+/K^+-transporting ATPase (EC 3.6.1.37); EDTA, ethylenediaminetetraacetic acid

nucleotide than is necessary for the rate dialysis procedure [10].

At 0°C and even in the presence of 10 mM EDTA, Na,K-ATPase is still able to hydrolyze ATP. This hydrolysis is not detected by the rate dialysis procedure. Centrifugation procedures, however, are sufficiently time-consuming for appreciable hydrolysis of ATP to occur. Obviously, this is not the case with ADP. Although ADP binds to Na,K-ATPase with lower affinity than ATP, measurements of ADP binding by the centrifugation method are a valid and useful alternative in qualitative and quantitative studies of the nucleotide-binding sites of Na,K-ATPase.

New observations and interpretations of nucleotide binding to Na,K-ATPase are supported by similar studies on ouabain binding. This is irrespective of the drawbacks in assessing [^{3}H]ouabain binding, which have been dealt with in a preliminary report [11].

It should be emphasized that the nucleotide-binding sites which are being studied are those that have high affinities for ATP or ADP. They are present in every enzyme preparation in exactly the same number as specific ouabain-binding and vanadate-binding sites [12]. Attempts have been made to determine the ADP- and ouabain-binding capacities of the most purified preparations.

Materials and Methods

Enzyme preparation. Pig kidney enzyme was prepared according to Jørgensen [13], i.e., selective extraction of plasma membranes with SDS in the presence of ATP, followed by isopycnic zonal centrifugation. The specific activity of the enzyme was 28–37 U(mg protein)$^{-1}$. It had an ADP-binding capacity of 3.2 to 3.6 nmol(mg protein)$^{-1}$ as measured with the method of Lowry et al. [14] using bovine serum albumin as a standard.

In a few cases a microsomal preparation was used. Crude microsomes from pig kidney outer medulla containing Na,K-ATPase were washed twice by centrifugation in an ice-cold solution of 250 mM sucrose, 12.9 mM imidazole, 0.625 mM EDTA, pH 7.15 (37°C). The microsomes were then activated by mixing enzyme suspension with SDS to give final concentrations of 3 mg protein and 0.4 mg SDS per ml. The Na,K-ATPase activity after standing overnight at room temperature is the maximally attainable and it is stable [8]. This activated enzyme preparation was washed three more times with the above solution. The final protein concentration was about 2.5 mg protein ml^{-1}. The specific activity was 13–17 U(mg protein)$^{-1}$ [14].

Protein determination. To evaluate the protein determination in our laboratory it was arranged that we and five other laboratories measured protein on two different purified Na,K-ATPase preparations from shark rectal gland and one albumin solution. The coefficient of variation (S.D. %) among laboratories was 5%, both with the original Lowry method and with the Peterson method [15]. The latter gave 10–15% higher protein concentrations than the original Lowry method. Furthermore, N-content was estimated by a Perkin-Elmer 240 Elemental Analyzer after combustion. Two different enzyme solutions with different specific activities were used. A buffer solution, which was the same as that in which the enzymes were suspended, served as control.

Na,K-ATPase activity. The activity was measured in the presence of 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+} and 3 mM ATP [16]. All activities mentioned in the text are those which can be inhibited by ouabain (approx. 97–99% of the total. They are given as U(mg protein)$^{-1}$. 1 U is equivalent to the hydrolysis of 1 μmol ATP per min.

Reagents. The reagents used were of analytical reagent grade whenever possible. SDS, Tris and imidazole were obtained from Sigma, EDTA from Merck, sucrose from British Drug House and the sodium salts of ADP and ATP from Boehringer. [^{14}C]ADP was obtained from NEN and [^{3}H]ouabain from The Radiochemical Centre, Amersham.

Nucleotides for use in binding experiments and for assays of activity were purified and converted to their Tris salts by chromatography on DEAE-Sephadex [1,10] or on Dowex 50W-X8. [^{3}H]Ouabain was purified by means of a minor modification of the method of Hansen [17]. Briefly, ouabain and kidney ATPase were allowed to react under conditions where only a fraction of the glycoside is bound. The ouabain-enzyme complex was washed free of unbound ouabain and bound ouabain was released into water by heating to 65°C for 30 min. Insoluble material (enzyme, etc.) was removed from the mixture by centrifugation.

ADP-binding assay. A centrifugation method was used [10] to assay ADP binding. To the enzyme solution was added one twelfth of a volume containing 150 mM Tris-HCl, NaCl 750 mM, EDTA 142.5 mM. To 1300 μl of this was added 200 μl of Tris-HCl 150 mM (pH 6.3 at 37°C) containing labelled and unlabelled ADP in varying concentrations. The final concentrations of reagents in the binding assay were: sucrose 200 mM, Tris 30 mM, EDTA 10 mM and Na^+ 50 mM. When binding was performed in the presence of K^+ NaCl was replaced by equimolar amounts of KCl to keep the ionic strength constant. The final pH at 37°C was 7.2. Aliquots of 75 or 100 μl were pipetted into counting vials for the determination of total radioactivity and the assay mixtures were then centrifuged for 3/4 or 1 h at 0–2°C at 100000 × *g*, which was sufficient to sediment the enzyme (and the enzyme-nucleotide complex). 75 or 100 μl aliquots of the supernatants were then withdrawn for determination of free

nucleotide. Bound nucleotide was calculated as total minus free. No unspecific binding was observed (see Fig. 3).

Binding of ouabain. Unless otherwise stated ouabain binding was determined by incubating the enzyme with varying concentrations of ouabain + ^{3}H-ouabain in the presence of 3 mM Mg^{2+}, 3 mM phosphoric acid neutralized with 2-amino-2-methyl-1,3-propanediol, 10 mM imidazole (pH 7.25) and 175 mM sucrose at 37°C for 90 min. The mixture was cooled to 0°C, at which temperature the enzyme-ouabain complex was found to be stable [18]. An aliquot was centrifuged at $100000 \times g$ for 60 min at 0°C. The concentration of bound ligand was estimated as the difference between the concentration of ouabain in the non-centrifuged aliquots and in the supernatants of the corresponding centrifuged preparations.

Preliminary experiments disclosed the presence of a small component of unspecific binding (a large number of sites with low affinity). This component was characterized by experiments with zonal enzyme and ouabain concentrations up to 40 times the enzyme concentration and with heat-denatured zonal enzyme (Fig. 4a and 4b).

Analysis of binding data. The results of binding data are presented in the form of Scatchard plots with concentration of bound ligand [B] plotted against [B]/[F], where [F] represents the concentration of free ligand. The data were fitted to the following model:

$$[B] = a \cdot [F]/(b + [F])$$

for straight-lined isotherms in Scatchard plots, and

$$[B] = a \cdot [F]/(b + [F]) + c \cdot [F]/(d + [F])$$

for the data shown in figures with upward curved isotherms in Scatchard plots. a and c denote the concentration of binding sites, and b and d are the corresponding site-dissociation constants. When $a = c$, $c/(a + c) = 1/2$.

A non-linear least-squares fit of these equations was applied to the experimental data, weighted so as to take into account that the coefficient of variation in the binding experiments was found to be constant. When all the binding points in an experiment had an average of at least two values, the S.E. entered into the weighting factor.

Results

Binding of ADP

Pig kidney enzyme treated with SDS has been used in the study of ADP binding, both with and without zonal centrifugation.

In Fig. 1a is shown the ADP binding to zonal enzyme in the presence of 20 mM K^+ and 30 mM Na^+.

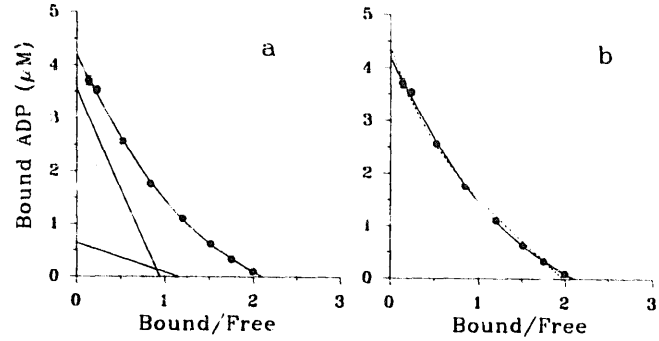

Fig. 1. (a) Scatchard plot of ADP binding to zonal enzyme from pig kidney in the presence of 20 mM K^+ and 30 mM Na^+. The curve is the best fit to the experimental data. The two straight lines are computed as the best fit to a model that assumes that the sample contains two independent classes of ADP sites. $c/(a + c) = 0.153$. (b) The same data as in Fig. 1a. The solution of the data to a model which contains equal concentrations of the two components, (i.e., $a = c$) is shown by the dashed line.

It appears that the curve is upward concave and the analysis gives a ratio of $c/(a + c)$ of 0.153. The plot for a ratio of 1:2 is also shown as the dashed line in Fig. 1b. The average deviation from the data is 2% and 3%, respectively. The concentration of sites using the best fit is 3.58 nmol sites per mg protein. The Na,K-ATPase activity is 32.5 U(mg protein)$^{-1}$.

The ADP binding to zonal enzyme was now studied in the presence of potassium at 15, 25 and 35 mM (Fig. 2). The ratio of $c/(a + c)$ for the best fit is 0.084. For a ratio of $c/(a + c)$ of 1:2, no solution to the data is found for $K^+ = 25$ and 35 mM. The concentration of sites is 3.23 nmol(mg protein)$^{-1}$. The Na,K-ATPase activity is 28.9 U(mg protein)$^{-1}$.

Finally, an enzyme that had not been subjected to zonal centrifugation was studied. The K^+- and Na^+-concentrations were both 25 mM. The binding data are shown in Fig. 3. The ratio of $c/(a + c)$ is 0.123, which lies between the values found with zonal enzyme. The

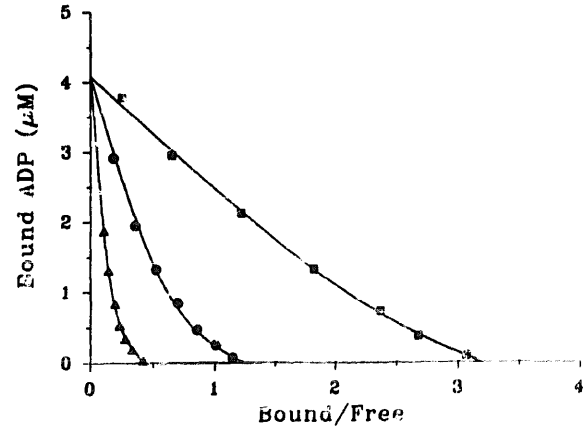

Fig. 2. Scatchard plot of ADP binding to zonal enzyme from pig kidney at K^+ and Na^+ concentrations of (15+35), (25+25) and (35+15) mM from right to left. The ratio of $c/(a + c)$ is 0.084 and the curves are all fitted to this value. With K^+ 25 and 35 mM there is no solution to $a = c$.

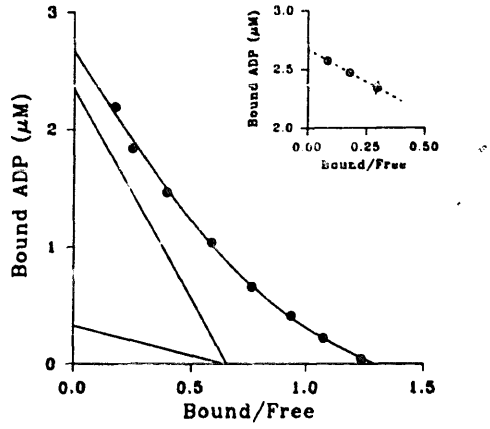

Fig. 3. Scatchard plot of ADP binding to pig kidney Na,K-ATPase not subjected to zonal centrifugation, in the presence of 25 mM K^+ and 25 mM Na^+. The points (●) are the average of 2–6 experiments with the S.E. shown as bars. Unless visible, S.E. bars were smaller than the diameter of the symbol. $c/(a+c) = 0.123$, i.e., between the two from zonal preparations. There is no solution to $a = c$. The inset shows the ADP binding at no K^+ added and with high concentrations of free ADP. No unspecific binding is observed (cf. Fig. 4 which shows the unspecific binding of ouabain).

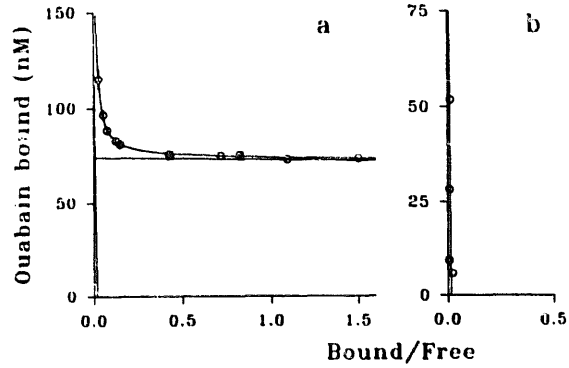

Fig. 4. (a) Scatchard plot of ouabain binding to pig kidney zonal Na,K-ATPase illustrating unspecific binding. Binding was performed as described under Materials and Methods. The points (○) are the average of 2–4 experiments with the S.E. shown as bars. Two classes of binding sites were assumed. The two straight lines are computed as the best fit to a two-site model. The dissociation constants (the slopes of the straight lines) are 1.12 nM for the high-affinity binding and about 8000-times higher for the unspecific binding. (b) Scatchard plot showing unspecific binding of ouabain. The zonal enzyme was heated to 65°C for 10 min. The line shown is almost vertical pointing to unspecific binding.

data cannot be fitted to a ratio of $c/(a+c)$ of 1:2. The concentration of sites is 1.37 nmol(mg protein)$^{-1}$ and the Na,K-ATPase activity is 13 U(mg protein)$^{-1}$.

The result with the three enzyme preparations was 12 ± 2%, which is significantly different from 1/2 or 50% ($P < 0.005$).

The inset in Fig. 3 shows [^{14}C]ADP binding in the absence of K^+ and shows that no unspecific binding is observed (cf. Fig. 4 for ouabain binding).

Maximal concentration of sites per mg protein

The ADP-binding capacity from seven preparations with a specific activity of 33.7 ± 0.6 (mean ± S.E.) U(mg protein)$^{-1}$ (Lowry method) was 3.61 ± 0.01 (mean ± S.E.) nmol(mg protein)$^{-1}$. The average turnover was 9335 min^{-1}. The analysis of N-content and the protein concentration based on this assay and the analysis of protein concentration (Lowry) is shown in Table I, where a nitrogen content in the protein moiety of 16.6% was used [19]. The binding stoichiometry is very close to 1 per 2 α-subunits (theoretically 3.4 nmol(mg protein)$^{-1}$)). Calculation according to Peterson's method [15] yields 3.2 nmol(mg protein)$^{-1}$.

TABLE I

Determination of protein concentration by nitrogen assay and by the method of Lowry et al. in Na,K-ATPase preparations

	N content (mg/ml)	Protein concentration (mg/ml)	
		nitrogen assay	Lowry assay
Buffer	0.0408	–	–
Enzyme solution I	0.3667	1.96	2.02
Enzyme solution II	0.3742	2.01	2.03

Binding of ouabain

All the experiments concerning ouabain binding have been made with zonal enzyme.

It is seen from Fig. 4a that the unspecific binding of ouabain appears when the concentration of free ouabain is above 100 nM. For free concentrations below 100 nM, the binding curve can be used to estimate the total high-affinity binding for ouabain. The concentration of high-affinity sites is 3.4 nmol(mg protein)$^{-1}$ corresponding to a Na,K-ATPase activity of 33.5 U(mg protein)$^{-1}$. The full straight lines show the correct resolution of the data into two components. The dissociation constant for the high-affinity component is 1.12 nM, and that for the unspecific binding is about 8000-times higher. In order to show that unspecific binding occurs, enzyme was heated to 65°C for 10 min. The concentration of free ouabain was then from 289 to 4312 nM. A nearly vertical line appears in Fig. 4b.

Very often a downward deviation from the straight line is obtained as can be seen in Fig. 5. This is probably due to radiolytic decomposition of [^{3}H]ouabain. Data with [^{3}H]ouabain that had been stored for one year (○) and after purification (□) are consistent with a radiolytic decomposition of no less than 7–8%. Also shown are binding isotherms obtained with a second batch of [^{3}H]ouabain before purification (◇) and after purification and storage for about 14 days (△). Freshly purified [^{3}H]ouabain is needed for accurate Scatchard analysis of binding data since the radiochemical is unstable when stored in

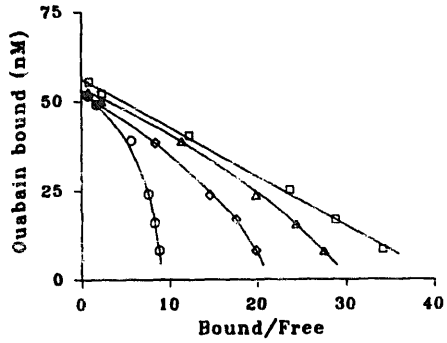

Fig. 5. Radiolytic decomposition of [^{3}H]ouabain. Data obtained with [^{3}H]ouabain that had been stored for one year (○) and after purification (□) are consistent with radiolytic decomposition of [^{3}H]ouabain of no less than 7–8%. Also shown are binding isotherms obtained with a second batch of [^{3}H]ouabain before purification (◇) and after purification and storage for about 14 days (△). Downward concave curves might falsely be interpreted as positive cooperativity between sites.

frozen buffer. Using relatively low concentrations of free ouabain and freshly purified [^{3}H]ouabain the binding capacity may be estimated as the intercept on the ordinate. An upward-curved Scatchard plot under these circumstances will be indicative of another problem.

In the presence of sodium (70 mM) the straight line is converted to an upward-curved isotherm (Fig. 6). Binding of ouabain to the enzyme is not at equilibrium after $2\frac{1}{2}$ h (inset in Fig. 6) so an incubation time of 3 h was used followed by centrifugation for 1 h. Even 3 h of preincubation might not be sufficient for equilibrium to be obtained. Longer incubation times were not used since a time-dependent decrease in Na,K-ATPase activity would be expected to occur.

It is therefore concluded that, even with freshly purified [^{3}H]ouabain, a correct resolution into two components cannot be obtained.

Analysis of the binding data in Fig. 6 according to

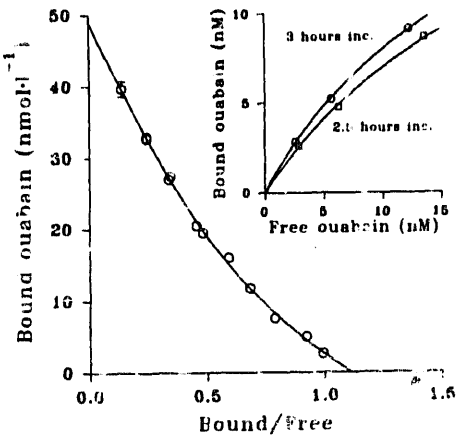

Fig. 6. Scatchard plot of ouabain-binding data obtained during a 3 h preincubation and in the presence of 70 mM Na^+. It can be seen in the inset that equilibrium is not achieved after $2\frac{1}{2}$ h preincubation. For further explanation see text.

the model presented in the above section on 'analysis of binding data' gives values for a and c of 39.2 nM and 9.2 nM, respectively. c thus amounts to 19% of the total sites. Equilibrium has probably not been attained, but this will be most pronounced at the small values of free ouabain, meaning that c will be slightly overestimated. The true value of c is probably smaller and it might well be in the same order as that determined in the ADP-binding experiments.

Discussion

Under appropriate experimental conditions, both impure preparations of Na,K-ATPase as well as highly purified membrane-bound enzyme showed an apparent heterogeneity in the properties of their nucleotide-binding sites. With the rate dialysis technique, the binding curves could easily be fitted to a model requiring that each molecule of Na,K-ATPase contains (a minimum of) two nucleotide-binding sites, that in appearance or in fact, show interaction with each other [5,8]. The curves could be fitted to a ratio of $c/(a+c)$ of 1:2.

However, if the centrifugation method is used, the solution of the curves by fitting the data which have been obtained in several enzyme preparations and with several duplicates, shows that the ratio of $c/(a+c)$ is not 1:2, i.e., that $a \neq c$. Setting $c/(a+c) = 1:2$ gives in some cases a solution to the curves which are obtained with the lowest K^+ concentrations, but this solution is not the best one. In other cases with high K^+ no solution is obtained (Figs. 2 and 3). Indeed, a ratio of 12% (±2%) resulted, which is significantly different from 50%. This ratio was the same for impure and more purified enzyme preparations. The reason for this finding could be that in the centrifugation experiments, binding at a lower concentration of free ADP has been measured, thereby giving results which are closer to the B/F-axis. Furthermore, with the rate dialysis technique equilibrium might not have been attained within 8 min with the low concentrations of nucleotide. It must therefore be assumed that at least two inhomogeneous enzymes are present.

The binding of ouabain to purified Na,K-ATPase is compatible with a straight line in the Scatchard plot when facilitated by (Mg^{2+} + P_i) and no sodium is added (Figs. 4 and 5). Labelled ouabain undergoes radiolysis and must therefore be used within a few days of purification. Since ouabain binds unspecifically, the concentration of free ouabain must not be too high if the binding capacity is to be estimated from extrapolation. When sodium is added to the incubation medium the binding isotherm becomes upward concave (Fig. 6). In the absence of sodium and potassium the binding reached equilibrium but in the presence of sodium alone equilibrium has not quite been achieved, even after $2\frac{1}{2}$ h of preincubation + 1 h of centrifugation.

The question arises as to, whether the enzyme has lost any activity after 3 h incubation at 37°C. Analysis of the upward curved isotherm shows that 19% of the sites have high-affinity. This proportion would be lower, if equilibrium had been reached, and would be similar to that seen with ADP.

Could the finding here be due to different isoforms of the enzyme? The conclusion has usually been that α_1-mRNA is predominantly or exclusively expressed in rat kidney [21,22]. However, in human kidney a high contribution by α_3-mRNA was found [23]. Arystarkhova et al. were able to isolate the α_3-isoform from pig kidney by means of monospecific antibodies [24]. Immunological studies of rat kidney with monoclonal antibodies indicated the presence of only α_1 [25]. Shyjan and Levenson [26] developed monospecific antibodies for each of the three α-isozymes in rats. The affinity-purified antisera used revealed that the α_1 subunit was the only one abundantly present.

The monospecific antibodies developed by Shyjan and Levenson was used by Hansen [27]. He measured the spectrophotometric absorbance signal for semi-quantitative determination of isozymes. Comparable binding of the individual antisera to isoenzymes on microplates coated with Na,K-ATPase and of the peroxidase-coupled anti-IgG used for detection of bound antibodies was assumed. According to this procedure, the distribution of α_1, α_2 and α_3 in pig kidney Na,K-ATPase would be 84 %, 12% and 4%, respectively. This means that the presently determined value of 12% for component c may reflect the existence of α_2 and α_3 in the enzyme. The distribution would then have been three components instead of two.

The value of 10–20% cannot reflect an enzyme with lower affinity for K^+, since binding of the K^+ congener Tl^+ to the enzyme is compatible with a straight line in the Scatchard plot, i.e., homogeneity of all K^+-sites [28].

By analyzing the nitrogen content in two preparations with different specific activities the protein concentration was found to be correct within 3%. Furthermore, in a number of experiments with 10 different preparations, the Lowry method and nitrogen assays appeared to agree within 3%, the standard error in each case being ±2% [19]. The amino acid analysis gave a lower estimate of the protein concentration than the Lowry and nitrogen assays. The maximal yield of amino acids is strongly dependent on the particular preparation procedure of the sample for hydrolysis and on the hydrolysis conditions. The highest yield is achieved when the most favourable conditions for protein solubilization are ensured.

It is suggested that measurements obtained with the Lowry method are correct. These results favour a model with one ATP-, one ADP- and one ouabain-binding site per $(\alpha\beta)_2$ over a model with two binding sites for membrane-bound Na,K-ATPase. Although it may be argued that 3.6 is larger than the theoretical 3.4 nmol mg^{-1}, the values are so close and so far from 6.8 nmol mg^{-1} that it is not easy to explain that our preparations should contain significantly less than 100% active ATPase. Optimal demasking of latent enzymatic activity of microsomes can be obtained without any change in the number of ouabain-binding sites [12]. It has been investigated whether the SDS treatment during the preparation of purified Na,K-ATPase could result in denaturation and disappearance of binding sites. Neither ouabain- or ADP-binding sites nor phosphorylation sites were altered by incubation with SDS [29].

Radiation inactivation studies suggest that dimeric α-subunit association is a structurally, and therefore also functionally, important feature of Na,K-ATPase. Furthermore, a single α-peptide exhibits all the partial reactions investigated, i.e., ATP, ADP, vanadate and ouabain binding with original affinity and K-pNPPase activity. The target size of the α-peptide was equal to the molecular mass of an α-monomer [30,31]. It is also interesting that unmodified occlusion of the K^+ congener, Tl^+, seems to be retained only by the intact α-peptides in assemblies that can catalyze a full Na,K-ATPase cycle. It is speculated that this indicates a close connection between full Na,K-ATPase activity and the ability to perform Na,K-transport by the individual α-peptides in these dimeric structures. It also suggests, that the K^+-ATP antagonism, which is prominent in nucleotide binding experiments with the membrane-bound Na,K-ATPase, but much less pronounced in solubilized enzyme [6], reflects normal K^+-occlusion by the α-peptides in an (α_2)-dimer.

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