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ATP BINDING TO SOLUBILIZED ($Na^+ + K^+$)-ATPase

THE ABOLITION OF SUBUNIT-SUBUNIT INTERACTION AND THE MAXIMUM WEIGHT OF THE NUCLEOTIDE-BINDING UNIT

JØRGEN JENSEN and PAUL OTTOLENGHI

Institute of Physiology, University of Aarhus, 8000 Aarhus C (Denmark)

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Membrane-bound ($Na^+ + K^+$)-ATPase from pig kidney outer medulla shows apparent heterogeneity in its ATP-binding site population when assays are carried out in the presence of K^+. This finding has been interpreted as being due to interaction between (at least) two subunits, each containing an ATP-binding site. Treating the membrane-bound enzyme with the detergent, $C_{12}E_8$, has been shown to solubilize enzymatically active $\alpha\beta$-protomers. We show that in the dissolved enzyme all ATP-binding sites in the population are identical both in the absence and in the presence of K^+, which would be consistent with an abolition of subunit-subunit interaction. This supports previous suggestions that enzyme solubilized by $C_{12}E_8$ is monomeric and that the membrane-bound enzyme is not. Differential extraction of enzyme-containing membranes with $C_{12}E_8$ yielded preparations with an ATP-binding capacity of up to 5.8 nmol per mg protein, measured by the method of Lowry et al. (Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275), with bovine serum albumin as standard. Evidence is presented that makes it likely that preparations with an ATP-binding capacity of 7.5 nmol per mg protein (as determined by the above-mentioned assay) will be obtainable. This corresponds to an $\alpha\beta$-protomer molecular weight of 133000 which approximates closely to the minimum value found in the literature for an $\alpha\beta$-protomer (i.e., 126000).

Introduction

We have recently provided evidence which we believe proves that, in their membrane-bound form, ($Na^+ + K^+$)-ATPase molecules are dimers containing two nucleotide-binding sites and two ouabain-binding sites [1]. The evidence for the above suggestion developed from the old observation that K^+ induces an apparent heterogeneity in a nucleotide-binding site population that was homogeneous in the absence of K^+ [1–3]. With ouabain-binding sites on the other hand, a homogeneous population of sites becomes apparently heterogeneous in the presence of Na^+ [1]. Some form of interaction between subunits, each of which possesses a nucleotide site and an ouabain site, was shown to be responsible for the apparent heterogenity.

Brotherus et al. [4] have shown that the molecular weight of ($Na^+ + K^+$)-ATPase from pig kidney medulla solubilized with the detergent $C_{12}E_8$ is 170000 or less: this value cannot accommodate more than a single α-peptide. Since it is probable that there is only one nucleotide site and only one

Abbreviations: $C_{12}E_8$, octaethyleneglycol mono-*n*-dodecyl ether; Tes, *N*-(tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid.

ouabain site per α-peptide, this soluble enzyme should only contain one nucleotide site and one ouabain site. Thus, if our suggestion of a dimeric membrane-bound enzyme reflects reality, treatment of membranous enzyme preparations with detergent should abolish the functional subunit interactions seen in nucleotide-binding studies concomitantly with the abolition of the structural interactions that lead to solubilization and monomerization. The first subject of this note is the description of just such an effect.

The second item in this communication was the outcome of graded treatment of membrane-bound enzyme with the detergent $C_{12}E_8$: it resulted in the differential extraction of protein from the membranes and led to preparations with a considerably increased number of ATP-binding sites per mg protein when compared to enzyme prepared according to the standard method of Jørgensen [5].

Materials and Methods

Membrane-bound ($Na^+ + K^+$)-ATPase was isolated from pig kidney outer medulla by the method of Jørgensen [5], i.e., selective extraction of plasma membranes with SDS in the presence of ATP, followed by isopycnic zonal centrifugation. The enzyme was kept in 250 mM sucrose, 12.9 mM imidazole, 0.625 mM EDTA and at a pH of 7.15 (measured at 38°C). The enzyme had an ($Na^+ + K^+$)-ATPase activity [6] of about 30 μmol ATP split/min per mg protein. Before measurement of ATP binding, the enzyme was sedimented by centrifugation for 30 min at $100000 \times g$ at 0°C. The pellet was suspended at a protein concentration of about 0.35 mg/ml in 10 mM Tes, 1 mM EDTA, 1 mM dithiothreitol and $C_{12}E_8$ in concentrations varying between 0 and 1.2 mg/ml. After 30 min at room temperature the nonsolubilized residue of samples to which $C_{12}E_8$ had been added was removed by centrifugation for 30 min at $100000 \times g$ at 0°C, and the supernatant was then used for binding studies. The control sample without $C_{12}E_8$ was used directly for ATP-binding site determination. The above treatment of enzyme-containing membranes with $C_{12}E_8$ is one that has been shown by Brotherus et al. [4] to solubilize and monomerize ($Na^+ + K^+$)-ATPase.

Binding experiments. The rate-dialysis technique of Colowick and Womack [7] as described by Nørby and Jensen [8] was used with the following specifications. The binding assay contained 1200 μl of the above-mentioned enzyme suspension or solution, 100 μl of 63 mM EDTA and 200 μl of 300 mM Tris with varying concentrations of mixtures of [^{14}C]ATP and unlabelled ATP. The final concentrations in the assay were 5 mM EDTA, 40 mM Tris, 8 mM Tes, 0.8 mM dithiothreitol, 120 mM $Na^+ + K^+$ and between 0.2 and 7.5 μM ATP and a pH of 7.46 at 0°C. The experimental procedure included collection of eight successive 0.5-min samples whereby it could be assured that equilibrium conditions existed. The determination of one complete binding isotherm took a maximum of 2 h. No measurable decrease in enzymatic activity was observed during such a period of time, even at the highest concentrations of $C_{12}E_8$ used.

Protein determinations. In all enzyme samples used for nucleotide-site determinations, protein was measured in each of the aliquots corresponding to each of the points on an ATP-binding isotherm. All protein concentrations were thus determined from between 4 and 20 individual measurements, using the method of Lowry et al. [9] after precipitation with trichloroacetic acid and using a standard curve with bovine serum albumin. The albumin standard was made up by using an extinction coefficient, $E^{1\,cm}_{1\%}$ (At 279 nm), of 6.67 [10]. The presence of detergent or of the other reagents in the medium used to dissolve the membrane-bound enzyme had no effect on the color yield of the albumin standard or of the membrane protein. Furthermore, the sum of the amounts of protein assayed after solubilization of enzyme with $C_{12}E_8$ in the soluble fraction + the insoluble residue gave complete recoveries of the protein contained in the sample before detergent treatment.

Reagents. Bovine serum albumin was obtained from Behringwerke (Marburg), SDS, Tris, Tes, dithiothreitol and imidazole were from Sigma, EDTA from Merck (Darmstadt) and sucrose from BDH. ATP was obtained as its Na^+ salt from Boehringer and [^{14}C]ATP as its ammonium salt from The Radiochemical Centre (Amersham). Labelled and unlabelled ATP were converted to their Tris salts by chromatography on DEAE-Sep-

hadex [8]. $C_{12}E_8$ was obtained from Nikko Chemicals, Tokyo.

Results

The evidence for subunit-subunit interaction in $(Na^+ + K^+)$-ATPase was based on the K^+-induced curvature in nucleotide-binding isotherms in Scatchard plots obtained with enzyme preparations that were not particularly pure (they had a nucleotide-binding capacity of about 1 nmol per mg protein [1]). Fig. 1 shows that much purer enzyme, prepared according to well established procedures [5], also shows a K^+-dependent apparent heterogeneity of the ATP-binding site population (i.e., a curvature in the binding isotherms of the Scatchard plots in the figure).

Brotherus et al. [4] have described conditions under which membrane-bound enzyme can be recovered in active, soluble form and with a protein molecular weight of 170000 or less. Using the same type of enzyme preparation as these authors and using all other experimental conditions that they described as yielding active enzyme with such small molecular weights, we obtained preparations that were used in the following experiments.

In Fig. 2 are compared the ATP-binding properties, in the presence of 120 mM NaCl, of crude microsomal enzyme, highly purified membrane-bound enzyme, and the latter enzyme dissolved

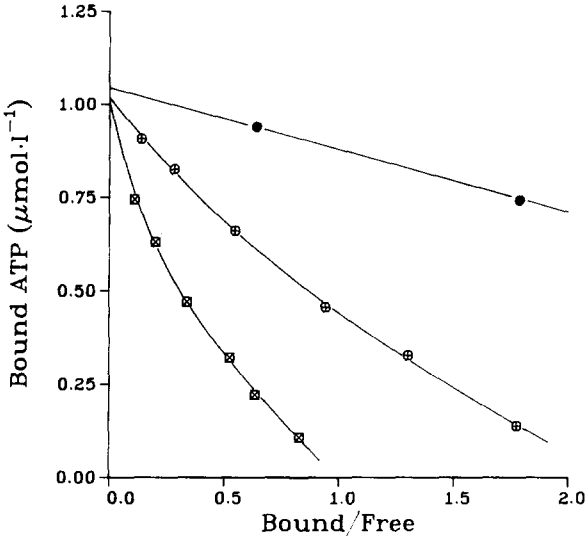

Fig. 1. Scatchard plots of isotherms for the binding of ATP to highly purified, membrane-bound $(Na^+ + K^+)$-ATPase in the presence of 120 mM Na^+ (only the first portion of the isotherm is shown here) (●); 80 mM Na^+ + 40 mM K^+ (⊕); and 60 mM Na^+ + 60 mM K^+ (⊠). In the binding assays, the enzyme preparation was present at a concentration of 0.32 mg protein per ml, giving an ATP site capacity of 3.3 nmol per mg protein. The curved isotherms were fitted to the experimental points by nonlinear regression analysis assuming that each molecule of enzyme has two ATP-binding sites that show apparent negative cooperativity [1]. The straight-line isotherm was calculated by linear regression. This latter isotherm can be seen in its full range in Fig. 2. The concentration of binding sites (E_t) in the assay mixture and the calculated apparent microscopic dissociation constants for ATP (K_{diss}) were found to be the following. (●) $E_t = 1.04$ μM; $K_{diss} = 0.17$ μM. (⊕) $E_t = 1.02$ μM; $K_{diss(1)} = 0.46$ μM; $K_{diss(2)} = 0.77$ μM. (⊠) $E_t = 1.01$ μM; $K_{diss(1)} = 1.02$ μM; $K_{diss(2)} = 2.71$ μM.

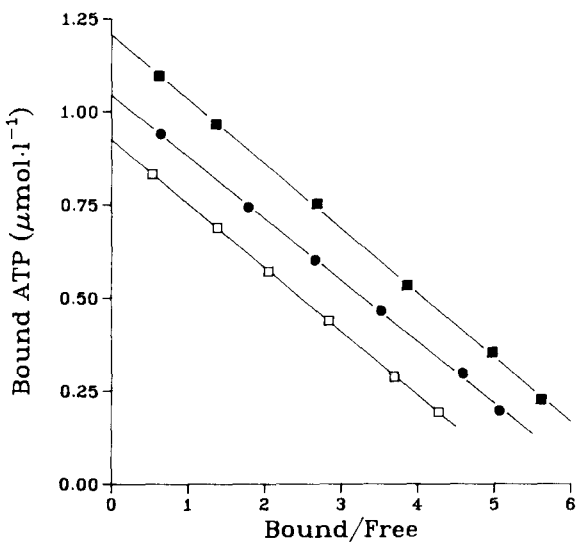

Fig. 2. A comparison of the binding of ATP to three enzyme preparations in the presence of 120 mM Na^+. (■) Crude microsomal $(Na^+ + K^+)$-ATPase with an ATP-binding capacity of approx. 1 nmol per mg protein. (●) Purified membrane-bound $(Na^+ + K^+)$-ATPase with an ATP-binding capacity of 3.3 nmol per mg protein. (□) Enzyme solubilized from the purified, membrane-bound preparation by using 3 mg $C_{12}E_8$ per mg protein and centrifuging off the undissolved residue. The same concentration of membrane-bound enzyme was used in the previous experiment and in this one; about 90% of the original binding sites for ATP were solubilized and the binding capacity of the dissolved material was 4.1 nmol per mg protein. The last curve is also shown in Fig. 3. The concentration of ATP-binding sites and apparent dissociation constants for ATP of the three preparations were the following. (■) $E_t = 1.21$ μM; $K_{diss} = 0.17$ μM. (●) $E_t = 1.04$ μM; $K_{diss} = 0.17$ μM. (□) $E_t = 0.92$ μM; $K_{diss} = 0.17$ μM. Thus, the affinity for ATP of the three preparations is the same.

with 3 mg $C_{12}E_8$ per mg protein as described in Materials and Methods. As can be seen, the affinities of all three preparations for ATP are nearly identical; the concentrations of detergent used dissolved about 85% of the membrane-bound ATP-binding sites and up to about 85% of the membrane protein. These values are essentially the same as those found by Brotherus et al. [4].

While the addition of K^+ to an ATP-binding system containing membrane-bound enzyme induces curvature in the binding isotherms of Scatchard plots (Fig. 1), after dissolution of the enzyme with $C_{12}E_8$ all traces of such curvature disappear (Fig. 3): no evidence remains of nucleotide-site heterogeneity (real or apparent) under conditions in which the enzyme is known [4] to exist in a molecular form that cannot accommodate dimers of α-peptides (or of $\alpha\beta$-protomers). It should be of interest to note that even in the presence of 120 mM K^+, ATP still binds to this dissolved enzyme with reasonably high affinity (and a K_{diss} of 1.2 μM).

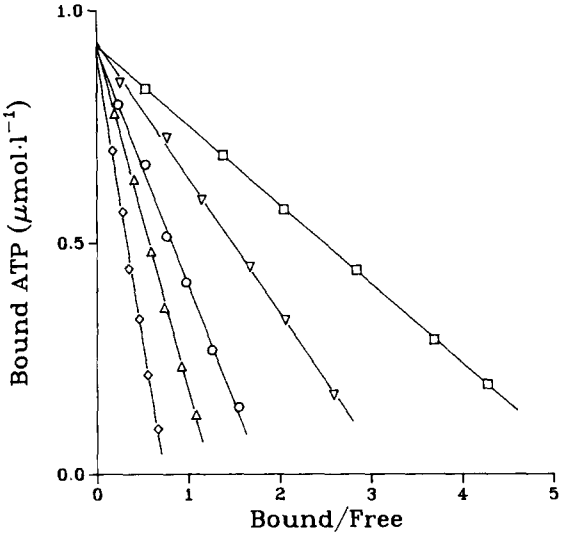

Fig. 3. The effect of varying the ($Na^+ + K^+$) composition of the binding assay on the isotherms for the binding of ATP to purified and detergent-solubilized ($Na^+ + K^+$)-ATPase. For each of the experiments shown, 0.4 mg per ml membrane-bound enzyme of Fig. 2 was dissolved by treatment with 1.2 mg per ml $C_{12}E_8$ and the particulate residue was removed by centrifugation. The isotherms shown were obtained with 0.22 mg protein per ml assay mixture that contained also the following concentrations of Na^+ and K^+. (□) 0 mM K^+ and 120 mM Na^+; (▽) 16 K^+ and 104 mM Na^+; (○) 30 mM K^+ and 90 mM Na^+; (△) 40 mM K^+ and 80 mM Na^+; (◇) 120 mM K^+ and 0 mM Na^+. Note that the isotherms are all straight lines, in contrast to those obtained in Fig. 1 with membrane-bound enzyme. Note also that the binding capacity is the same in all these experiments (E_t = 0.90–0.93 μM): this shows that the ionic composition of the solubilization and assay media does not influence the enzyme's binding capacity for ATP and it also gives a measure of the reproducibility of the solubilization and assay procedures. The dissociation constants for ATP for the curves in the figure, taken from right to left, were: 0.17, 0.29, 0.51, 0.75 and 1.23 μM. Note that the affinity of soluble enzyme for ATP remains high even in the presence of 120 mM K^+ (K_{diss} = 1.23 μM).

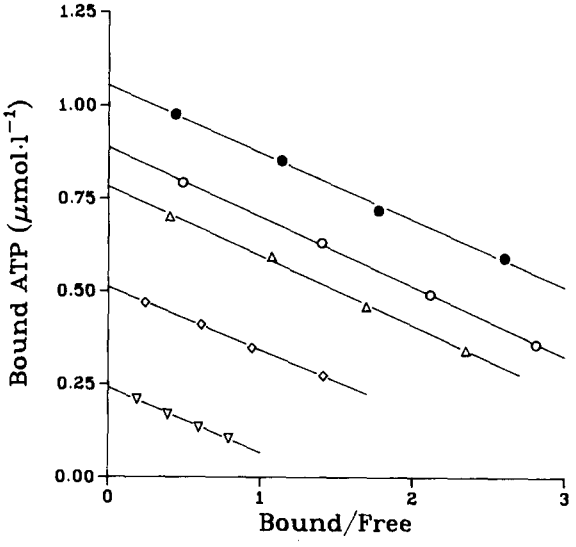

Fig. 4. (●) ATP-binding isotherm obtained with a purified membrane-bound ($Na^+ + K^+$)-ATPase containing 0.33 mg protein per ml (0.265 mg per ml in the binding assay). The remaining isotherms were determined on soluble enzyme obtained by treating the above membrane-bound enzyme with various amounts of $C_{12}E_8$. (▽) 0.2 mg per ml (= 0.6 mg per mg protein); (◇) 0.3 mg per ml (= 0.9 mg per mg protein); (△) 0.4 mg per ml (= 1.2 mg per mg protein); (○) 0.6 mg per ml (= 1.8 mg per mg protein). The highest concentrations of detergent used have dissolved 85% of the total membrane-bound enzyme. The ATP-binding isotherms were obtained in the presence of 120 mM Na^+ and absence of K^+ (in the ATP-binding assay, the enzyme and $C_{12}E_8$ were diluted to 0.8 of the above concentrations as described in Materials and Methods). These conditions were chosen so as to allow comparison with undissolved enzyme which only gives straight-line isotherms in the absence of K^+. The following data apply to the curves shown. (●) E_t = 1.05 μM; K_{diss} = 0.18 μM. (○) E_t = 0.89 μM; K_{diss} = 0.19 μM. (△) E_t = 0.78 μM; K_{diss} = 0.19 μM. (◇) E_t = 0.51 μM; K_{diss} = 0.17 μM. (▽) E_t = 0.24 μM; K_{diss} = 0.17 μM. The dissociation constants of all the enzyme preparations shown are thus the same.

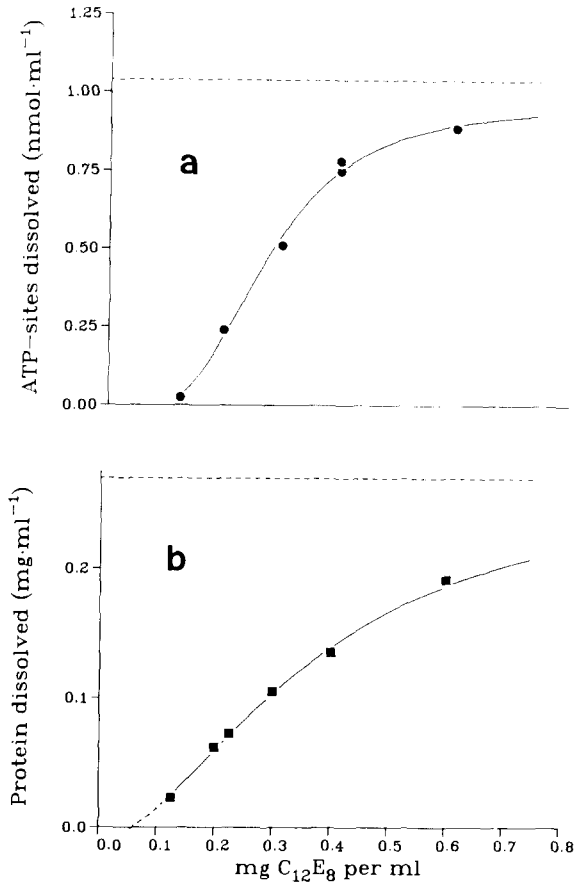

Fig. 5. The solubilization of (a) ATP-binding sites and (b) protein from membranous preparations of $(Na^+ K^+)$-ATPase as a function of the concentration of $C_{12}E_8$. The membrane-bound enzyme used had a specific activity of 30 μmol ATP split/min per mg protein before treatment with $C_{12}E_8$. Protein concentrations are averages of between 4 and 20 determinations made on each sample. The site capacities were obtained from the Scatchard plots of Fig. 4, as well as from two other experiments. The reproducibility of the determinations of both protein concentration and site capacity was good to less than 2%. The extrapolated intercept on the abscissa (0.06 mg $C_{12}E_8$ per ml) can be considered to be related to the critical micelle concentration. Values for the critical micelle concentration of $C_{12}E_8$ of about 0.05 mg per ml have been reported by Helenius et al. [11]. The experimental points of panel a were fitted to the following mathematical expression:

$$A = \frac{\alpha X^n - \beta}{X^n + \gamma}$$

where A = concentration of ATP-binding sites extracted by concentration X of $C_{12}E_8$ and $\alpha, \beta\gamma$ and n are constants. $\alpha = 0.961$, $\beta = 4.456 \cdot 10^{-4}$, $\gamma = 1.192 \cdot 10^{-2}$ and $n = 3.4$. The experimental points of panel b were fitted to a similar expression:

$$P = \frac{aX^m - b}{X^m + c}$$

where P = concentration of protein solubilized by concentration X of $C_{12}E_8$ and a, b, c and m are constants. $a = 0.27$, $b = 1.606 \cdot 10^{-3}$, $c = 0.169$ and $m = 1.8$. The horizontal, stippled lines represent binding capacity and protein concentration in the membrane-bound enzyme preparation before the addition of $C_{12}E_8$.

In the experiments described in Fig. 2 and 3, a constant amount of 1.2 mg $C_{12}E_8$ was used to dissolve enzyme from 0.4 mg membrane-bound protein suspended in 1 ml. The following describes the differential extraction of protein and enzyme from the same highly purified membrane-bound enzyme preparations used above as a function of the concentration of $C_{12}E_8$ (at a constant protein concentration of 0.33 mg per ml).

The enzyme-containing membranes were treated with $C_{12}E_8$ at room temperature as described in Materials and Methods; soluble enzyme was recovered after removal of all undissolved material by centrifugation. Fig. 4 shows isotherms for the binding of ATP to soluble enzyme obtained at various $C_{12}E_8$/protein ratios: the affinity of enzyme for ATP remains, in all cases, the same as that of untreated membrane-bound enzyme.

Fig. 5a and b shows the progressive extraction of ATP-binding sites and of protein as the ratio of $C_{12}E_8$/membrane protein is increased. It can be noticed that the shape of the two curves is not the same. At low and at high $C_{12}E_8$ concentrations, more protein than ATP-binding sites is solubilized while in the middle range of $C_{12}E_8$ concentrations, the opposite is the case. Table I shows the ATP-binding capacity of the dissolved protein as obtained at each of the detergent concentrations used. The highest value actually obtained was 5.8 nmol sites per mg protein measured with the method of Lowry et al. [9] (sample 4, Table I) but it is obvious that this sample also contains some protein with much lower ATP-binding capacity (e.g., corresponding to that of sample 1, Table I) that dissolves in the low range of $C_{12}E_8$ concentrations.

In theory, it should be possible to perform an experiment consisting of two sequential extractions of enzyme-containing membranes with deter-

gent. The first extraction should dissolve material with low ATP-binding capacity and the second should then yield material with higher site capacities. Under the experimental conditions used here, such an experiment would prove to be difficult as will become clear from Fig. 6. An estimate of the minimum amount of protein per mol of ATP sites that is extractable with detergent can still be made from an analysis of dP/dA vs. $C_{12}E_8$ concentration (where P = concentration of protein extracted by $C_{12}E_8$ and A = concentration of ATP sites extracted at the same detergent concentration). dP/dA can be calculated as the ratio of dP/dX to dA/dX (where X represents $C_{12}E_8$ concentration). dP/dX and dA/dX are, in turn, obtainable by differentiating the mathematical models that describe the results plotted in Fig. 5b and a.

Fig. 6 shows a plot of dP/dA as a function of $C_{12}E_8$ concentration: the minimum value of dP/dA gives an estimate of the maximum molecular mass of an ATP-binding unit as 133 kDa. This corresponds to a minimum binding capacity for ATP of 7.5 nmol per mg protein (as determined using the method of Lowry et al. [9]).

The sharpness of the approach to the minimum of the function in Fig. 6 would appear to render sequential extractions with $C_{12}E_8$ experimentally impractical under the conditions used in this study.

It may be added that polyacrylamide gel electrophoresis of the sample from Table I with the highest binding capacity contained both α- and β-peptides in a ratio that was not higher than that of membrane-bound enzyme (as judged from the amount of Coomassie blue bound).

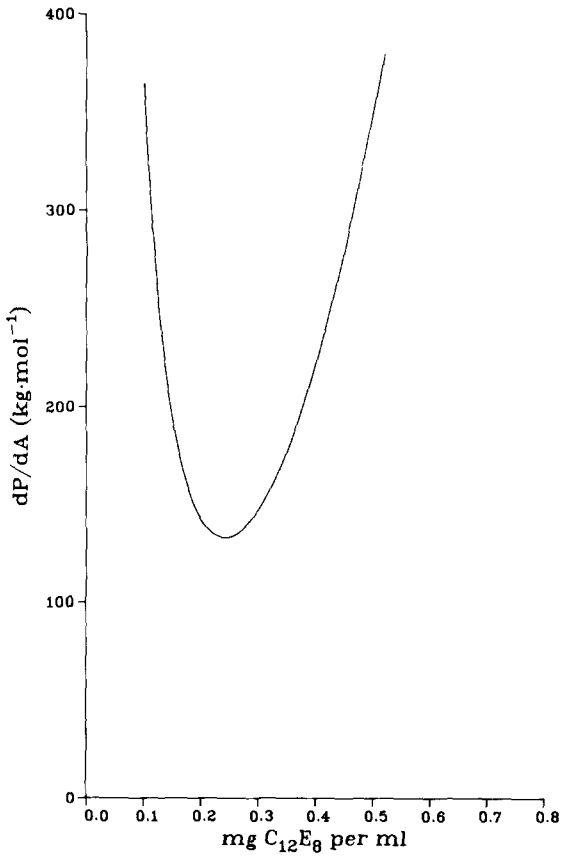

Fig. 6. A plot of dP/dA vs. $C_{12}E_8$ concentration. dP/dA is calculated as the ratio of the derivatives (dP/dX and dA/dX) belonging to the equations describing the solubilization of protein and ATP sites as a function of the detergent concentration (see text to Fig. 5):

$$dP/dA = 1000\frac{m(ac+b)X^{m-1}(X^n+\gamma)^2}{n(\alpha\gamma+\beta)X^{n-1}(X^m+c)^2}$$

where the factor 1000 converts the units employed in Fig. 5 into kg protein per mol sites; all other symbols have been defined in Fig. 5. The computed minimum value of dP/dA is 133 kg protein per mol ATP site. This gives a maximum value for the molecular mass of the protein unit bearing an ATP-binding site as 133 kDa. The ATP-binding capacity of such a unit is 7.5 nmol sites per mg protein (as determined using the method of Lowry et al. [9]).

TABLE I

THE ATP-BINDING CAPACITY OF MEMBRANE-BOUND ENZYME AND OF ENZYME DISSOLVED BY VARIOUS CONCENTRATION RATIOS OF $C_{12}E_8$ TO PROTEIN (EXPTS. 1–6)

Values of ATP-binding site capacities and of protein concentrations were measured as described in Materials and Methods.

Experiment		$C_{12}E_8$-to-protein ratio	ATP-binding capacity (nmol sites per mg protein)
Membrane-bound enzyme		0	3.7; 3.9
Dissolved enzyme	1	0.375	1
	2	0.6	3.9
	3	0.9	4.8
	4	1.2	5.7; 5.8
	5	1.8	4.6
	6	3.0	4.1

Discussion

In the presence of K^+, the nucleotide-binding site population in preparations of membrane-bound $(Na^+ + K^+)$-ATPAse shows an apparent heterogeneity that manifests itself as a curvature of binding isotherms plotted in the form of Scatchard plots. This behavior has been demonstrated to be the consequence of interaction between structural units, each of which must contain an ATP-binding site [1].

As mentioned above, Brotherus et al. [4] showed that dissolving membrane-bound $(Na^+ + K^+)$-ATPase with $C_{12}E_8$ gives rise to units with a molecular weight of 170000 or less and they suggested that these units are protomers of one α- and one β-peptide. Since this low molecular weight form of the enzyme is enzymatically active [4], the $\alpha\beta$-protomer must possess an ATP-binding site.

From a synthesis of the above two statements it was to be expected that dissolving $(Na^+ + K^+)$-ATPase in $C_{12}E_8$ would abolish interaction between ATP-site-containing subunits. The straight-line isotherms of the binding of ATP in the presence of K^+ to enzyme dissolved in $C_{12}E_8$ (Fig. 3) show that all ATP-binding sites in the preparation have identical affinity for ATP and are evidence that subunit-subunit interaction has been abolished by the detergent treatment. These findings demonstrate that membrane-bound enzyme and $C_{12}E_8$-dissolved enzyme differ and, if the dissolved enzyme is protomeric, our suggestion that the membrane-bound ATPase is at least dimeric has now been given added significance.

If an $\alpha\beta$-protomer is to be capable of binding one molecule of ATP or of ouabain or a vanadate ion, the binding capacity of a pure preparation of enzyme will have to be between 6 and 8 nmol per mg protein, depending on the molecular weights chosen for the peptides composing an $\alpha\beta$-protomer [12–15]. The latter figure is probably to be preferred, since it is based upon molecular weights determined by ultracentrifugation under conditions where peptide polymerization is prevented [15].

Until recently, the binding capacity for the above-mentioned ligands as well as the phosphorylation capacity of most of the best preparations (in most cases purified according to the standard procedures described by Jørgensen [5]) attained values of about 4 nmol per mg protein (measured by the method of Lowry et al. [9], with bovine serum albumin as standard [13,16–20], and in one case [21], the same purification procedure yielded membrane-bound enzyme with 5.1 nmol phosphorylation sites per mg protein. Finally, there is a report [22] that enzyme composed exclusively of α-peptide and with 7.8 nmol phosphorylation sites per mg protein could be obtained by the use of a different method of preparation and of protein assay. Then, within the last couple of years, it has been recognized that the use of bovine serum albumin as a standard for the protein determination according to the method of Lowry et al. [9] may result in an overestimation of enzyme protein, the discrepancy being anything between 10% [14,20] and 90% [23], depending on the author. Thus, estimates of nucleotide-binding capacity could be increased by a factor of as much as nearly two, without any change in the degree of purification.

In the present paper we report that the differential extraction with $C_{12}E_8$ of a standard preparation of highly-purified membrane-bound ATPase has yielded enzyme with an ATP-binding capacity of 5.8 nmol per mg protein (as determined using the method of Lowry et al. [9]) and still containing apparently normal proportions of α- and β-peptide components. If enzyme can be prepared with a binding capacity of 7.5 nmol per mg protein according to the above-mentioned assay, as should be possible from the results presented in Fig. 6, one must perforce conclude that the preparations of $(Na^+ + K^+)$-ATPase available so far cannot be both completely pure (with respect to protein composition) and completely alive (from the point of view of surviving nucleotide-binding sites).

The calculated site capacity of 7.5 nmol per mg protein (Fig. 6) corresponds to one nucleotide site per 133000 kDa. If the method of protein determination [9] as used here overestimates protein by no more than 10%, the calculated molecular weight of the unit bearing a high-affinity nucleotide site would lie between 121000 and 133000. Values within this range would be consistent with an $\alpha\beta$-protomer molecular weight of 126200 ± 7600 as determined by Freytag and Reynolds [15]. On the other hand, if the method of protein determination of Lowry et al. [9] over-

estimates enzyme protein by more than 10%, as found by some authors [13,23], each $\alpha\beta$-protomer will have to be assigned at least two identical, high-affinity, ATP-binding sites.

Acknowledgments

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