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# BINDING OF ATP TO BRAIN MICROSOMAL ATPase

# DETERMINATION OF THE ATP-BINDING CAPACITY AND THE DISSOCIATION CONSTANT OF THE ENZYME–ATP COMPLEX AS A FUNCTION OF $K^+$ CONCENTRATION

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SUMMARY

1. The binding of the ATP molecule to brain microsomal ($Na^+ + K^+$)-activated ATPase was studied by a rapid dialysis rate technique.

2. The experiments were performed at 2° in the presence of 10 mM EDTA to minimize hydrolysis of ATP. The ionic strength was 0.073 M, pH 7.4.

3. The specific activity of the preparations and the $Na^+ + K^+$ activity/$Mg^{2+}$ activity ratio were changed by heat denaturation.

4. The dissociation constant of the enzyme–ATP complex (*E*–ATP) was 0.12 $\mu$M.

5. The $Mg^{2+}$ requirement for binding of ATP to the enzyme, if any, was much lower than that for hydrolysis of ATP. The binding seemed to be independent of $Na^+$.

6. Increasing the concentration of $K^+$ up to 3 mM led to an increase in the apparent dissociation constant of *E*–ATP towards a maximum (0.7 $\mu$M). The effect of $K^+$ could be described by a model involving the formation of the following complexes: *E*–ATP, $K^+$–*E* and $K^+$–*E*–ATP.

7. Proportionality was found between the ATP-binding capacity (nmoles/mg protein) and ($Na^+ + K^+$)-ATPase activity ($\mu$moles $P_i$ released per mg protein per h), suggesting that binding to ($Na^+ + K^+$)-ATPase was measured. The catalytic center activity based on this assumption was $7000 \pm 470$ $min^{-1}$.

## INTRODUCTION

During the last years much evidence has been obtained relating ($Mg^{2+} + Na^+ + K^+$)-activated ATP hydrolysis by microsomal preparations to active transport of cations across cell membranes. Details of the intermediate steps involved in this ATP hydrolysis are needed to understand the mechanism of active cation transport and have therefore been the subject of many investigations (for references see SKOU[1], ALBERS[2], HEINZ[3] and WHITTAM AND WHEELER[4]). The results so far seem to indicate the existence of a ($Mg^{2+} + Na^+$)-dependent phosphorylation[5–20] of a compound in the microsomal particles, and it is generally assumed that this is preceded by a $Mg^{2+}$-dependent formation of an enzyme–ATP complex.

The present study is concerned with the binding of the ATP molecule to the enzyme system. The application of the rapid dialysis rate technique developed by COLOWICK AND WOMACK[21] and the observation that the $Mg^{2+}$ requirement, if present, for the binding of ATP is much lower than for hydrolysis, made it possible to characterize the binding process quantitatively in terms of the number of binding sites and dissociation constant. Information about the enzyme–substrate interaction has hitherto been obtained only in an indirect way from kinetic studies[6,19,22–29].

## MATERIALS AND METHODS

### *Reagents*

The reagents were the purest obtainable and were purchased from Merck (imidazole, HCl, KCl, $MgCl_2$, EDTA and $Na_2HPO_4$), Sigma (Trizma-base), Analar (sucrose) and Boehringer (disodium salt of ATP).

Labelled ATP ([$\gamma$-$^{32}$P]ATP, henceforth [$^{32}$P]ATP) and $^{32}P_i$ were obtained regularly from The Radiochemical Centre, Amersham, England. ATP, [$^{32}$P]ATP and $^{32}P_i$ were purified and converted to their Tris salts as described below. The specific activity of the [$^{32}$P]ATP used in the binding studies was always higher than 5 C/mmole, in most cases higher than 10 C/mmole.

### *Preparation of ATPase*

Preparations of ATPase from ox brain were obtained essentially as described by SKOU AND HILBERG[17]. The sediment after the last centrifugation at 100000 × *g* was resuspended by gentle homogenization in cold 12.5 mM imidazole–HCl buffer (pH 7.1 at 37°) containing 250 mM sucrose, to give a protein concentration of 2.5–4.4 mg/ml. The specific activity of these stock enzyme solutions ($\mu$moles $P_i$ per mg protein per h) determined as described below were: $Mg^{2+}$ + $Na^+$ + $K^+$ activity, 170–220; $Mg^{2+}$ activity, 28–36. A few enzyme preparations were washed by successive centrifugations at 100000 × *g* and resuspension in the above-mentioned imidazole buffer. This reduced the content of inorganic contaminants (see below) but did not affect the specific activity.

### *$K^+$ and $Na^+$ in the enzyme preparations*

Preliminary experiments revealed that most, if not all, of the $K^+$ and $Na^+$ in the stock enzyme solutions was present in the supernatant after centrifugation of the enzyme solutions at 100000 × *g* for 30 min. The concentrations in the supernatant determined by flame photometry (Eppendorph) were: $K^+$, 50–400 $\mu$M, $Na^+$, 130–650 $\mu$M.

### *Protein*

The protein concentration of the enzyme solutions and "upper chamber solutions" (see below) was determined as follows: The solutions were diluted by a factor of 10 with water and placed at 0–4°. Trichloroacetic acid was added to give a concentration of 5 % trichloroacetic acid, and protein was determined on the precipitate by the method of LOWRY *et al.*[30].

### *ATPase activity*

ATPase activity was determined by the coupled ATPase assay described by NØRBY[31]. The composition of the reaction mixture was:

(1) $Mg^{2+}$ activity, 3 mM ATP, 3 mM $Mg^{2+}$, 50 $\mu$l ATPase per 3 ml, 1 mM phosphoenolpyruvate, 30 $\mu$g pyruvate kinase per ml, 30 $\mu$g lactate dehydrogenase per ml and 120 $\mu$M NADH($+H^+$). The ionic strength was 0.14 mM (adjusted with Tris buffer), temperature 37°, pH 7.35–7.40. The $Mg^{2+}$ activity thus obtained was corrected to an ionic strength of 0.29 M. The correction factor (0.8) was determined from repeated measurements of $Mg^{2+}$ activity at ionic strengths, 0.14 and 0.29 M.

(2) $Mg^{2+} + Na^+ + K^+$ activity, conditions as for $Mg^{2+}$ activity except for the ATPase concentration (4.5 $\mu$l ATPase per 3 ml) and the addition of 130 mM NaCl + 20 mM KCl, ionic strength 0.29 M.

*Purification of ATP, [$^{32}P$]ATP and $^{32}P_i$ by chromatography*

The substance to be purified was dissolved in 5–10 ml 100 mM Tris buffer (pH 8.3) and applied to a column of DEAE-Sephadex A25 (Pharmacia). The length and cross-sectional area of the column was 1.5 cm and 0.7 $cm^2$, respectively. The flow was 20 ml/h. After washing with 3 ml 100 mM Tris (pH 8.3), the compounds could be stepwise eluted by 30 ml 100 mM Tris (pH 6.3) (removing $P_i$ and, in an overlapping peak, AMP), 30 ml 150 mM Tris (pH 6.3), ADP, and 300 mM Tris (pH 6.3), which gave a sharp ATP peak. The temperature was 4–10°, and the elution was followed by measurement of $^{32}P$ and $A_{259\,nm}$ on the fractions.

*Determination of $^{32}P_i$ in commercial and purified [$^{32}P$]ATP*

This was done by a modification of the two-phase separation method of BERENBLUM AND CHAIN[32] and LINDBERG AND ERNSTER[33]. Silicotungstic acid reagent "SIW" (Fisher Scientific Co., Merck) and isobutanol–benzene (1:1, by vol.) were mixed at low temperature and allowed to separate. 4 ml "SIW" (thus saturated with isobutanol–benzene) and 300 $\mu$l 1 mM $Na_2HPO_4$ were pipetted into a tube on an icebath. The sample (50–100 $\mu$l) was added, and the mixture allowed to stand first for 10 min on ice, then for 10 min at room temperature, whereafter 0.5 ml 10 % ammonium molybdate and 5 ml isobutanol–benzene were added. The tube was shaken for 15 sec, the phases allowed to separate, and appropriate amounts of the upper phase (usually 1–2 ml) and of the lower phase (50–100 $\mu$l) were then transferred to vials for counting as described below.

Following this rigidly standardized procedure, we repeatedly found a $^{32}P_i$ content of 0.5 % in even the most purified [$^{32}P$]ATP preparation, based on radioactivity measurements. This was therefore considered to be a blank value due to hydrolysis of ATP in the strong acid milieu during the extraction procedure.

The counts percentage of $^{32}P_i$ in the commercial [$^{32}P$]ATP samples, up to 25 %, was found to increase with the duration of storage of the freeze-dried samples. Following chromatographic purification, the [$^{32}P$]ATP solution in 300 mM Tris (pH 6.3) was perfectly stable at −18°. During binding studies the solutions were kept on ice for 3–4 h, and no increase in $^{32}P_i$ was seen in this period provided care was taken not to contaminate the solution with ATPase.

*Binding studies*

(*a*) *Apparatus.* The binding of ATP to brain microsomes was measured by the dialysis rate technique described by COLOWICK AND WOMACK[21]. The inner diameter of the cylindrical dialysis cell was 19 mm, the height of the upper and lower chamber

being 10 and 6 mm, respectively. The volume of the magnetic stirrers was 0.5 $cm^3$. The membranes were cut from Visking seamless cellulose tubing (Union Carbide Co.). The thickness of the membranes was 0.001 inch, the average pore radius, 24 Å. The membrane material was equilibrated with buffer before use. The inner diameter of the outlet tubing from the lower chamber was 1 mm, the length about 80 mm. The perfusion buffer reservoir, the inlet tube and the dialysis cell were all thermostated at 2°. A constant perfusion flow through the lower chamber (4 ml/min) was obtained by means of a Mariotte bottle. Under these conditions the net fluid flow from lower to upper chamber is negligible.

(*b*) *Typical [$^{32}P$]ATP-binding experiment.* 1.4 ml enzyme solution (in standard experiments, 1.4 ml 12.5 mM imidazole buffer, pH 7.1, at 37°), 100 µl 1.2 M imidazole buffer containing 150 mM EDTA (pH 7.1) at 37°, 20 µl 300 mM Tris (pH 6.3) at 37°, containing from zero to 0.2 mM unlabelled ATP, and 40 µl [$^{32}P$]ATP in 300 mM Tris (pH 6.3) at 37°, were pipetted into the upper chamber giving 1.56 ml solution with an ionic strength of 0.073 M. The composition of the perfusion buffer was similar to that of the solution in the upper chamber except that it was free of enzyme protein and ATP. The experiment was started by introduction of [$^{32}P$]ATP into the upper chamber, and the effluent from the lower chamber was collected continuously in 2-ml fractions directly into vials containing 10 ml scintillation fluid for measurement of radioactivity (see below). The duration of the experiment was 10–12 min. The mixture of perfusion buffer and scintillation fluid thus obtained was turbid at room temperature but clear and homogenous at 0–4°, at which temperature the counting took place.

*Counting of $^{32}P$ radioactivity*

All the countings were performed on glass vials containing 10 ml of the following scintillator solution[34]: 2 l toluene, 1.25 l Triton X-100, 0.5 l ethanol, 5 g 2,5-diphenyloxazole and 0.25 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (Packard). The counting took place in a Packard liquid scintillation spectrometer Model 3003 or 3320, window 50–1000, gain 1.8 %, giving an efficiency of 100 %. Preliminary experiments revealed that [$^{32}P$]ATP is adsorbed to the walls of the glass vials when dissolved in the above scintillation fluid, whereby the efficiency of the counting is considerably reduced. This was prevented by addition of 15 µl 5 M $H_2SO_4$ to the vials containing [$^{32}P$]ATP ($^{32}P_i$ is not adsorbed).

## RESULTS

*General problems concerning the procedure used in the ATP-binding experiments*

One of the first problems encountered was hydrolysis of ATP when incubated with the highly concentrated enzyme solution at 37°. Preliminary experiments showed that the rate of hydrolysis could be sufficiently lowered (see below) by decreasing the temperature to 2° and by adding EDTA to a concentration of 10 mM. The effect of EDTA on the hydrolysis is probably due to chelation of the small amounts of $Mg^{2+}$ contaminating the enzyme preparations.

The results of a typical ATP-binding experiment, illustrated by Fig. 1, reveal several characteristic features of the system. The transient state of the system during the first 4 min is omitted from the figure. The standard curve (Curve 1, no enzyme in the upper chamber) indicates that the rate of diffusion of ATP through the membrane

is proportional to the concentration of free ATP in the upper chamber, since a 500-fold increase in this concentration does not change the diffusion rate of [$^{32}$P]ATP. The fact that Curve 8, in which the concentration of ATP is very large relative to the concentration of enzyme, and Curve 1 reach the same level (within the experimental error) furthermore shows that the presence of enzyme does not influence the permeability of the membrane for ATP. From the intercept of the standard curve with the ordinate axis and the amount of [$^{32}$P]ATP in the upper chamber, the dialysis rate constant can be calculated. It is so low that only 0.92 $^{0}/_{00}$ of the free ATP passes from the upper to the lower chamber per min.

The position of the Curves 2–7 indicates that ATP is bound to the enzyme. The calculation of the amount of free and bound ATP is, however, complicated by the steady, although slow, increase in counts/min per sample in the effluent. This increase is consistent with a slow hydrolysis of [$^{32}$P]ATP to ADP and $^{32}P_i$, since it was shown that the diffusion rate constant of $P_i$ is 3–4 times higher than that of ATP and since it could be demonstrated that $^{32}P_i$ is not bound to the enzyme.

Experiments (not shown) in which the ATP concentration was increased from a low to a very high value, relative to the concentration of enzyme, confirmed this assumption and directly demonstrated that the binding of [$^{32}$P]ATP was reversible. In such experiments the addition of ATP increased the counts/min per sample of

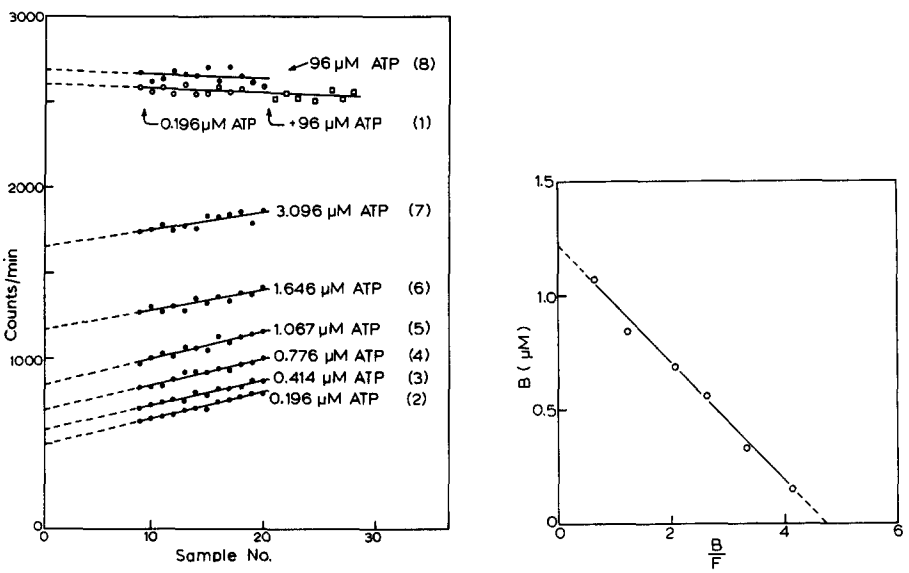


Fig. 1. Typical ATP-binding experiment showing counts/min per sample of effluent as a function of time (sample No.) in "steady state". The experimental conditions were as described in MATERIALS AND METHODS. In experiments symbolized by ● (Curves 2–8) the protein concentration in the upper chamber was 3.09 mg/ml. The enzyme preparation used contained 179 µM $K^+$ and 448 µM $Na^+$ and had a specific activity of 30.2 ($Mg^{2+}$) and 201 µmoles $P_i$/mg protein per h ($Mg^{2+}$ + $Na^+$ + $K^+$). The standard experiment without enzyme is shown by ○ and □ (Curve 1). Total concentration of ATP in the upper chamber is given for each curve. In all experiments the upper chamber contained $3.64 \cdot 10^6$ counts/min per ml of [$^{32}$P]ATP. The linear regression lines shown were calculated by the method of least squares. The intercepts of these lines with the ordinate axis are used in the calculation of free and bound ATP (*cf.* text and Fig. 2).

Fig. 2. Scatchard-type plot of data derived from Fig. 1 as described in the text. The equation for the regression line shown in the figure is: $B = (1.23 \pm 0.02) - (0.258 \pm 0.008) \cdot (B/F)$.

effluent to a value higher than that in a corresponding experiment without enzyme. The difference could, however, be accounted for by the degree of hydrolysis measured directly on the upper chamber solution or calculated from the slope of the curve (counts/min per sample *versus* time) before the addition of ATP.

The above-mentioned circumstances required: (1) separate binding experiments for each concentration of ATP, in contrast to the stepwise addition method described by COLOWICK AND WOMACK[21] and (2) an extrapolation of the results to zero time (Fig. 1) in order to estimate the value for counts/min per sample of effluent which would have been obtained in the absence of hydrolysis. These values are then, together with the concentration of ATP, used in the calculation of free and bound ATP (see ref. 21). For example, it appears from Fig. 1 that the counts/min per sample are 2602 in the standard experiment and 701 in Expt. 4. The fraction of free and bound ATP in Expt. 4 is therefore 0.269 and 0.731, respectively. This corresponds to a concentration of free ATP (F) of 0.209 $\mu$M and a concentration of bound ATP (B) of 0.567 $\mu$M.

If the [$^{32}$P]ATP used contains even small amounts of $^{32}P_i$, the extrapolated values for counts/min per sample are too high since $^{32}P_i$ is not bound. Correction for this is easily performed when the ratio between the dialysis rate constants of $^{32}P_i$ and [$^{32}$P]ATP is known.

*Calculation of the dissociation constant for the enzyme–ATP complex and the concentration of binding sites*

The results of the ATP-binding studies were all found to fit well with the following simple model:

$$E + \text{ATP} \rightleftharpoons E\text{–ATP}$$

At equilibrium,

$$\frac{[E]' \cdot [\text{ATP}]}{[E\text{–ATP}]} = K'_{\text{diss}} \tag{1}$$

or

$$\frac{([E_t] - [E\text{–ATP}]') \cdot [\text{ATP}]}{[E\text{–ATP}]'} = K'_{\text{diss}} \tag{2}$$

in which $[E_t]$, $[E\text{-ATP}]'$ and [ATP] denote the concentration of total binding sites, bound ATP (B) and free ATP (F), respectively. $K'_{\text{diss}}$ is called the apparent dissociation constant for reasons discussed below.

Eqn. 2 is transformed to:

$$\text{B} = [E_t] - K'_{\text{diss}} \cdot \frac{\text{B}}{\text{F}} \tag{3}$$

and the results are plotted in a Scatchard-type plot (Fig. 2). $K'_{\text{diss}}$ is obtained from the slope and $[E_t]$ from the intercept with the ordinate axis.

*Relationship between ATPase activity and number of ATP-binding sites*

The ATPase activity, expressed as $\mu$moles $P_i$/ml enzyme preparation per h, in the six native enzyme preparations investigated varied within the following ranges: $Mg^{2+}$ activity, 73–121; $Mg^{2+} + Na^+ + K^+$ activity, 415–800; $Na^+ + K^+$ activity

($Mg^{2+} + Na^+ + K^+ - Mg^{2+}$), 352–679. These variations were mainly due to variations in the protein concentration, the specific activity being relatively constant (see MATERIALS AND METHODS). The ratio $Na^+ + K^+$ activity/$Mg^{2+}$ activity was likewise rather constant, 4.5–5.6. The results of these experiments are represented by open circles in Figs. 3–5.

The relationship between ATPase activity and number of binding sites was also studied in experiments where the specific activity of the enzyme preparation was varied by heat denaturation, keeping the protein concentration constant. Heat denaturation at 45° for 70–180 min (symbolized by squares in Figs. 3–5) generally reduced the specific activity, but the ratio $Na^+ + K^+$ activity/$Mg^{2+}$ activity was slightly increased to 6.14–6.70. On the contrary, heat denaturation at 50° for 12–190 min (triangles in Figs. 3–5) reduced the specific activity as well as $Na^+ + K^+$ activity/$Mg^{2+}$ activity ratio, the latter to 4.75–3.18.

One experiment (not given in Figs. 3–5) showed that [$^{32}$P]ATP did not bind to trichloroacetic acid-precipitated enzyme protein.

It appears from Figs. 3–5 that the ATP-binding capacity (nmoles/mg protein) correlates poorly with the specific $Mg^{2+}$ activity but well with the $Mg^{2+} + Na^+ + K^+$

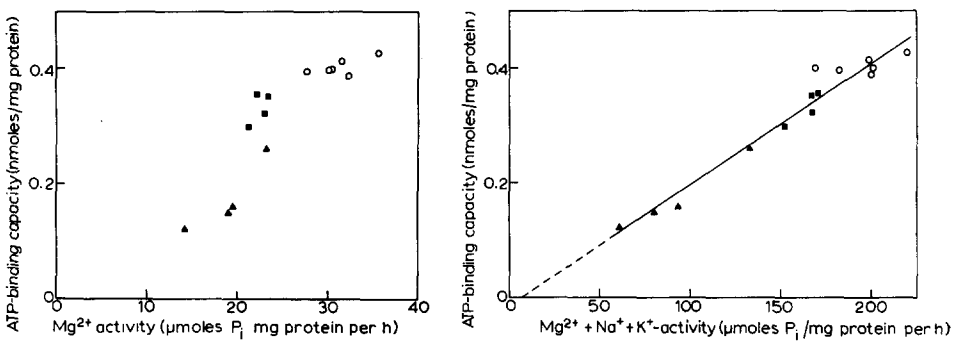


Fig. 3. Relationship between ATP-binding capacity and $Mg^{2+}$ activity for various enzyme preparations. ○, native; ■, heated to 45° for 70–180 min; ▲, heated to 50° for 12–190 min.

Fig. 4. Relationship between ATP-binding capacity and $Mg^{2+} + Na^+ + K^+$ activity. For explanation of symbols see Fig. 3. The regression line shown was calculated by the method of least squares: $Y = (2.12 \pm 0.13) \cdot 10^{-3} X - (0.016 \pm 0.022)$.

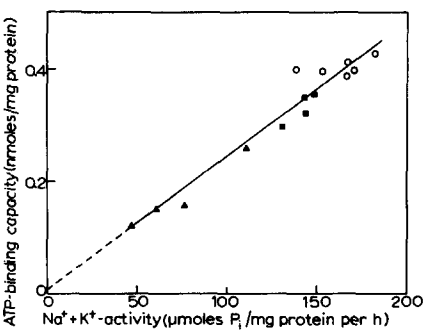


Fig. 5. Relationship between ATP-binding capacity and $Na^+ + K^+$ activity ($Mg^{2+} + Na^+ + K^+$ activity – $Mg^{2+}$ activity). For explanation of symbols see Fig. 3. The regression line shown was calculated by the method of least squares: $Y = (2.38 \pm 0.16) \cdot 10^{-3} X + (0.004 \pm 0.022)$.

and the $Na^+ + K^+$ activity. The regression lines shown in Figs. 4 and 5 have intercepts with the ordinate axis which are not significantly different from zero, indicating proportionality between binding capacity and $Mg^{2+} + Na^+ + K^+$ as well as $Na^+ + K^+$ activity for the enzyme preparations used in this study.

If it is assumed that the ATP-binding measured occurs at the catalytic center, the ATPase catalytic center activity (ATP molecules transformed per ATP-binding site per min) is equal to the reciprocal value of the slope of the regression line times 1000/60: 7850 ± 480 ($Mg^{2+} + Na^+ + K^+$ activity, Fig. 4) or 7000 ± 470 ($Na^+ + K^+$ activity, Fig. 5). These values represent the molecular activities if there is one catalytic center per ATPase molecule.

*The effect of the $K^+$ concentration on $K'_{diss}$*

Determination of $K'_{diss}$ on the six native enzyme preparations mentioned above revealed a variation of this value between 0.175 and 0.35 $\mu$M, the lowest values being obtained for two washed preparations. Heat denaturation as described did not change $K'_{diss}$ significantly. The variations in $K'_{diss}$ could, however, be ascribed to variations in the concentration of $K^+$.

TABLE I

THE EFFECT OF $K^+$ ON THE APPARENT DISSOCIATION CONSTANT OF ENZYME–ATP COMPLEX, $K'_{diss}$

The same enzyme preparation was used in all the experiments. KCl was added to the upper chamber solution as well as to the perfusion buffer in order to keep the concentration constant during each experiment. The concentration of $Na^+$ was 104 $\mu$M in all experiments.

| $K^+$ ($\mu M$) | $K'_{diss}$ ($\mu M$) |
|---|---|
| 34 | 0.177 |
| 564 | 0.384 |
| 1090 | 0.487 |
| 2150 | 0.607 |
| 3220 | 0.604 |

The effect of $K^+$ is illustrated by the data given in Table I, which shows that addition of $K^+$ at constant concentration of $Na^+$ increases $K'_{diss}$. It should be pointed out that a rectilinear relationship between B and B/F, like that shown in Fig. 2, was obtained with 34 $\mu$M as well as with 3220 $\mu$M $K^+$. The slope of the line was changed (Table I), but the intercept with the ordinate axis, $[E_t]$, was not altered significantly by the addition of $K^+$.

In Fig. 6 the data (1) from experiments with various enzyme preparations (representing varying $K^+$ and $Na^+$ concentrations, symbolized by crosses) and (2) from experiments in which the $K^+$ concentration was varied at constant $Na^+$ concentration (symbolized by squares and circles) are assembled. The fact that the former appear to illustrate the same quantitative relationship as the latter, in spite of a variation in $Na^+$ concentration from 104 to 588 $\mu$M, seems to indicate that $Na^+$ has little if any effect on $K'_{diss}$ relative to $K^+$ under these conditions. This was supported by one experiment (not shown) in which the $Na^+$ concentration was increased from 0.1 to 3.2 and further to 33 mM without any significant effect on the ATP-binding level.

For these reasons the relationship illustrated by Fig. 6 is considered to be due to a specific effect of $K^+$. It is seen that $K'_{diss}$ approaches a maximum value as the concentration of $K^+$ is increased, suggesting the formation of a $K^+$–enzyme–ATP

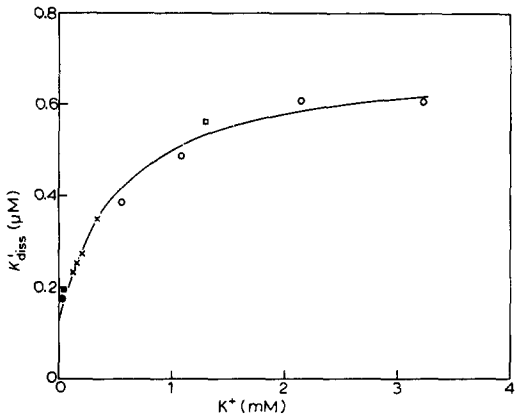


Fig. 6. Relationship between the apparent dissociation constant of enzyme–ATP complex and $K^+$ concentration. Squares and circles represent values from experiments with two enzymes preparations, without (closed symbols) and with (open symbols) addition of $K^+$. The results shown by × are from four different preparations. The curve represents the best fit of Eqn. 10 to the observations (see text).

complex ($K^+$–*E*–ATP) with a higher dissociation constant than the *E*–ATP complex. The model for ATP-binding described above was therefore extended to:

$$E + \text{ATP} \rightleftharpoons E\text{–ATP}$$

$$E + K^+ \rightleftharpoons K^+\text{–}E$$

$$K^+\text{–}E + \text{ATP} \rightleftharpoons K^+\text{–}E\text{–ATP}$$

$$E\text{–ATP} + K^+ \rightleftharpoons K^+\text{–}E\text{–ATP}$$

giving the following equations for equilibrium:

$$\frac{[E]\cdot[\text{ATP}]}{[E\text{–ATP}]} = K^{E\text{–ATP}} \tag{4}$$

$$\frac{[E]\cdot[K^+]}{[K^+\text{–}E]} = K^{K^+\text{–}E} \tag{5}$$

$$\frac{[K^+\text{–}E]\cdot[\text{ATP}]}{[K^+\text{–}E\text{–ATP}]} = K^{K^+\text{–}E\text{–ATP}} \tag{6}$$

$$\frac{[E\text{–ATP}]\cdot[K^+]}{[K^+\text{–}E\text{–ATP}]} = K^{E\text{–ATP}\text{–}K^+} = \frac{K^{K^+\text{–}E\text{–ATP}} K^{K^+\text{–}E}}{K^{E\text{–ATP}}} \tag{7}$$

$$[E_t] = [E] + [K^+\text{–}E] + [E\text{–ATP}] + [K^+\text{–}E\text{–ATP}] \tag{8}$$

The concentration of bound ATP (B) in this model is represented by the sum [*E*–ATP] + [$K^+$–*E*–ATP]. Combination of Eqns. 4–8 leads to:

$$B = [E_t] - \frac{(K^{K^+\text{–}E} + [K^+])K^{E\text{–ATP}} K^{K^+\text{–}E\text{–ATP}}}{K^{E\text{–ATP}}\cdot[K^+] + K^{K^+\text{–}E} K^{K^+\text{–}E\text{–ATP}}} \cdot \frac{B}{F} \tag{9}$$

which shows that

$$K'_{diss} = \frac{(K^{K^+-E} + [K^+])K^{E-ATP}K^{K^+-E-ATP}}{K^{E-ATP}\cdot[K^+] + K^{K^+-E}K^{K^+-E-ATP}} \quad (10)$$

It appears from Eqns. 9 and 10 (1) that a rectilinear relationship between B and B/F should be obtained at constant $K^+$ concentration, (2) that $K'_{diss}$ should increase with increasing $K^+$ concentration and (3) that $[E_t]$ should be independent of the $K^+$ concentration. This is in agreement with the observations. The possible influence of a $K^+$–ATP-complex formation on the model will be discussed later.

*Estimation of* $K^{E-ATP}$, $K^{K^+-E-ATP}$, $K^{K^+-E}$ *and* $K^{E-ATP-K^+}$

The dissociation constants which gave the best fit of Eqn. 10 to the observations were estimated by means of a digital computer (method of least squares on a transformed problem).

The values for the different constants (*cf.* Eqns. 4–7) were: $K^{E-ATP} = 0.12\ \mu M$, $K^{K^+-E} = 87\ \mu M$, $K^{K^+-E-ATP} = 0.69\ \mu M$ and $K^{E-ATP-K^+} = 500\ \mu M$. The curve shown in Fig. 6 is calculated from these constants and Eqn. 10.

## DISCUSSION

To understand the mechanism of the hydrolysis of ATP in the presence of ($Mg^{2+} + Na^+ + K^+$)-activated ATPase, it is important to obtain information about the binding of ATP to the enzyme system. This requires that the binding process may be studied under conditions where the rate of hydrolysis of ATP is nil or very small. The procedure used in the present investigation takes advantage of the preliminary observations that ATP at 0° and at very low concentrations of $Mg^{2+}$ (high EDTA concentration) will bind to brain microsomes. Moreover, the binding constant appeared to be of a magnitude so as to permit the use of the rapid rate-dialysis technique described by COLOWICK AND WOMACK[21]. The small amount of ATP hydrolysis even under these conditions could easily be corrected for.

The binding of ATP has been studied here with [$^{32}$P]ATP, labelled in the $\gamma$-position, as tracer. Since incubation of ($Mg^{2+} + Na^+ + K^+$)-ATPase with this tracer under various conditions leads to the formation of phosphorylated protein ($E \sim {}^{32}P$)[5–20], the binding of $^{32}P$ observed by us need not *a priori* represent a binding of the ATP molecule as such. However, experiments like that described in the first section of RESULTS, where the concentration of unlabelled ATP was increased to a very large value relative to the enzyme concentration at the end of a binding experiment, indicated that the compound bound to the enzyme was ATP. The addition of unlabelled ATP increased the isotope concentration in the effluent from the lower chamber to a level which, when corrected for the small amount of hydrolysis during the experiment, was equal to that expected from the amount of [$^{32}$P]ATP added. This would not have been the case had the isotope liberated been $^{32}P_i$ since the permeability of the membrane for $P_i$ was 3 times that for ATP.

Two of the observations made during this study seem to indicate that the binding of ATP is confined to the ($Mg^{2+} + Na^+ + K^+$)-activated ATPase. Firstly, the binding capacity is directly proportional to the $Na^+ + K^+$ activity (Fig. 5) and

the $Mg^{2+} + Na^+ + K^+$ activity (Fig. 4), whereas it shows a poor correlation with the $Mg^{2+}$ activity. Secondly, the attainment of linear Scatchard-type plots (Fig. 2) allows the binding, within the ATP concentration used, to be described by a simple model involving only one dissociation constant. If the other enzymes and proteins most certainly present in the brain microsome fraction were to contribute significantly to the ATP binding, it would require them to have approximately the same heat-denaturation characteristics and approximately the same protein–ATP dissociation constant. According to the arguments given above we feel justified to assume that we are dealing with the binding of ATP to the $(Mg^{2+} + Na^+ + K^+)$-activated ATPase. Furthermore, the molecular activities ($7000 \pm 470$ $(Na^+ + K^+)$ or $7850 \pm 480$ $(Mg^{2+} + Na^+ + K^+)$ ATP molecules transformed per ATP-binding site per min) are close to those found in phosphorylation experiments on enzyme preparations from various tissues[15,19,35].

From the concentration of $Mg^{2+}$ in the enzyme solutions (about 50 $\mu$M) (pH 7.1, 37°) and the association constant for $Mg^{2+}$–EDTA[36], it can be calculated that the concentration of free $Mg^{2+}$ was less than 5 nM. Increasing the concentration of total $Mg^{2+}$ by 100 $\mu$M (leading to a 3-fold higher $Mg^{2+}$ concentration) increased the hydrolytic activity by a factor of 3–4 but did not affect the binding of ATP (unpublished observation). It may therefore be concluded: (1) that the concentration of $Mg^{2+}$–ATP is practically zero so that binding of free ATP is measured, (2) that if $Mg^{2+}$ is required for the binding the enzyme–$Mg^{2+}$ dissociation constant is extremely low, and (3) that the $Mg^{2+}$ requirement for binding is much lower than that for hydrolysis.

Comparison between the dissociation constants for the enzyme–ATP complexes obtained in the present study and the constants published by other investigators characterizing the relationship between ATP concentration and rate of hydrolysis (apparent $K_m$, $K_{0.5}$, *etc.*) is complicated for a number of reasons. Such constants are dependent on but generally not equal to the enzyme-substrate dissociation constant[37]. Furthermore, the simultaneous presence of $MgATP^{2-}$, $Mg^{2+}$ and $ATP^{4-}$ in hydrolysis experiments makes the interpretation of the constants even more difficult, since the interaction of the enzyme with all the three compounds must be taken into consideration[38]. Most of the kinetic studies published have been performed with ATP concentrations higher than 0.1 mM[23–27,29]. The authors of these studies find $K_m$ values for $MgATP^{2-}$, which is considered to be the "true" substrate, around $2 \cdot 10^{-4}$ M. It appears, however, from the experiments of SAITO[19] and of NEUFELD AND LEVY[28] that the enzyme system behaves quite differently at low ($1 \cdot 10^{-6}$ M) and at high ($1 \cdot 10^{-4}$ M) ATP concentrations. At low ATP concentrations like those used in the present study, NEUFELD AND LEVY[28] found $K_m$ for the hydrolysis of ATP to be about 0.2 $\mu$M (total $Mg^{2+}$, 5 mM), and SAITO[19] obtained a $K_m$ value of 3.3 $\mu$M (total $Mg^{2+}$, 1 mM). SAITO[19] and POST *et al.*[6] in studying the formation of phosphorylated intermediates during the hydrolysis of ATP determined the ATP concentration for half-maximum effect to be 3.6 and 0.5 $\mu$M, respectively. From his experiments SAITO[19] estimated the enzyme–ATP dissociation constant to be 0.93 $\mu$M in the presence of 1 mM $Mg^{2+}$ (in terms of free ATP this corresponds to about 0.1 $\mu$M[39]). With the above reservations in mind it seems reasonable to conclude that the enzyme–ATP dissociation constants measured in the present investigations, 0.1–0.7 $\mu$M (depending on the $K^+$ concentration) characterize an early step in the ATP hydrolysis, the binding of free ATP. It

cannot be excluded that binding of $MgATP^{2-}$ to the enzyme also can take place. It should be mentioned in this connection that HEINZ AND HOFFMAN[40] and SHAMOO *et al.*[20] concluded from experiments with [$^{14}$C]ATP and [$^{32}$P]ATP that binding of unsplit ATP to erythrocyte ghosts and microsomes from mucosal cells of freshwater turtle bladder, respectively, can take place. Both groups claim that the binding depends on the presence of divalent cations (2 mM $Mg^{2+}$ or 1 mM $Ca^{2+}$, ref. 40; 3 mM $Mg^{2+}$, ref. 20). The binding process was, however, not studied in detail.

The interpretation of the influence of $K^+$ on ATP binding and *vice versa* (Fig. 6 and the model related to this figure) is partly based on a hypothesis with regard to the formation of a $K^+$–enzyme and $K^+$–enzyme–ATP complex. Furthermore, the formation of a $K^+$–ATP complex has been ignored in the model. The ratio $K^+$–ATP/free ATP is very small when calculated from a stability constant of about 15 $M^{-1}$ (ref. 41) but amounts to about 0.6 at 3 mM $K^+$ if the recently published stability constant of 200 $M^{-1}$ (ref. 42) is used. The effect of $K^+$, however, cannot be ascribed solely to the formation of a $K^+$–ATP complex, even if the last-mentioned stability constant is applied. There is therefore no doubt that $K^+$ does increase the enzyme–ATP dissociation constant, an observation which requires a reconsideration of the role of $K^+$ in the ($Mg^{2+}$ + $Na^+$ + $K^+$)-activated hydrolysis of ATP.

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