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IDENTIFICATION OF INTACT ATP BOUND TO $(Na^+ + K^+)$-ATPase

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SUMMARY

1. Native and ouabain-treated microsomes of turtle bladder epithelial cells incubated at 0° with [U-^{14}C]ATP form a Mg^{2+}-dependent, acid-stable complex with ^{14}C in a cooperative homotropic manner.

2. The bound ^{14}C is readily identifiable as intact ATP by first cleaving the ^{14}C from the ^{14}C-labeled microsomal precipitate and by the subsequent chromatographic recovery of ^{14}C-labeled ATP in the supernatant fluid.

3. The formation of *E*–ATP is ouabain inhibitable only in the presence of $Mg^{2+} + Na^+ + K^+$; and conversely the formation of *E*–ATP in the ouabain-treated enzyme in the presence of Mg^{2+} is inhibitable by addition of Na^+ and K^+ together. This suggests that at least part of the *E*–ATP complex is an integral part of the $(Na^+ + K^+)$-ATPase system.

4. The bond between the enzyme and ATP, probably a covalent one, resists increases in ionic strength and osmolality as well as increases in hydrogen bond dissolution.

5. The stoichiometric relations and turnover numbers of *E*–ATP and phosphoproteins are estimated with respect to their relative contributions to the overall catalyzed rate of hydrolysis of ATP.

INTRODUCTION

Previously reported data demonstrated the presence of a Mg^{2+}-dependent, Na^+-sensitive, acid-stable, ^{14}C-labeled complex between ATP and a precipitate of microsomal protein which had been incubated with [8-^{14}C]ATP[1]. The presumptive conclusion was that the ^{14}C-labeled protein was an *E*–ATP complex which precedes the formation of *E*–P in the sequence of $(Na^+ + K^+)$-ATPase reactions[1,2].

Present studies, presented partially in abstract form[3,4], examine some of the properties of the acid-stable *E*–ATP complex under a different set of conditions than those used previously. To increase the detectable binding of ATP to protein, the concentration of ATP was reduced and the specific activity of the ^{14}C-labeled ATP ([U-^{14}C]ATP) was increased. This technique permitted: (a) the identification of the ^{14}C bound to microsomes; (b) a determination of the ouabain sensitivity of the Mg^{2+}-dependent *E*–ATP complex formed with or without Na^+ and/or K^+ in the incubation

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fluid; and (c) a comparison of P binding by microsomes incubated in the presence of [γ-^{32}P]ATP with ATP binding by microsomes incubated in the presence of [U-^{14}C]-ATP.

METHODS

Materials

Tris salt of ATP, disodium ATP and ouabain were obtained from Sigma Chemical Company, St. Louis, Mo. Cellulose MN 300–polyethyleneimine impregnated was obtained from Brinkman Instrument Inc., Westbury, New York. [γ-^{32}P]ATP, as the sodium salt (specific activity varies from 1 to 10 C/mmole), and [U-^{14}C]ATP, as the ammonium salt (specific activity 568 mC/mmole), were obtained from Amersham/Searle Company, Ill. Microsomal fractions, isolated from mucosal epithelial cells removed from the urinary bladders of fresh water turtles (*Pseudemy scripta*), were prepared as previously described[5] and kept refrigerated at $-30°$.

Assay of initial rates of hydrolysis

In the standard assay procedure, final concentrations in the incubation mixture were as follows: 0.01 mM [γ-^{32}P]ATP (specific activity 5 C/mmole), 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 40 mM Tris–HCl (pH 7.3) as a buffer, 0.1 mM EDTA–Tris, inhibitors when indicated, and 4 μg of microsomal protein in a final volume of 100 μl.

The assay media less ATP were allowed to preincubate in tubes (5 mm $\times$ 50 mm) for 10 min at 38° before initiating the hydrolysis by the addition of [γ-^{32}P]ATP. The resulting mixture was incubated at 0° for 10 min prior to termination of the reaction by addition of 25 μl of 25 % (w/v) of $HClO_4$. The rest of the procedure for determining the P_i released was carried out as has been previously described[1,2]. The amount of microsomal protein was determined by the method of LOWRY *et al.*[6].

Assay of ^{14}C labeling of microsomal proteins

An aliquot of microsomal proteins, 0.04–0.20 mg, was incubated in an ice bath for 50 sec (unless otherwise indicated) in a medium containing 0.01 mM or otherwise as indicated of [U-^{14}C]ATP (specific activity 568 mC/mmole) 3mM $MgCl_2$, 40 mM Tris–HCl (pH 7.3) as buffer, 0.1 mM EDTA–Tris, 85 mM NaCl, 15 mM KCl, and inhibitors when indicated, in a final volume of 100 μl. Reaction was terminated by addition of 100 μl of 25 % (w/v) $HClO_4$ after which the mixture was centrifuged at 15000 $\times$ *g* for 15 min at 0°.

The $HClO_4$ precipitate was washed and centrifuged 5 times or more with 200 μl aliquots of a cold (0°) solution containing 5 % $HClO_4$, 0.015 M disodium ATP, and 0.05 M NaH_2PO_4. After each centrifugation, the supernatant was checked for radioactivity. The repeated washings and centrifugations were stopped only after little or no radioactivity was detected in the supernatant. The precipitates were then transferred into a scintillation vial containing 10 ml of 90 % formic acid. The precipitate together with the formic acid in the vial was left for 0.5 h. Then 10 ml of a toluene scintillation counting solution containing 50 % absolute ethanol was added, and the radioactivity, measured in the Beckman scintillation counter. The amount of protein labeling was estimated from counts of ^{14}C in the known amount of protein together with the specific activity of [U-^{14}C]ATP.

Assay of ^{32}P labeling of protein

Microsomal pellets, 0.04–0.20 mg were incubated in the same medium as described above, except 0.01 mM [γ-^{32}P]ATP (specific activity 5 C/mmole), instead of [U-^{14}C]ATP was used.

Treatments applied to microsomal precipitates

Having formed and isolated the washed, acid-stable, ^{14}C-labeled microsomal precipitates, aliquots of 0.04–0.2 mg (measured as microsomal protein) were subjected, to any one of the following treatments.

(a) Exposure for 10 min at 38° to 200 μl of 0.8 M hydroxylamine at pH 1.0, removal of the hydroxylamine-containing supernatant, and a subsequent exposure for 10 min at 38° to 0.2 % $(NH_4)_2\,MoO_4$. Then the mixture was centrifuged at 15000 $\times\, g$ for 15 min, and aliquots of the supernatant were applied to the cellulose–polyethyleneimine strip for identification of the nucleotides, ATP, ADP and AMP.

(b) Exposure for 10 min at 38° to 200 μl of any one of the following: 0.8 M NaCl, 0.8 M sucrose, or 0.8 M guanidine–HCl, after which the washed precipitate was analyzed for ^{14}C.

The solvent system used for the chromatographic separation of nucleotides was 0.25 M KH_2PO_4 (pH 3.4). After chromatographic separation on the cellulose–polyethyleneimine paper, each spot, identified from its location on the paper strip relative to that of nucleotide standards (ATP, ADP and AMP) treated in the same manner, was cut out and immersed in 10 ml of the toluene solution in which the counts were determined. Concurrently, standard solutions of [U-^{14}C]ATP, subjected to the same chemical treatment (*e.g.* NH_2OH and MoO_4^{2-} at pH 1.3 or otherwise as indicated) as were the microsomal precipitates, were also applied to the same cellulose–polyethyleneimine plates.

RESULTS

General comments

As expected, the amount of acid-stable ^{14}C binding to microsomes incubated with a low concentration of ATP of high specific activity (in the ^{14}C label) was greater than that previously encountered in microsomes incubated with a high concentration of ATP of low specific activity[1]. As a matter of fact, the previously reported binding of [8-^{14}C]ATP was detectable only after rapid millipore filtration with repeated washing of the acid-precipitated microsomes, but was not detectable after ultracentrifugation with repeated washing of the same acid-precipitated microsomes. In the present work, the acid-stable precipitate of microsomes retained a high degree of radiolabeling even after prolonged ultracentrifugation with repeated washings of the labeled precipitate.

Kinetic pattern of Mg^{2+}-dependent ATP binding

The amount of ATP bound was estimated from the specific activity of the [U-^{14}C]ATP and the number of ^{14}C counts/mg of protein in the acid-precipitated, washed microsomes. The values of the Mg^{2+}-dependent binding to be shown had been corrected for the amount of binding to the $HClO_4$-"killed" microsomes which amounted to no more than 15 % of that bound to enzymatically intact microsomes. An

alternative blank correction which could have been made for binding to intact microsomes incubated in the absence of Mg^{2+} (see below), amounted to no more than 28 % of that bound to microsomes incubated in the presence of Mg^{2+}.

A plot of values of the amount of ATP bound *versus* time of incubation of microsomes with [U-^{14}C]ATP (chemical concentration, 0.01 mM) at 0°, demonstrates the dependence of the binding reaction on the presence of Mg^{2+}. (Fig. 1). The amount of ATP bound to each mg protein (initially less than 3 μmoles of ATP bound per mg of microsomal protein) reached a maximal level of 16 pmoles within 2 sec, and remained at this level for the next 50 sec of incubation (see the uppermost plot in Fig. 1). Concomitantly, the amount of ATP bound per mg of protein in the absence of Mg^{2+} was: no more than 4.0 $\pm$ 0.5 in the presence of Na^+ alone; and no more than 2.0 $\pm$ 0.5 in the presence of K^+ alone, as shown in the two lower plots of the figure. Not shown is the corresponding amount of ^{14}C binding, in the presence of Tris buffer but in the absence of Mg^{2+}, Na^+, and K^+, which amounted to no more than 2.0 $\pm$ 0.4 pmoles/mg protein.

The data of Fig. 1 indicate unequivocally the formation of a Mg^{2+}-dependent, acid-stable, ^{14}C-labeled intermediary complex during the incubation of microsomes with [U-^{14}C]ATP. What follows is a study of the amount of this binding as a function of ATP concentration and the sensitivity of this binding to Na^+, K^+, and ouabain.

In Fig. 2 is shown a plot of values of the amount of ATP bound per mg of microsomal protein after 10 sec of incubation of 0° *versus* ATP concentration.

For substrate concentrations of less than 0.01 mM, the kinetic pattern resembled that expected of cooperative homotropic kinetics, a suspicion confirmed by the Hill plot of the data (see below). For substrate concentrations in excess of 0.02 mM the pattern was that characteristic of saturation kinetics with an apparent v_{max} of 140 pmoles/mg and with a small amount of substrate-induced inhibition appearing at ATP concentrations of 0.17 mM or more. Kinetic parameters obtained graphically from a Hill plot of the data were as follows: $K_h = 3.6 \cdot 10^{-4}$; $K_{s,0.5} = 0.025$ mM; and $n = 2.2$.

In subsequent experiments of this report, the concentration of ATP in the in-

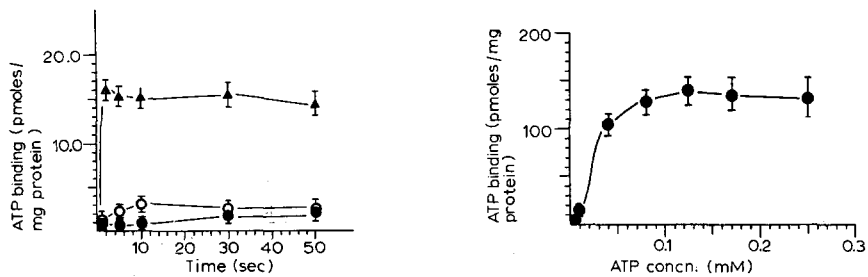

Fig. 1. Amount of ATP binding of native microsomal protein *versus* time of incubation with [U-^{14}C]ATP at 0°. ▲—▲, Mg^{2+}; ○—○, Na^+ (or blank); ●—●, K^+. The term blank indicates the presence of the buffer system only. The values presented are the mean $\pm$ S.E. from 6 different experiments on 6 batches of microsomes. The complete description of ionic concentrations as well as that of other conditions can be found in METHODS.

Fig. 2. Amount of ATP binding of native microsomal protein *versus* ATP concentration in the presence of Mg^{2+}. Time of incubation was 50 sec at 0°. The values presented are the mean $\pm$ S.E. from 6 different experiments on 3 batches of microsomes. A complete description of ionic concentrations and the other conditions is found in METHODS.

cubation fluid was always set at 0.01 mM. This concentration resulted in optimal levels of ^{14}C labeling of the microsomes without any significant degree of depletion of ATP *via* the overall hydrolysis at 0°, for the 50-sec period.

The negligible degree of depletion of ATP, less than 3 % of the amount initially available during the incubation of 0°, was demonstrated in ten parallel experiments where the microsomes were incubated with [γ-^{32}P]ATP in order to compare the amount of P_i released hydrolytically with the total amount of substrate available in each flask (see below for further details on this point).

Effect of higher substrate concentration on binding

In three preliminary experiments on microsomes incubated for 50 sec with [U-^{14}C]ATP at 0°, the amount of ATP bound to protein was measured in the presence of 0.5, 1.0, and 1.0 mM ATP. In two out of the three experiments, (0.5 and 1.0 mM ATP), the amount of ATP binding per mg of protein after 50 sec of incubation in the presence of Mg^{2+} + Na^+ or of Mg^{2+} + Na^+ + K^+ was one-third less than that in the presence of Mg^{2+} alone. In the third experiment (1.0 mM ATP), the amount of binding in the presence of Mg^{2+} + Na^+ was 50 % greater than that in the presence of Mg^{2+} alone. Though fragmentary, these findings provide only a partial confirmation of previously reported binding data at high concentrations (1.0 mM) of ATP. However, in the previously reported experiments, [8-^{14}C]ATP instead of [U-^{14}C]ATP was the label; and the acid-precipitated microsomes were obtained as a millipore residue rather than as a centrifugal pellet. Obviously, further experimentation is required to examine the effects of Na^+ and/or K^+ on ATP binding as a function of ATP concentration.

Mg^{2+}-dependent binding in presence of Na^+ and/or K^+

Fig. 3 gives a plot of values of ATP bound per mg of protein *versus* time of incubation of microsomes with [^{14}C]ATP at 0°.

Data of Fig. 3a were taken from 4 experiments, each one of which included measurements of ^{14}C binding in the presence of Mg^{2+} alone, Mg^{2+} + Na^+, and Mg^{2+} + K^+. It can be seen that the addition of either Na^+ alone or K^+ alone had no detectable effect on the amount of Mg^{2+}-dependent ^{14}C labeling measured at any incubation time from 2 to 50 sec.

Thus, the presently reported pattern of Mg^{2+}- or of (Mg^{2+} + Na^+)-dependent binding of [U-^{14}C]ATP in the presence of 0.01 mM ATP was different from that reported previously with [8-^{14}C]ATP in the presence of 1.0 mM ATP; and such differences, ascribable to the concentration of substrate, as well as to the method of obtaining acid precipitated microsomes, have been detailed in the previous section.

Data of Fig. 3b were taken from 6 experiments, each one of which included measurements of ^{14}C binding in the presence of Mg^{2+} alone and in the presence of Mg^{2+} + Na^+ + K^+. The amount of ATP bound in the presence of Mg^{2+} alone was not significantly different from that in the presence of Mg^{2+} + Na^+ + K^+ throughout the whole time-course of incubation from 0 to 50 sec.

Effect of ouabain

Not shown are data on the mean values of the amounts of ATP binding after 20 and 50 sec of incubation of microsomes and [U-^{14}C]ATP at 0° with and without

ouabain in the presence of Mg^{2+} or $Mg^{2+} + Na^+$ or $Mg^{2+} + K^+$ in 6 experiments. Under none of these conditions did ouabain treatment of the protein result in any discernible change in the ATP binding.

Fig. 4 gives a plot of values of ATP bound per mg of protein *versus* the time of incubation of native and of ouabain-treated microsomes with [U-^{14}C]ATP at 0° in presence of $Mg^{2+} + Na^+ + K^+$. Of interest is the fact that the pretreatment of the microsomes with ouabain resulted in a significant reduction (approx. 50 %) in the amount of ATP bound to the protein after 5, 10, 30 and 50 sec of incubation. This finding contrasted with lack of a discernible effect of ouabain when either Na^+ alone or K^+ alone was added to the Mg^{2+}-containing incubation fluid. Nevertheless, the ouabain sensitivity in the presence of $Mg^{2+} + Na^+ + K^+$ may be correlated with analogous findings on the catalyzed rate of overall hydrolysis of ATP in microsomes from turtle bladder[1,2,5] and from other tissues[7,8] where the inhibitory effects of ouabain are also detected only in the presence of all three cations, Mg^{2+}, Na^+ and K^+. In this connection, the ouabain sensitivity (shown in Fig. 4) suggests that at least 50 % of the ^{14}C-labeled protein is an integral part of the $(Na^+ + K^+)$-ATPase molecule.

An alternative interpretation of the data in Fig. 4 is that the addition of Na^+ and K^+ together causes a decrease in the Mg^{2+}-dependent binding of ATP to the oua-

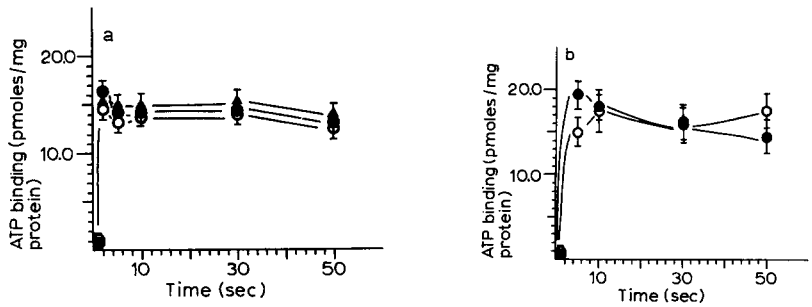

Fig. 3. Amount of ATP binding of native microsomal protein *versus* time of incubation with [U-^{14}C]ATP at 0°. (a) Data on the mean amount of binding ± S.E. from 6 different experiments in the presence of Mg^{2+} (▲—▲), $Mg^{2+} + Na^+$ (●—●) and $Mg^{2+} + K^+$ (○—○). (b) Data on the mean amount of binding ± S.E. from 6 different experiments in the presence of Mg^{2+} (○—○) and $Mg^{2+} + Na^+ + K^+$ (●—●). The rest of the conditions are those described in METHODS.

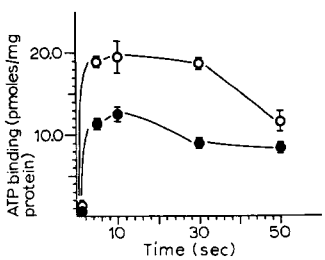

Fig. 4. Amount of ATP binding of ouabain-treated microsomal protein *versus* time of incubation with [U-^{14}C]ATP at 0° in the presence of $Mg^{2+} + Na^+ + K^+$ (○—○) and $Mg^{2+} + Na^+ + K^+$ + ouabain (●—●). Concentration of KCl was 15 mM and of ouabain, $1 \cdot 10^{-3}$ M. The total ionic system and ouabain together with the enzyme (less [U-^{14}C]ATP) were preincubated for 10 min at 38°, after which the mixture was brought to 0° prior to initiating the reaction with [U-^{14}C]ATP. Each of the values presented is the mean ± S.E. from 6 different experiments on 3 batches of microsomes. The complete description of the conditions can be found in METHODS.

bain-treated microsomes. This effect may be analogized to the Na^+- or K^+-induced decreases in the ATPase activity of native or of ouabain-treated microsomes at incubation temperatures of 50–60° (ref. 9).

Identification of the ^{14}C bound to microsomes

Previous data, reported in abstract form[3,4], showed a hydroxylamine-induced cleavage of the ^{14}C-labeled protein complex derived from microsomes incubated with [U-^{14}C]ATP. Exposure of the ^{14}C-labeled, microsomal precipitates for 10 min to hydroxylamine at pH 7.0 removed practically all of the ^{14}C label from the precipitates; but only 21 % of the ^{14}C split off the protein could be identified as intact ATP.

However, it was discovered empirically that exposure of the ^{14}C-labeled microsomal precipitates to hydroxylamine at pH 1.3, followed by exposure of the precipitate to $(NH_4)_2MoO_4$ also at pH 1.3 (and not *vice versa*) resulted in the removal of over half of the bound ^{14}C; and in the chromatographic identification and recovery of 80 % of the supernatant ^{14}C in the form of intact ATP[3,4].

Table I presents the results of chromatographic analysis of the ^{14}C-labeled nucleotides appearing in the supernatant fluid after the NH_2OH–MoO_4^{2-} treatment of the pre-formed ^{14}C-labeled, acid-precipitated microsomes; and that of the ^{14}C-labeled nucleotides appearing after NH_2OH–MoO_4^{-2} treatment of [U-^{14}C]ATP (in the absence of microsomes).

It can be seen that 57 % of the ^{14}C appearing in the supernatant fluid after the NH_2OH–MoO_4^{2-} treatment of the labeled microsomes was chromatographically identical to ATP and that the remaining 43 % was chromatographically identical to ADP and AMP.

If the acid nature and the chemical reactivity of the supernatant had no effect on the ATP appearing therein, then at least 57 % of the [^{14}C]ATP in the supernatant must have been bound to the protein in the form of unsplit ATP. However, when pure ATP was exposed for the same length of time to the same acid environment with NH_2OH and MoO_4^{2-}, 27 % was broken down to ADP and AMP (see second row, Table I). Therefore, the amount of ATP (57 %) released from the microsomes into the acidic supernatant must be 27 % less than that initially cleaved from the protein,

TABLE I

NUCLEOTIDES RELEASED INTO THE SUPERNATANT FLUID AFTER ACID–HYDROXYLAMINE–MOLYBDATE TREATMENT OF THE *E*–ATP COMPLEX IN THE ACID-PRECIPITATED MICROSOMAL PROTEINS

The acid precipitated *E*–ATP complex was treated with 0.8 M NH_2OH (pH 1.0) at 38° for 10 min followed by treatment with 0.2% $(NH_4)_2MoO_4$ in 0.25 M H_2SO_4 at 38° for 10 min. The ATP standard was treated similarly. The conditions required for obtaining the ^{14}C-labeled microsomal complex are described in METHODS. Each value shown is the mean of 4 determinations; and the standard errors were less 4% of the mean value in all cases.

	Relative amounts of nucleotides released		
	ATP (%)	*ADP* (%)	*AMP* (%)
Acid-precipitated *E*–ATP complex	57	21	22
ATP (standard) (originally 99% ATP)	73	16	11

which means that about 80% of the ^{14}C removed from the microsomal protein is intact ATP; and that the amount of ATP must have been bound to the protein in the form of an intact ATP molecule.

Data in Table I suggest that hydroxylamine first reacts in an unspecified manner with either the enzyme–substrate complex, *E*–ATP, or with the free enzyme protein prior to substrate complexation, but not with free ATP. Then the NH_2OH–*E*–ATP complex is broken down by $(NH_4)_2MoO_4$ into free ATP and other products. Neither one of the reagents, (NH_2OH or MoO_4^{2-}) by itself attacks free ATP, as shown by experiments where pure ATP, remained intact (and was recovered chromatographically) after exposure to $(NH_4)_2MoO_4$ in 0.25 M H_2SO_4, or after exposure to NH_2OH at pH 1.0.

Bond strength between ATP and the protein and between P and the protein

The demonstrated complexing between ATP and the protein could have been due to the formation of hydrophobic bonds, hydrogen bonds, covalent bonds, or electrostatic interactions. Accordingly, tests were performed on the ^{14}C and ^{32}P complexes of acid-precipitated microsomes which had been previously incubated with [U-^{14}C]ATP or [γ-^{32}P]ATP.

To test for hydrophobic bonding, the amounts of ATP and of P bound to paired aliquots of the acid precipitated, labeled microsomes were determined before and after a 10-min incubation at 0°; and before and after an identical incubation at 38°. Twenty such paired experiments (at the two temperatures) were performed on microsomal precipitates at pH 1.3, and twenty more at pH 7.0.

Table II presents data on the effect of exposure of the labeled precipitate to 38° less that of exposure to 0° on the binding of ATP and of P to the acid-precipitated, labeled microsomes previously incubated in the native state with [U-^{14}C]-ATP and [γ-^{32}P]ATP, respectively.

TABLE II

THERMAL DEGRADATION (BETWEEN 0 AND 38°) OF ^{14}C- AND OF ^{32}P-LABELED MICROSOMAL PRECIPITATES AFTER 10 min EXPOSURE TO MEDIA OF pH 1.3 AND 7.5 PAIRED EXPERIMENTS AT pH 1.3 AND IN 20 ADDITIONAL PAIRED EXPERIMENTS AT pH 7.0

Microsomes were labeled in their native states (before $HClO_4$ precipitation) by incubation for 50 sec at 0° with [U-^{14}C]ATP or with [γ-^{32}P]ATP at pH 7.3 and under the ionic conditions denoted in first column.

Incubation of native microsomes	*Amount of bound complex (pmoles/mg)*	*Percentage breakdown at 38° less that at 0°* in labeled microsomal precipitates after 0 min exposure to*	
		pH 1.3	*pH 7.5*
[U-^{14}C]ATP, 0.01 mM			
Mg^{2+}	15 ± 1	21 ± 2	30 ± 2
[γ-^{32}P]ATP, 0.01 mM			
Mg^{2+}	143 ± 3	0	0
$Mg^{2+} + Na^+$	290 ± 4	10 ± 1	15 ± 1
ΔNa^+	147 ± 3	20 ± 1	30 ± 1

* No detectable degradation of either ^{14}C-labeled protein (*E*–ATP) or of ^{32}P-labeled protein (*E*–P) occurred after the 10 min exposure to pH 1.3 or 7.0 at 0°.

At 0°, incubation of the ^{14}C- or ^{32}P-labeled microsomal precipitates for 10 min in a medium of pH 1.3 or pH 7.0 resulted in no detectable removal of either label. At 38°, incubation of ^{14}C-labeled precipitates in a medium of pH 1.3 resulted in the removal of 21 ± 2 % ($P < 0.001$); and incubation in a medium of pH 7.0, in the removal of 30 ± 2 % ($P < 0.001$) of the bound ^{14}C. Incubation of the ^{32}P-labeled precipitates in a medium of pH 1.3 resulted in the removal of none of the Mg^{2+}-dependent P bound and in the removal of 10 ± 1 % ($P < 0.001$) of the ($Mg^{2+} + Na^+$)-dependent P bound, or about 20 % of the Na^+-induced increment of the bound P. Similar incubation of the ^{32}P-labeled precipitates at pH 7.0 resulted in the removal of none of the Mg^{2+}-dependent P bound, and in the removal of 15 ± 1 % ($P < 0.001$) of the ($Mg^{2+} + Na^+$)-dependent P bound or of approx. 30 % of the Na^+-induced increment of the bound P.

Despite the apparent similarity in the thermal sensitivity of *E*–ATP and the Na^+-dependent *E*–P, concomitant data on ^{14}C-labeled nucleotides in the supernatants of the ^{14}C-labeled microsomal precipitates showed that only 15 % of the ^{14}C label was intact ATP and the remaining 85 %, AMP and ADP. This means that the heat-induced cleavage of the bond between the γ-phosphoryl group of intact ATP and the protein was approx. 15 % of the heat-induced cleavage of the Na^+-induced bond between the γ-phosphate group alone and the protein. This may have some bearing on the relative stoichiometry of the ATPase sequence (see later).

Apart from the stoichiometry, the thermal lability suggests that there was no hydrophobic bonding between ATP and the protein or between P and the Na^+ portion of the protein. It remains possible, however, the part of the bonding between P and the protein is hydrophobic in nature.

Not shown are data on the stability of the pre-formed *E*–ATP and *E*–P in the microsomal precipitates at 38° for 10 min at pH 7.3 with respect to increases in the osmolality (sucrose, 0.8 M), in the ionic strength (NaCl, 0.8 M), and with respect to increases in the presence of hydrogen bond breaking reagents (guanidine–HCl, 0.8 M). None of these treatments reduced the amount of *E*–ATP or that of *E*–P, suggesting that the complexes between ATP and the protein or between P and the protein were probably formed by covalent bonds rather than by hydrogen bonds or interionic attractions.

Crude stoichiometry

It was of interest to compare the amount of *E*–ATP complex determined from ^{14}C binding with that of the total phosphoprotein determined from ^{32}P binding when the microsomes were incubated at 0° for 50 sec with low concentrations (0.01 mM) of ATP. Accordingly, 6 experiments were performed with [γ-^{32}P]ATP at these low ATP concentrations in the presence of Mg^{2+}, $Mg^{2+} + Na^+$, and $Mg^{2+} + Na^+ + K^+$.

Results, in terms of pmoles of P bound per mg of microsomal protein were as follows. In the presence of Mg^{2+} alone, 150; in the presence of $Mg^{2+} + Na^+$, 300; and in the presence of $Mg^{2+} + Na^+ + K^+$, 150. Each value represents the approximate mean estimated from the data of 6 experiments.

The aforementioned data on phosphoprotein also show that the amount of phosphate bound to the microsomes, 150–300 pmoles/mg, was over 10-fold greater than the average amount of intact ATP bound (16–20 pmoles/mg) under the same conditions of incubation.

Double labeling of microsomes

The radio labeling of microsomes incubated in the presence of Mg^{2+} and of $Mg^{2+} + Na^+ + K^+$ with both [γ-^{32}P]ATP and [U-^{14}C]ATP provided a doubly labeled protein which was precipitated in the usual way with $HClO_4$. Both radioisotopes were measured in the scintillation counter immediately, and again, 3 months after the experiment to allow for the decay of the ^{32}P which would permit more precise estimates of the bound ^{14}C.

Data on simultaneous double labeling were obtained from two experiments. In each experiment, four replicate determinations were made under each one of the two ionic conditions. It was found that each mgm of microsomal precipitate (previously incubated in the presence of Mg^{2+} alone and in the presence of $Mg^{2+} + Na^+ + K^+$) was simultaneously complexed with 16 pmoles of ATP and 146 pmoles of phosphorous.

In summary, during the time required to form one molecule of *E*–ATP, there are formed 10 molecules of phosphoprotein in the presence of Mg^{2+} alone or in the presence of $Mg^{2+} + Na^+ + K^+$ (as estimated from data on simultaneous double labeling of microsomal precipitates). Similarly, during the time required to form one molecule of *E*–ATP, there are formed 20 molecules of phosphoprotein in the presence of $Mg^{2+} + Na^+$ (as estimated from data on single labeling of microsomal precipitates).

Estimated turnover numbers

In addition to the relative stoichiometry, the turnover numbers of *E*–ATP and *E*–P at 0° were estimated from the overall rate of hydrolysis of ATP, catalyzed by microsomes under the same conditions of temperature, ionic environment, substrate concentration and incubation time.

Table II shows data on the rate of P_i release in 6 experiments on microsomes incubated at 0° with 0.01 mM ATP. In the presence of Mg^{2+}, the activity was 208 nmoles/mg protein per h; and consequently, the $(Na^+ + K^+)$-induced increment of ATPase activity amounted to 43.

Data on $(Na^+ + K^+)$-ATPase at 0° may be related, *via* the turnover number, to the amount of *E*–ATP formed, assuming that half of the *E*–ATP formed was the first step in the $(Na^+ + K^+)$-ATPase sequence. The estimated turnover number of *E*–ATP at 0° was approx. 7 per sec. The turnover of the Na^+-induced increment of phosphoprotein at 0° amounted to about 0.1 per sec.

TABLE III

ATPase ACTIVITY AFTER 10 min OF INCUBATION AT 0° IN THE PRESENCE OF 0.01 mM ATP

Each of the values shown is the mean ± S.E. from 6 different experiments on 3 batches of microsomes. The complete description of ionic concentrations and the rest of the conditions can be found in METHODS.

Ionic condition	*ATPase (0°) (nmoles/mg per h)*
Mg^{2+}	208 ± 8
$Mg^{2+} + Na^+ + K^+$	251 ± 9
$\Delta (Na^+ + K^+)$	43 ± 6
$P [\Delta (Na^+ + K^+) = 0]$	<0.001

DISCUSSION

The Mg^{2+}-dependent, ouabain-sensitive formation of *E*–ATP together with the observed mutual interactions of the ouabain-treated microsomes with Na^+ and K^+ together suggests that half of the *E*–ATP is an integral part of the ($Na^+ + K^+$)-ATPase activity, and consequently is of physiological significance. That the bond between ATP and the microsomal protein is covalent or a strong type of chlathyrate or chelate is suggested by the stability of the pre-formed *E*–ATP complex when exposed to high levels of osmolality and ionic strength as well as to high concentrations of agents which break hydrogen bonds.

The estimated stoichiometric relations at 0° among *E*–ATP, phosphoprotein and the rate of P_i formation show that the turnover of the Na^+-induced increment of the total phosphoprotein (*i.e.* *E*–ATP + $E \sim P$ + *E*–P) is approx. 1/70th that of *E*–ATP. Either the turnover of non-ATP phosphoprotein (*e.g.* $E \sim P$ + *E*–P) is the rate-limiting step in the overall hydrolysis of ATP, or the non-ATP phosphoprotein is not in direct series with the intermediary steps of the ($Na^+ + K^+$)-ATPase sequence.

Of further interest is that the turnover of the Na^+-induced moiety of total phosphoprotein (0.1 per sec) is consistent with the estimated rate of the ouabain-sensitive moiety of Na^+ transport in erythrocytes at 0° (ref. 10).

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REFERENCES

1 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 484.
2 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 495.
3 W. A. BRODSKY AND A. E. SHAMOO, *Biophys. Soc. Abstr.*, 11 (1971), 287a.
4 A. E. SHAMOO AND W. A. BRODSKY, *Biophys. Soc. Abstr.*, 11 (1971) 288a.
5 R. E. SOLINGER, C. F. GONZALES, Y. E. SHAMOO, H. R. WYSSBROD AND W. A. BRODSKY, *Am. J. Physiol.*, 215 (1964) 18.
6 O. H. LOWRY, J. V. PASSONEAU, F. X. HASSELBERGER AND D. W. SCHULZ, *J. Biol. Chem.*, 239 (1964) 18.
7 J. C. SKOU, *Phys. Rev.*, 45 (1965) 596.
8 R. W. ALBERS, *Ann. Rev. Biochem.*, 36 (1967) 727.
9 A. E. SHAMOO, T. P. SCHILB AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 225 (1971) 254.
10 J. F. HOFFMAN, *J. Gen. Physiol.*, 54 (1969) 3435.