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THE INTERMEDIARY COMPLEXES FORMED BY $(Na^+ + K^+)$-DEPENDENT ATPase

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SUMMARY

1. The microsomal pellet obtained from the isolated mucosal cells of bladders of freshwater turtles contains a $(Na^+ + K^+)$-ATPase activity.

2. Mg^{2+}-dependent ^{32}P-labeling of the native and ouabain-treated protein by [γ-^{32}P]ATP at 0° amounts to 0.29 and 0.19 nmole P per mg protein per 50 sec, respectively.

In the native protein, addition of Na^+ increased this labeling by 158%; and addition of $Na^+ + K^+$, decreased the labeling of the protein to the underlying Mg^{2+}-dependent level.

In the ouabain-treated protein, addition of Na^+ increased Mg^{2+}-dependent labeling by 220%; but addition of $Na^+ + K^+$ resulted in no change in labeling which remained at the $Mg^{2+} + Na^+$-dependent level.

3. Mg^{2+}-dependent ^{14}C labeling of the native microsomal protein by [^{14}C]ATP at 0° amounts to 0.72 nmole/mg protein per 50 sec; and addition of Na^+ decreases the Mg^{2+}-dependent labeling by 46%.

4. The Na^+-induced decrement in microsomal binding of [^{14}C]ATP simultaneously with the Na^+-induced increment in binding of [^{32}P]ATP suggests the formation and breakdown of a Na^+-sensitive enzyme–ATP complex.

INTRODUCTION

The reactions of microsomal ATPase proceed through the formation and breakdown of a phosphorylated intermediate which has properties of a high energy phosphate complex, and which is usually detected as ^{32}P-labeled acid-stable protein after incubation of the microsomes at 0° with [γ-^{32}P]ATP[1]. The effects of Na^+, K^+ and ouabain on the phosphorylation[2–5] and dephosphorylation[4–8]; on the exchange reaction between ATP and ADP; and on the overall hydrolysis[9,10] have been considered relevant to the Na^+ transport mechanism.

The purpose of the present study was to characterize some of the intermediate

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steps between ATP and the microsomal proteins of the epithelial cells of the turtle bladder, a known Na^+ transporting system[11] which possesses a $(Na^+ + K^+)$-stimulated, ouabain-inhibited ATPase activity[12], and which satisfies five out of eight criteria given by SKOU[4] for isolation of a transport system.

Details studied included: the rate and cation dependency of ^{32}P labeling and of ^{14}C labeling of microsomal proteins which had been incubated with [γ-^{32}P]ATP and with [^{14}C]ATP, respectively; the effects of certain inhibitors (*i.e.* ouabain and *N*-ethylmaleimide); and the nucleotide specificity of the protein binding process.

METHODS

Materials

Tris salt of ATP, disodium ATP, ouabain, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, L-histidine·HCl, and imidazole grade I were obtained from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]ATP as the ammonium salt, [^{14}C]ATP, as the sodium salt, and $^{32}P_i$ in 0.02 M HCl were obtained from International Chemical and Nuclear Corp., Calif. Microsomal fractions, isolated from mucosal epithelial cells removed from the urinary bladders of fresh water turtles (*Pseudemys scripta*) were prepared as previously described[12] and kept refrigerated at $-30°$.

Assay of initial rates of hydrolysis

For rapid spot checks of ATPase activity in different fractions, the micro-technique of LOWRY *et al.*[13] as recommended by ALBERS *et al.*[14] was used. However, this method is not generally recommended for routine use in K^+-sensitive reactions[14] because the pyruvate kinase activity itself is K^+ sensitive[15,16].

In the standard assay procedure, final concentrations in the incubation mixture were as follows: 3 mM [γ-^{32}P]ATP (specific activity $1 \cdot 10^5$ counts/min per μmole), 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.3) as a buffer, 0.1 mM EDTA–Tris, inhibitors when indicated, and 4 μg of enzyme protein in a final volume of 100 μl.

The assay media less ATP were allowed to pre-incubate in tubes (5 mm × 50 mm) approx. 0.5 h at 38° before initiating the hydrolysis by addition of the [γ-^{32}P]ATP. The resulting mixture was incubated at 38° (or in some cases at 0°) for 10–20 min prior to termination of the reaction by addition of 25 μl of 25 % (w/v) of $HClO_4$.

After centrifugation of the $HClO_4$-treated mixture at 15000 × *g* for 15 min at 0°, 100 μl of the supernatant fluid were analyzed for either $^{32}P_i$ or, in some cases, P_i, by a technique according to BERENBLUM AND CHAIN[17] modified along the lines suggested by WEIL-MALHERBE AND GREEN[18]. After extraction of the phosphomolybdate complex into the isobutanol layer, the procedure was considered as terminated with respect to radioisotopic counting of $^{32}P_i$, since no color development is required for such an end-point measurement.

An aliquot (50 μl) of the isobutanol layer was added to the naphthalene–dioxane scintillation counting fluid[19] for measurement of ^{32}P in a Beckman scintillation counter. The amount of ATP hydrolyzed was calculated from the data on ^{32}P counts and specific activity of [^{32}P]ATP in the reaction mixture. The rate was normalized with respect to the amount of microsomal protein, determined by the method of LOWRY *et al.*[20].

The $(Na^+ + K^+)$-stimulated ATPase is defined as that activity measured in the presence of $Mg^{2+} + Na^+ + K^+$, less than that measured in the presence of Mg^{2+} alone. The $(Na^+ + K^+)$-stimulated part is inhibited by ouabain, the Mg^{2+}-dependent part is not. Therefore, $(Na^+ + K^+)$-stimulatable ATPase is often called ouabain-sensitive ATPase, and Mg^{2+}-dependent ATPase is often called ouabain-insensitive ATPase.

Assay of ^{32}P-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 1 mM [^{32}P]ATP (specific activity, $1 \cdot 10^5$ counts/min per nmole), 85 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.3) as buffer, 0.1 mM Tris–EDTA, KCl and inhibitors when indicated, in a final volume of 100 μl. Reactions were terminated by addition of 100 μl of 10% (w/v) $HClO_4$, after which the mixture was centrifuged at 15000 $\times$ *g* for 15 min at 0°. At this step, hydrolysis at 0° was measured when desired by removing an aliquot of the supernatant and treating it as described above.

The $HClO_4$ precipitate was washed twice with 200-μl aliquots of a cold (0°) solution containing 5% $HClO_4$, 0.05 M disodium ATP, and 0.05 M NaH_2PO_4. The entire 400 μl of the resulting suspension was filtered by suction through millipore paper with a pore size of 0.45 μ; after which an additional 25 ml of the same cold washing solution was placed on the same paper and filtered by suction.

The volume desired for washing was pre-determined by increasing the volume of washing solution (from 5 to 50 ml) in order to determine at what point further increases in volume produced no change in radioactivity of the filtrate. Filter paper, so treated, was immersed into 10 ml of toluene scintillation counting solution, and radioactivity was measured in the Beckman scintillation counter.

Two types of blanks were obtained: the first, by filtering and washing the microsome-free incubation mixture (filter control); and the second, by filtering and washing the same incubation mixture containing microsomes previously denatured by treatment with $HClO_4$, ($HClO_4$-killed control). In all cases, the radioactivity of the filter control was much less than that of the $HClO_4$-killed control.

Another type of blank was obtained, but not routinely, by filtering and washing the same incubation mixture containing microsomes previously denatured by immersing the mixture into a boiling water bath for 3–5 min (heat-killed control). The average value of labeling of the heat-killed microsomes was the same as that of $HClO_4$-killed microsomes. Consequently the latter was used routinely as the base line control for nonspecific labeling in all of the experiments to be reported herein. The amount of protein labeling was estimated from the counts of ^{32}P in the protein together with the specific activity of [γ-^{32}P]ATP.

Assay of [^{14}C]ATP labeling of protein

Microsomal pellets, 0.05–0.20 mg, were incubated in the same medium as described above, except that 1 mM [^{14}C]ATP (specific activity $5 \cdot 10^6$ counts/min per μmole), instead of [^{32}P]ATP was used.

$^{32}P_i$ labeling of protein

5 mM $^{32}P_i$ (specific activity $1 \cdot 10^5$ counts/min per nmole), instead of 3 mM ATP,

was added to the standard microsomal fraction and carried through the entire procedure described for ^{14}C- and ^{32}P-labeled ATP in order to determine the rate of $^{32}P_i$ labeling of the microsomal proteins.

RESULTS

ATPase activity with [γ-^{32}P]ATP

The hydrolytic activity of the native and of the ouabain-treated microsomal pellet in the presence of Mg^{2+} alone, $Mg^{2+} + Na^+$, and $Mg^{2+} + Na^+ + K^+$ was determined from the rate of release of $^{32}P_i$. As expected, the simultaneous addition of Na^+ and K^+ to the incubation mixture increased the Mg^{2+}-dependent activity from 11.0 to 21.7 μmoles/mg protein per h in the native microsomes, but not in the microsomes which had been preincubated with ouabain.

Such data, derived from the use of labeled [^{32}P]ATP were consistent with our previous data, derived from the use of non-radioactive ATP[12,21]; and consistent with data of others[22] who used an ADP assay for measuring ATPase activity in the microsomal pellet from turtle bladder.

^{32}P labeling from [γ-^{32}P]ATP

Table I presents mean values and statistical parameters for the cation-sensitive rate of ^{32}P labeling of native and of ouabain-treated microsomal proteins. Values are normalized with respect to the Mg^{2+}-dependent labeling rate, 0.29 nmole/mg protein per 50 sec taken as 100%.

In the native microsomal protein, addition of Na^+ increased the mean labeling

TABLE I

^{32}P LABELING OF NATIVE AND OUABAIN-TREATED MICROSOMES INCUBATED WITH [γ-^{32}P]ATP FOR 50 SEC AT 0° UNDER THE CATIONIC CONDITIONS DESIGNATED IN THE FIRST COLUMN

Each tube contained approx. 200 μg of microsomal protein in a native or ouabain-treated system. Final concentration of each constituent of the incubation mixture was: 50 mM Tris–HCl, 85 mM Na^+, 15 mM K^+, 3 mM Mg^{2+}, 3 mM [γ-^{32}P]ATP (specific activity $1 \cdot 10^6$ counts/min per μmole), 0.1 mM Tris–EDTA, and 1 mM ouabain. Final volume, 100 μl; final pH 7.3; temperature, 0°; time of incubation, 50 sec. Reaction was terminated by adding 100 μl 10% (w/v) cold $HClO_4$ (final concentration, 5%).

	Conditions for labeling	*% Phosphorylation (Mg^{2+}-dependent as 100%)*	
		Native	*Ouabain*
A.	Mg^{2+}	100 ± 21 $n = 7$	64 ± 16 $n = 6$
B.	$Mg^{2+} + Na^+$	258 ± 49 $n = 7$ B − A $P < 0.02$	205 ± 47 $n = 4$ B − A $P < 0.05$
C.	$Mg^{2+} + Na^+ + K^+$	126 ± 49 $n = 4$ C − A $P < 0.6$	203 ± 41 $n = 4$ C − A $P < 0.05$

activity by 158 % in 7 experiments ($P < 0.02$); and addition of $Na^+ + K^+$ produced little if any significant change ($P > 0.6$) above the Mg^{2+} level of labeling. Apparently, the addition of K^+ to the $Mg^{2+} + Na^+$ loaded protein induces a significant ($P < 0.02$) stripping of the protein-bound ^{32}P. Not shown are similar results on ^{32}P labeling of microsomes in an imidazole–histidine buffer system. The aforementioned data on native microsomes are similar to those found in other tissues[3,4,22].

In the ouabain-treated microsomal protein, the ^{32}P-labeling rate with Mg^{2+} alone was 64 % of the corresponding rate in the native protein.

A surprising and new finding in the ouabain-treated mixture was the fact that addition of Na^+ resulted in an increment of the Mg^{2+}-dependent ^{32}P labeling (from 64 to 205 %) similar in magnitude to that observed in the native microsomes of the turtle bladder, but greater than that reported by others in other tissues[3,4,23].

As expected, the K^+-induced stripping of ^{32}P from the ($Mg^{2+} + Na^+$)-treated microsomes was completely blocked by ouabain ($P > 0.6$). Such a blocking of dephosphorylation has been found in other microsomal systems treated with ouabain[3–5,23].

Not shown are parallel results on ouabain-treated microsomes in an imidazole–histidine buffer system. Unlike the ^{32}P binding in Tris buffer, addition of Na^+ failed to increase the Mg^{2+}-dependent labeling and addition of $Na^+ + K^+$ increased the Mg^{2+}-dependent labeling. These results are somewhat different from those of others[3–5,14,23].

Effect of N-ethylmaleimide

Four experiments were performed to determine the effects of *N*-ethylmaleimide on ^{32}P labeling in the presence of Mg^{2+} and Na^+. Mean values $\pm$ S.E. of ^{32}P labeling of the *N*-ethylmaleimide-treated microsomes, expressed as percentages of ^{32}P labeling of the native protein with Mg^{2+} alone, were as follows: (a) 134 $\pm$ 73 % in the presence of Mg^{2+} + *N*-ethylmaleimide, which was statistically the same as 100 % in the presence of Mg^{2+} alone; and (b) 396 $\pm$ 124 %, in the presence of $Mg^{2+} + Na^+$ + *N*-ethylmaleimide which was statistically greater than that in the presence of Mg^{2+} alone, indicating that the Na^+ stimulation of ^{32}P labeling of microsomes remained intact in the presence of *N*-ethylmaleimide.

Substrate specificity

Apparently, ATP is the preferred substrate for the ($Na^+ + K^+$)-dependent ATPase, as well as for Na^+-dependent phosphorylations and exchange reactions in the electric organ of the eel[9,23,24]. The technique was to determine first the rate of ^{32}P binding from 1 mM [^{32}P]ATP, and then determine the effect on such binding by adding 5 mM of other nucleotides to the incubation mixture. Results showed the following order of nucleotide preference: ATP > UTP > GTP > ITP > CTP. Thus, [^{32}P]ATP binding, corrected for the presence of equimolar amounts of CTP, ITP, GTP, and UTP, was 16, 12, 1, and 1 %, respectively, or conversely, the percentage inhibition of [^{32}P]-ATP binding was 84, 88, 99 and 99 %, respectively.

Hydrolysis at 0°

Data on microsomal ATPase activity have been derived from measurements at 38° and the addition of $Na^+ + K^+$ to the Mg^{2+}-containing system uniformly accelerates the reaction while the addition of Na^+ alone to the Mg^{2+}-containing system

stimulates the activity in some cases[1,2,25–28], but not in others[22,27,29–37]. Therefore, experiments were designed to correlate rate of labeling of microsomal proteins at 0° with the overall rate of hydrolysis at 0°.

Fig. 1 presents a plot of values of P_i liberated *versus* time of incubation at 0° in a microsomal pellet, one aliquot of which was exposed to Mg^{2+} alone, the other to $Mg^{2+} + Na^+$. There was no apparent difference in the amount of P_i released from either aliquot at any time during the 10 min of incubation. The amount of P_i liberated after 10 min, 110 nmoles/mg protein, was approx. 5 % of that liberated by incubation of the same microsomal system at 38° (ref. 21). The amount of ATP hydrolyzed in 50 sec at 0° at (10 nmoles/mg protein) was *ca.* 30 times greater than the average amount of Mg^{2+}-dependent labeling, and *ca.* 13 times greater than the average of the $(Mg^{2+} + Na^+)$-dependent labeling (see Table I and Fig. 1) suggesting a low turnover rate.

Time-course of phosphoprotein formation

(*a*) *Native microsomes.* In ^{32}P-binding experiments on crab nerve microsomes in 25 μM ATP, SKOU[4] found that the available ATP was almost completely hydrolyzed in 50 sec at 37°. During this hydrolysis, the $(Mg^{2+} + Na^+)$-dependent ^{32}P labeling of protein increased, reached a maximum in 10–20 sec and then decreased during the next 30 sec. We decided to design parallel experiments on ^{32}P, labeling as a function of time in bladder microsomes at 0° where the phosphoprotein formation could be measured when the extent of ATP hydrolysis was negligible (as shown in Fig. 1).

Native and ouabain-treated microsomes were incubated with [^{32}P]ATP. The amount of ^{32}P labeling of protein and the amount of $^{32}P_i$ liberated were determined in each incubation flask. Since subsequent results on overall hydrolysis (P_i liberated) at 0° were essentially the same as those shown in Fig. 1, they will not be displayed with the subsequent results on ^{32}P labeling.

Fig. 2 is a plot of values of ^{32}P-labeled microsomal protein formed as a function of time of incubation at 0° in one of four similar experiments on the native enzyme system.

The time-dependent pattern of the $(Mg^{2+} + Na^+)$-dependent labeling of microsomal proteins of the turtle bladder was qualitatively similar to that of $(Mg^{2+} + Na^+)$-dependent labeling of microsomes from crab nerve[4] and electric organ[23], as well as to that of Mg^{2+}-dependent labeling of the electric organ[23,32]. On the other hand, the time-

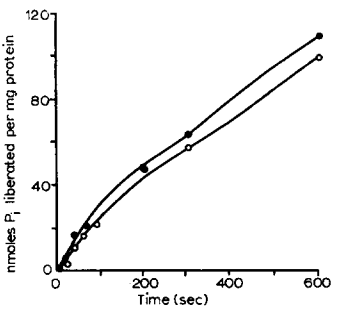

Fig. 1. Hydrolysis at 0° in the absence of K^+. Amount of $^{32}P_i$ released *versus* time of incubating of native microsomal proteins with [^{32}P]ATP. ●—●, P_i release in the presence of Mg^{2+} alone; ○—○, P_i release in the presence of $Mg^{2+} + Na^+$. Apart from the absence of K^+, the remaining constituents and volume in each incubation flask was the same as has been described for Table I.

dependent pattern of the Mg^{2+}-dependent labeling in turtle bladder differed from those of Mg^{2+}-dependent labeling of crab nerve microsomes[4] and kidney microsomes[3].

(b) *Ouabain pre-treated microsomes.* Fig. 3 was constructed from the results of one of four experiments on ouabain treated microsomal proteins incubated at 0° with [^{32}P]ATP. Data presented in the two plots are values of [^{32}P]ATP-labeled protein as a function of time of incubation.

The (Mg^{2+} + Na^+)-dependent labeling was greater than the Mg^{2+}-dependent labeling of the ouabain treated enzyme system throughout the 300 sec of incubation, a pattern which was qualitatively similar to that shown in Fig. 2. The ^{32}P labeling under both conditions (Mg^{2+} alone and Mg^{2+} + Na^+) increased, reached maximal levels at 100 and 160 sec, respectively, and then decreased. The time required to reach maximal levels of ^{32}P labeling in the presence of Mg^{2+} alone in the ouabain treated system was the same as that in the native enzyme system, *ca.* 100 sec. However, the time required for maximal ^{32}P labeling in the presence of Mg^{2+} + Na^+ in the ouabain treated system, 160 sec, was greater than that in the native system, 60 sec.

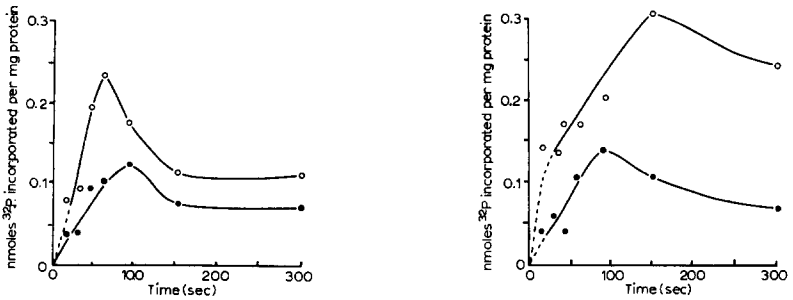

Fig. 2. Amount of ^{32}P labeling of native microsomal proteins *versus* time of incubation with [^{32}P]-ATP at 0°. ●—●, labeling in the presence of Mg^{2+} alone; ○—○, labeling in the presence of Mg^{2+} + Na^+. Final concentrations of each constituent of the incubation mixture was: 85 mM Na^+, 15 mM K^+, 3 mM Mg^{2+}, 3 mM [γ-^{32}P]ATP (specific activity $1 \cdot 10^9$ counts/min per μmole), 0.1 mM Tris–EDTA and 1 mM ouabain. Final volume, 100 μl; temperature, 0°. Reaction was terminated by adding 100 μl 10% (w/v) cold $HClO_4$ (final concentration, 5%). Each value of ^{32}P incorporated has been corrected for that incorporated by control microsomes which had been "killed" by $HClO_4$ just prior to the incubation. The control or non-specific labeling amounted to 0.07 nmole/mg protein.

Fig. 3. Amount of ^{32}P labeling of ouabain treated microsomal proteins *versus* time of incubation with [^{32}P]ATP at 0°. ●—●, labeling in the presence of Mg^{2+} alone; ○—○, labeling in the presence of Mg^{2+} + Na^+. Except for ouabain, the composition and volume of all incubation mixtures were the same as those described for Fig. 3.

The post-maximal decrease in ^{32}P labeling of the ouabain treated system was slower than that of the native system, a retardation which was more obvious in the presence of Mg^{2+} + Na^+ than in the presence of Mg^{2+} alone.

The effects of pre-treatment of the microsomal fraction with ouabain on the (Mg^{2+} + Na^+)-dependent ^{32}P labeling of the protein were: first, a "ouabain-shift", *i.e.* a prolongation of the time required to reach maximal levels of labeling; and second, a retardation in the rate of delabeling of the phosphoprotein. Neither one of these effects was found in the Mg^{2+}-dependent labeling or delabeling of the native proteins.

P_i binding

Presumably, the ^{32}P label on the protein comes mainly from [^{32}P]ATP. To test for the extent of $^{32}P_i$ binding, $^{32}P_i$, instead of ATP, was used in the incubation.

The mean values and standard errors of P_i binding to the native and ouabain treated microsomal proteins in four experiments were as follows: 0.002 ± 0.002 (Mg^{2+} alone) and 0.001 ± 0.001 (Mg^{2+} + Na^+) (nmole/mg protein per 50 sec) to the native protein and 0.007 ± 0.002 (Mg^{2+} alone) and 0.007 ± 0.002 to the ouabain treated protein. The values were of the order of 1–3 % of the corresponding amounts of the P binding from ATP. The extent of P_i binding was greater in the ouabain treated than in the native protein a finding in accord with that recently reported by SIEGEL *et al.*[33].

^{14}C labeling from [^{14}C]ATP

The next set of experiments, concerning the labeling of microsomal proteins from [^{14}C]ATP, was undertaken to obtain an independent check on the data of ^{32}P labeling and to seek evidence for the presence of an enzyme–ATP complex in the microsomal proteins. Accordingly, [^{14}C]ATP, instead of [^{32}P]ATP, was incubated with the microsomal pellet under the standard conditions described in METHODS.

Table II presents mean values for the absolute and relative labeling of native microsomal protein with ^{14}C after 50 sec of incubation with [^{14}C]ATP at 0° in six preliminary experiments.

Without exception, the addition of Na^+, to the Mg^{2+}-containing incubation mixture resulted in a clean-cut decrease in the Mg^{2+}-dependent ^{14}C labeling (from 0.72 to 0.39 nmole/mg per 50 sec) of the protein. The 46 % decrease of ^{14}C labeling in going from the Mg^{2+}-containing to the (Mg^{2+} + Na^+)-containing incubation mixture was in striking contrast to the 158 % increase of ^{32}P labeling of the protein under the same conditions of incubation (see Table III).

The findings on ^{14}C and ^{32}P labeling of the microsomal proteins are consistent with the concept that the first intermediate between the enzyme protein (E) and substrate (ATP) is E–ATP (measured as the ^{14}C-labeled protein). Na^+-induced acceleration of the intermediate steps in the hydrolysis could then result in an increased rate of accumulation of $E \sim P$ (measured as an increase in ^{32}P labeling of the protein) at the expense of the accumulation, but not the turnover rate of E–ATP.

Verification of the findings on ^{14}C and ^{32}P labeling was done as follows: (1)

TABLE II

^{14}C LABELING OF NATIVE MICROSOMAL PROTEINS INCUBATED WITH [^{14}C]ATP

Constituents (other than [^{32}P]ATP) and volume of the incubation mixtures were the same as has been described for Table I. [^{14}C]ATP concn., 1 mM.

Conditions for labeling	*Amount of [^{14}C]ATP binding**	
	Absolute (nmole/mg protein per 50 sec)	*Relative to Mg^{2+}*
Mg^{2+}	0.72 ± 0.22	1.00
Mg^{2+} + Na^+	0.39 ± 0.12	0.54
Statistical parameters	0.34 ± 0.11; $P < 0.02$ ($n = 6$)	

* Mean ± S.E. of individual paired differences, $(Mg^{2+})_1 - (Mg^{2+} + Na^+)_1$.

[^{32}P]ATP and [^{14}C]ATP were incubated together with cold 1 mM ATP, in one flask with the microsomal mixture described in METHODS and used throughout this work. Preliminary data from two experiments showed that the Mg^{2+}-dependent binding of ^{14}C was greater than that of ^{32}P; that the addition of Na^+ decreased ^{14}C binding (so that the ^{14}C activity ratio, $(Mg^{2+} + Na^+)/(Mg^{2+})$ approx. 0.5) and simultaneously increased ^{32}P binding (so that the ^{32}P activity ratio $(Mg^{2+} + Na^+)/(Mg^{2+})$ approx. 2.2). All three findings with the simultaneously added isotopic labels were about the same as those found with individually added isotopic labels (see Tables I and II). (2) In the next two experiments on ^{14}C labeling of the protein, the reaction was terminated solely by rapid filtration of the incubation mixture. No $HClO_4$ was used to "kill" the reaction in the flask or to wash the microsomal pellet remaining on the filter paper. The ^{14}C labeling pattern of this nearly native microsomal pellet with Mg^{2+} alone and with $Mg^{2+} + Na^+$ was essentially the same in absolute magnitude and in degree of the Na^+-induced decrement as that found in the $HClO_4$ treated microsomal pellet. Apparently the ^{14}C-labeled protein is acid-stable. This acid-stable type of ^{14}C binding was in contradistinction to the acid-labile type of ^{14}C binding of erythrocyte ghosts after incubation with [^{14}C]ATP as reported by HEINZ AND HOFFMAN[34].

Parenthetically, the [^{14}C]ATP, [^{32}P]ATP, and cold ATP were found to be chromatographically identical.

DISCUSSION

^{32}P labeling

The formation of intermediary protein complexes by the native and ouabain treated forms of microsomal ATPase from turtle bladders can be related to transport phenomena in the intact tissue and to ATPase activity in the isolated microsomal pellet. For example, ouabain, a known inhibitor of Na^+ transport in the intact system and of $(Na^+ + K^+)$-ATPase activity in the microsomes, has a 4-fold effect on reactions involving the phosphorylated intermediate (a) It blocks the K^+-induced stripping of ^{32}P from the protein in the presence of $Mg^{2+} + Na^+$; (b) It prolongs the time required for reaching the maximal level of ^{32}P labeling in the presence of $Mg^{2+} + Na^+$ ("ouabain-shift"); (c) It retards the post-maximal rate of the K^+-independent stripping (of ^{32}P) from the protein; (d) The fourth effect (really the absence of an effect) is that the Na^+-dependent increment of ^{32}P labeling of the (ouabain treated) protein was the same as that of the native protein during the first 50 sec of incubation at 0°. Na^+-induced increment was consistent with the found in crab microsomes incubated with [^{32}P]ATP at 37° for more than 40 sec, but was different from that in the same microsomes incubated for less than 40 sec[4], and different from that found at 0° in guinea pig kidneys[3] and electric organ of the eel[22]. As expected, ouabain did not affect the Mg^{2+}-dependent phosphorylation or dephosphorylation of the microsomal proteins.

[^{14}C]ATP labeling

The Na^+-induced decrement of the Mg^{2+}-dependent ^{14}C labeling of protein incubated with [^{14}C]ATP occurs simultaneously with an increment of the Mg^{2+}-dependent ^{32}P labeling of protein incubated with [^{32}P]ATP. These data can be considered as a direct demonstration of the formation and breakdown of a Na^+-sensitive complex

between ATP and microsomal protein, *i.e.* of the formation of a complex of the form, *E*–ATP in the initial step of the $(Na^+ + K^+)$-dependent ATPase reaction.

A tentative scheme, consistent with the present data on Na^+-sensitive isotopic labeling of microsomal protein incubated with either [^{32}P]ATP or with [^{14}C]ATP, is as follows:

$$E + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} E\text{–ATP} \tag{1}$$

$$E\text{–ATP} \overset{Mg^{2+},\ Na^+}{\rightleftharpoons} E \sim P + \text{ADP} \tag{2}$$

As far as we are aware, the patterns of protein labeling with ^{32}P and ^{14}C constitute the first evidence consistent with the existence of a Na^+-sensitive, acid-stable complex between unsplit ATP and the enzyme protein. This Na^+-sensitive complex has been sought for but not found in guinea pig kidney microsomes[3] and in electric organ microsomes[23]. Although HEINZ AND HOFFMAN[34] did find Mg^{2+}-dependent acid-labile [^{14}C]ATP binding in erythrocyte ghosts, they made no mention of any effect of Na^+.

Problems remaining

Further data are needed to elicit the characteristics of the subsequent reactions in the overall hydrolysis, which can be represented by

$$E \sim P \overset{Mg^{2+},\ Na^+}{\longrightarrow} E'\text{–}P \tag{3}$$

$$E'\text{–}P \overset{K^+}{\longrightarrow} E' + P_i \tag{4}$$

$$E' \rightleftharpoons E \tag{5}$$

where data from turtle bladder microsomes, like those from other tissues[3–5,9,23,33], are consistent with Reactions 3–5. In particular, the site of the Na^+ effect, consistent with present data, could be at Reactions 2 or 3, or at both 2 and 3.

Further data are also needed to determine the extent of binding of [^{14}C]ATP to ouabain-treated microsomes and to determine the effect of K^+ on this binding. In the case of binding of [^{14}C]ATP to ouabain treated microsomes, results from preliminary experiments suggest that the Na^+-induced decrement of Mg^{2+}-dependent ^{14}C labeling is still present.

Apart from identifying a reaction sequence, the stoichiometry of the reactions and the nature of the bonds formed and broken are unknown. Whereas, a phosphorylated intermediate has been recently characterized as a glutamyl-γ-phosphate residue in the enzyme[35,36], there is no available information on the identity of the bond or bonds between unsplit ATP and the enzyme.

The 5 chemical equations listed in the scheme are by no means the complete mechanistic picture of the $(Na^+ + K^+)$-dependent ATPase. Nevertheless, they are suggestive of the existence of ATPase coupling to the carrier operation and ion transport as well as to the energy metabolism of the cell.

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