BBA 46067

THE EFFECT OF HYDROXYLAMINE ON MICROSOMAL ATPase

ADIL E. SHAMOO

Institute for Medical Research and Studies and Laboratory of Biophysics, Mount Sinai School of Medicine of The City University of New York, 220 East 23rd Street, New York, N.Y. 10010 (U.S.A.)

(Received August 31st, 1970)

SUMMARY

The ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase, but not the Mg^{2+}-ATPase, is irreversibly inhibited when turtle bladder microsomes were incubated with hydroxylamine.

The Mg^{2+}-dependent or the ($Mg^{2+} + Na^+$)-dependent phosphorylation of ADP by the phospho-protein (the exchange reaction) is reversibly inhibited when the microsomes are incubated with hydroxylamine.

The Na^+-induced increment of ^{32}P-labelling of microsomes previously incubated with [γ-^{32}P]ATP is completely eliminated by hydroxylamine, but the Mg^{2+}-dependent ^{32}P-labelling of such microsomes is unaffected by hydroxylamine.

It is concluded that the phospho-enzyme formed during the Mg^{2+}-dependent hydrolysis does not contribute to the Mg^{2+}-dependent exchange reaction. Instead, the phosphoenzyme formed during the ($Na^+ + K^+$)-stimulated hydrolysis is apparently the only substance which phosphorylates ADP in the exchange reaction, even in the absence of Na^+ and/or K^+.

The hydroxylamine-sensitive nature of the sodium form of the phospho-enzyme in the ($Na^+ + K^+$)-stimulated ATPase sequence is consistent with the existence of an enzyme-acyl-phosphate bond of high internal energy with respect to that of ADP.

On the other hand, the hydroxylamine-resistant nature of the phospho-enzyme in the Mg^{2+}-ATPase sequence suggests the existence of a non-acyl type of enzyme phosphate bond with low internal energy relative to that of ADP.

INTRODUCTION

A phospho-protein complex, capable of phosphorylating ATP is formed during the ATPase catalyzed hydrolysis of ATP (refs. 1–7). This concept has been documented by data on ^{32}P binding to microsomes incubated with [γ-^{32}P]ATP (refs. 2–5); and by data on the phosphorylation of ADP by $E \sim P$ (*i.e.*, the exchange reaction) originating from the reaction between the microsomal enzyme and ATP (refs. 7–9). Two forms of this phosphoprotein (denoted $E \sim P$ for Mg^{2+}-dependent ATPase and $E' \sim P$ for the ($Na^+ + K^+$)-stimulated ATPase), each of which contributes to the exchange reaction, have been adduced on the basis of data from the turtle bladder[6,7]. If the two forms, $E \sim P$ and $E' \sim P$, were identical enzyme acyl phosphates, the presence of

hydroxylamine should: (a) block both the Mg^{2+}-dependent and the ($Mg^{2+}+Na^{+}+K^{+}$)-activated hydrolysis; (b) block both the Mg^{2+}-dependent and the ($Mg^{2+}+Na^{+}+K^{+}$)-activated exchange reactions; and (c) result in the release of ^{32}P from the acid stable ^{32}P-labelled protein which had been previously incubated with [γ-^{32}P]ATP (refs. 10–14). This is because the enzyme acyl phosphate reacts with hydroxylamine as follows:

$$E\text{–}C(=O)\text{–}O\text{–}P + NH_2OH \rightarrow E\text{–}C(=O)\text{–}NHOH + P_i,$$

where the acyl bond is transferred to hydroxylamine forming an enzyme hydroxamate and inorganic phosphate[15–19].

Data to be reported herein indicate that: (1) hydroxylamine inhibited completely the ($Na^{+}+K^{+}$)-stimulated increment of Mg^{2+}-activated ATPase, but not the Mg^{2+}-activated ATPase activity; (2) hydroxylamine, when present in the incubation medium, blocked completely both exchange reactions—namely, that due to "pure" Mg^{2+}-dependent ATPase and that due to ($Na^{+}+K^{+}$)-stimulated Mg^{2+}-ATPase; and (3) hydroxylamine treatment of precipitated ^{32}P-labelled phosphoprotein removed all of the bound ^{32}P due to the ($Na^{+}+K^{+}$)-stimulated ATPase.

The fact that the Mg^{2+}-dependent hydrolysis proceeded in the absence of any detectable Mg^{2+}-dependent exchange activity (in the presence of hydroxylamine) is a new finding which suggests that the Mg^{2+}-dependent exchange, when present, is not necessarily coupled to the Mg^{2+}-dependent ATPase activity.

METHODS

Materials

Tris-salt of ATP from Sigma Chemical Co., St. Louis, Mo., hydroxylamine-HCl from Eastman Kodak Co., Rochester, New York. [γ-^{32}P]ATP as the ammonium salt and [8-^{14}C]ADP from International Chemical and Nuclear Corporation, Calif. Microsomal fractions, isolated from mucosal epithelial cells removed from the urinary bladders of fresh water turtles (*Pseudemys scripta*) were prepared as previously described[20] and stored at −30°.

Assay of initial rates of hydrolysis

The final concentrations in the incubation mixture were as follows: 3 mM [γ-^{32}P]ATP (specific activity $1\cdot10^{5}$ counts/min per μmole), 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 40 mM Tris–HCl (pH = 7.3), 0.1 mM EDTA–Tris, hydroxylamine (freshly prepared at neutral pH) at the indicated concentration and 10 μg of microsomal proteins in a final volume of 100 μl.

The rate of P_i release, determined as previously described[6], was referred to the amount of microsomal protein, determined by the method of LOWRY *et al.*[21].

Data control tests showed that in the absence of microsomes, 0.8 M hydroxylamine reacted with 3 mM ATP at pH 7.3 in a volume of 100 μl to form P_i at the rate of 0.03 μmoles/h. Under the same conditions of no microsomes, the incubation of hydroxylamine with ADP yielded no detectable P_i.

Assay of exchange rate (transphosphorylation reaction)

The final concentrations in the incubation mixture were as follows: 5 mM Tris–ATP, 0.5 mM $MgCl_2$, 85 mM NaCl, 1.6 mM [^{14}C]ADP (specific activity $1\cdot10^{6}$ counts/min per μmole), 40 mM Tris–HCl (pH = 7.3), 0.1 mM EDTA–Tris, hydroxylamine

(freshly prepared at neutral pH) in the indicated concentration and about 5 μg of microsomal protein in a total volume of 30 μl. The amount of [^{14}C]ATP formed was estimated as previously described[7]. The exchange rate was expressed in terms of the [^{14}C]ATP formed as a percentage of the total [^{14}C]ADP present initially.

Assay of ^{32}P-labelling of microsomal proteins

Microsomal proteins, 0.04–0.20 mg, were incubated in an icebath for 50 sec. The incubation mixture contained 1 mM [γ-^{32}P]ATP (specific activity $1 \cdot 10^4$ counts/min per nmole), 85 mM NaCl (when indicated), 3 mM $MgCl_2$, 40 mM Tris–HCl (pH = 7.3) as buffer, 0.1 mM Tris–EDTA, hydroxylamine in concentrations up to 0.8 M as indicated, in a final volume of 100 μl. $HClO_4$, 100 μl of a 10 % (w/v) solution was added, after which the mixture was carried through the binding procedure previously described[3].

RESULTS

Effect of hydroxylamine on ATPase

Table I presents a summary of results derived from measurements of the hydrolytic activity of the native and of the hydroxylamine-treated microsomal pellet in the presence of the cations indicated in the first column.

TABLE I

ATPase activity of native and hydroxylamine-treated microsomes at 38°. The activity of the Mg^{2+}-dependent ATPase in the absence of Na^+ and K^+, 12 μmoles/mg protein per h was taken as 100 %. Each tube contained approx. 10 μg of microsomal protein in a native or hydroxylamine-treated system. Final concentration of each constituent of the incubation mixture was: 40 mM Tris–HCl, 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 3 mM [γ-^{32}P]ATP (specific activity $1 \cdot 10^5$ counts/min per μmole), 0.1 mM Tris–EDTA and 0.8 M hydroxylamine. Final volume, 100 μl, final pH 7.3, temperature 0°, time of incubation 10 min. Before the reaction was triggered by the addition of [γ-^{32}P]ATP, the incubation mixture was incubated for 10 min at 38° as a pre-treatment. After the reaction had been incubated for 10 min with [γ-^{32}P]ATP, the reaction was terminated by adding 25 μl of 25 % (w/v) cold $HClO_4$. Four experiments were performed on two different batches of microsomes; for each condition, the activity was estimated from three replicate determinations.

Experimental conditions	*ATPase activity (Mg^{2+} = 100 %)*	
	Native	*Hydroxylamine*
Mg^{2+}	100 ± 4	92 ± 4
Mg^{2+} + Na^+ + K^+	210 ± 5	93 ± 5

For the native protein, the ($Na^+ + K^+$)-stimulation of the Mg^{2+}-ATPase activity was similar to that previously reported[6]. No such stimulation occurred after the addition of either Na^+ or K^+ alone. The activity of the Mg^{2+}-dependent ATPase in the absence of Na^+ and K^+, 12 μmoles/mg protein per h was taken as 100 %.

For the hydroxylamine-treated enzyme, the Mg^{2+}-dependent ATPase activity was not markedly different from the Mg^{2+}-dependent ATPase activity of the native enzyme. The ($Na^+ + K^+$)-induced stimulation, characteristic of the native enzyme, did not occur in the hydroxylamine-treated enzyme—a finding in harmony with recent work on various tissues showing that the presence of hydroxylamine apparently inhibits completely the ($Na^+ + K^+$)-dependent moiety of the enzyme[13–15].

Thus, hydroxylamine completely blocks the $(Na^+ + K^+)$-stimulated Mg^{2+}-dependent ATPase at some unspecified intermediate step, without any concomitant effect on the Mg^{2+}-dependent ATPase activity.

Effect of hydroxylamine on exchange rate (transphosphorylation)

Table II, a summary of results on different batches of microsomes, presents values of relative activity of the exchange rate for native and for hydroxylamine-treated enzyme mixtures. Values of the exchange rate are expressed as percentages of the native Mg^{2+}-dependent exchange activity (average value = 10 μmoles/mg protein per h).

TABLE II

Exchange rate (transphosphorylation) of native and hydroxylamine-treated microsomes at 38°. The exchange rate is expressed as percentage of the native Mg^{2+}-dependent exchange activity (average value = 10 μmoles/mg protein per h). Each tube contained approx. 2 μg of microsomal protein in a native or hydroxylamine-treated system. Final concentration of each constituent of the incubation mixture was: 40 mM Tris–HCl, 85 mM NaCl, 0.5 mM $MgCl_2$, 5 mM Tris–ATP, 1.6 mM [^{14}C]ADP (specific activity $1 \cdot 10^6$ counts/min per μmole), 0.1 mM EDTA–Tris, and 0.1 M hydroxylamine, final pH = 7.3 and final volume 30 μl, before the triggering of the reaction with ATP and [^{14}C]ADP, the mixture was incubated at 38° for 10 min. Then the reaction was carried out at 38° for 10 min. The reaction was terminated by adding to the mixture 5 μl of 30% formic acid in ethanol. Six experiments were performed on three batches of microsomes. For each condition, the activity was estimated from three replicate determinations.

Experimental conditions	*Exchange rate*	
	Native	*Hydroxylamine (0.1 M)*
Mg^{2+}	100 ± 5	2 ± 2
Mg^{2+} + Na^+	105 ± 5	5 ± 3

In the native microsomal pellet, the Mg^{2+}, and the $(Mg^{2+} + Na^+)$-dependent exchange activities were similar to those previously published from this laboratory[7].

In the hydroxylamine-treated microsomal pellet, the Mg^{2+}-dependent exchange activity was 2% of the Mg^{2+}-dependent exchange rate of the native enzyme ($P < 0.001$), and the $(Mg^{2+} + Na^+)$-dependent exchange rate was 5% of the Mg^{2+}-dependent exchange rate of the native enzyme ($P < 0.001$). Thus, the presence of hydroxylamine almost completely blocked the Mg^{2+}-dependent exchange activity in presence or absence of Na^+.

Next, the effect of different concentrations of hydroxylamine on ATPase and exchange activities was studied to determine the nature of hydroxylamine interaction with the enzyme system.

ATPase activity versus hydroxylamine concentration

Fig. 1 is a plot of mean values ± S.E. of the relative activity of ATPase as a function of the logarithm of hydroxylamine concentration in six experiments, each on a separate batch of microsomes. The Mg^{2+}-dependent ATPase in the presence of hydroxylamine is expressed as percentage of the Mg^{2+}-dependent ATPase of the native

enzyme. The $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase in the presence of hydroxylamine is expressed as percentage of the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase of the native enzyme.

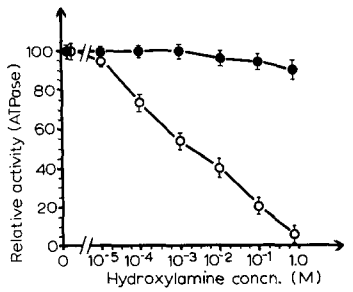

Fig. 1. The effect of hydroxylamine on ATPase activity. The constituent and the procedure is a described for Table I and METHODS. The values presented are the mean ± S.E. from six different experiments on six batches of microsomes. ●—●, Mg^{2+}-ATPase; ○—○, $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase.

Hydroxylamine, over a wide range of concentrations, did not appreciably affect the Mg^{2+}-dependent ATPase, but did inhibit the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase in a nearly linear manner with respect to the log of hydroxylamine concentration. The $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase was nearly zero at a hydroxylamine concentration of 0.8 M.

Effect of osmolality and ionic strength on ATPase activity

The next experiments were designed to compare the effects on ATPase activity of osmolality (by addition of equi-osmolal amounts of sucrose) and of ionic strength (by addition of equi-molar amounts of choline HCl and Tris–HCl) with that of hydroxylamine.

In six experiments, the mean level of the Mg^{2+}-dependent ATPase activity decreased to 80 % of its control level, while that of the $(Na^+ + K^+)$-stimulated activity decreased to 50 % of its control level after the sucrose concentration was raised to 0.8 M. The results on sucrose were similar to those of others[22].

In four other experiments, the mean level of Mg^{2+}-dependent ATPase activity decreased to 85 % of its control level, while that of the $(Na^+ + K^+)$-stimulated activity decreased to 50 % of its control level after the choline HCl concentration was raised to 0.8 M.

These results suggest that part, but not all of the inhibition from hydroxylamine could have been due to the increased osmolality and for the increased ionic strength of the incubation medium.

Exchange activity versus hydroxylamine concentrations

Fig. 2 is a plot of mean values ± S.E. of relative exchange activity as a function of the logarithm of the hydroxylamine concentration in three experiments, each on a separate batch of microsomes.

Both the Mg^{2+}-dependent and the $(Mg^{2+} + Na^+)$-dependent exchange rates

decreased with increasing concentrations of hydroxylamine; and reached near-zero levels when the concentration of hydroxylamine was 0.8 M. Apparently the Mg^{2+}-dependent exchange rate decreased more rapidly than did the ($Mg^{2+} + Na^+$)-dependent rate as the concentration of hydroxylamine was increased.

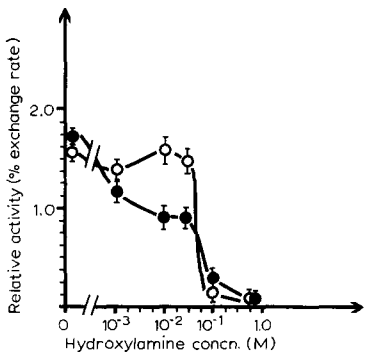

Fig. 2. The effect of hydroxylamine on exchange activity (transphosphorylation). The constituents and the procedure are as described for Table II and METHODS. The values presented are the mean ± S.E. from six different experiments on six batches of microsomes. ●—●, Mg^{2+}; ○—○, $Mg^{2+} + Na^+$.

It is important to note that hydroxylamine, at 0.8 M, inhibits the Mg^{2+}-dependent exchange almost completely without appreciably affecting the Mg^{2+}-dependent ATPase activity. In other words, in the presence of hydroxylamine, the overall Mg^{2+}-dependent hydrolysis occurs in the absence of any detectable exchange reaction.

Effect of sucrose on exchange reaction

Not shown, are control data on the effects of added sucrose (at concentrations isosmotic to those of the added hydroxylamine) which showed that the Mg^{2+}-dependent exchange rate remained at its control level when the added sucrose concentration was 0.4 M; and decreased to 50 % of its control level when the added sucrose concentration was raised to 0.8 M.

In other words, at 0.8 M sucrose, the degree of inhibition of either the Mg^{2+}-dependent or the ($Mg^{2+} + Na^+$)-dependent exchange rate was similar to that of the ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase, but dissimilar to that of the Mg^{2+}-dependent ATPase (which was practically uninhibited) under the same conditions. This fits the notion that the ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase molecule is an integral part of the Mg^{2+}-dependent exchange enzyme—even in the absence of Na^+ and/or K^+. From a practical view-point, the inhibitory effect of hydroxylamine (at concentration of 0.4 M) on the exchange reactions cannot be attributed to osmolality.

Pre-treatment of microsomes with hydroxylamine

Microsomal proteins, 2–3 mg, were incubated at 38° with 0.1 M hydroxylamine (pH = 7.3) for 10 min. Then the excess hydroxylamine was removed by re-centrifuging the microsomes at 65000 × *g* for 1 h and discarding the supernatant. This suspension was used as the source of microsomes in the following set of experiments,

which involved the incubation of a hydroxylamine-treated enzyme mixture in the absence of hydroxylamine.

Table III presents a summary of results on the ATPase activity and the exchange rate of such a hydroxylamine-pre-treated enzyme under the ionic conditions designated in the first column.

TABLE III

ATPase and exchange activities of microsomes previously treated with 0.1 M hydroxylamine. The values of activity are expressed in terms of μmoles/mg protein per h. Microsomes were incubated with 0.1 M hydroxylamine for 10 min at 38°. Afterwards, the excess of hydroxylamine was removed by centrifugation, then the microsomal pellet was resuspended in 1 mM EDTA. These microsomes were used as a source of enzyme. The assay procedure for ATPase and exchange activities was carried out according to that in METHODS. Four experiments were performed on two different batches of microsomes. For each condition, the activity was estimated from three replicate determinations.

Experimental conditions	*ATPase (μmoles/mg protein per h)*	*Exchange rate (% exchange)*
Mg^{2+}	5.0 ± 0.5	12.0 ± 2.0
$Mg^{2+} + Na^+$	—	11.0 ± 2.0
$Mg^{2+} + Na^+ + K^+$	5.1 ± 0.5	—

ATPase. The mean value of the Mg^{2+}-dependent ATPase activity of the hydroxylamine-treated microsomes was 5 μmoles/mg protein per h. The $(Na^+ + K^+)$-stimulated part of ATPase was abolished ($P<0.001$) in the hydroxylamine-treated microsomes just as it was in the microsomes incubated in the presence of hydroxylamine. The results shown in Tables I and III and Fig. 1 suggest that hydroxylamine treatment of the enzyme produces an irreversible inhibition of the $(Na^+ + K^+)$-ATPase without inducing any change (not even a reversible one) in the Mg^{2+}-dependent ATPase.

Exchange rate. When the hydroxylamine to which the enzyme had been exposed was removed from the incubation medium, neither the Mg^{2+} nor the $(Mg^{2+} + Na^+)$-dependent exchange activity was abolished. This contrasts sharply with the blockade of such activity when the enzyme was treated with and incubated in the presence of hydroxylamine. Thus, hydroxylamine induces a reversible blockage of the exchange reactions, because its presence guarantees the cleavage of the phosphorylated intermediate as soon as it is formed, thereby eliminating the exchange reaction. The subsequent removal of hydroxylamine allows for the formation of the phosphorylated intermediate (even in this pretreated enzyme with no detectable $(Na^+ + K^+)$-activity) and thus the exchange reaction is restored.

Effect of hydroxylamine on ^{32}P-binding

Table IV presents mean values ± S.E. on the formation of ^{32}P-labelled phosphoprotein in native and hydroxylamine-treated microsomes after incubation at 0° for 50 sec under the ionic conditions designated in the first column. In these experiments,

TABLE IV

^{32}P-Binding of native and hydroxylamine-treated microsomes at 0°. The values of ^{32}P-binding are expressed in terms of nmoles/mg protein. Each tube contained approx. 0.1–0.2 μg of microsomal protein in a native or hydroxylamine-treated system. Final concentrations of each constituent are those described in METHODS, that of hydroxylamine 0.8 M. Before the triggering of the reaction with [γ-^{32}P]ATP, the mixture was incubated for 10 min at 38°. The rest of the procedure is as described in METHODS. Four experiments were performed on two different batches of microsomes. For each condition, the activity was estimated from three replicate determinations.

Experimental conditions	^{32}*P-binding (nmole/mg protein)*	
	Native	*Hydroxylamine*
Mg^{2+}	0.6 ± 0.1	0.2 ± 0.1
Mg^{2+} + Na^+	1.6 ± 0.2	0.1 ± 0.1

the microsomes were incubated for 10 min at 38° with or without 0.8 M hydroxylamine prior to starting the reaction by the addition of [γ-^{32}P]ATP.

In the native microsomes, the addition of Na^+ increased the ^{32}P-binding from 0.6 to 1.6 nmole/mg protein in 50 sec ($P<0.02$), in confirmation of what had been reported previously from this laboratory[6]. The presence of hydroxylamine decreased the Mg^{2+}-dependent ^{32}P-binding from 0.6 to 0.2 nmole/mg protein per 50 sec, a decrease which was not statistically significant ($P<0.1$). Addition of Na^+ to the hydroxylamine-treated microsomes failed to cause a significant change in ^{32}P-binding ($P>0.9$) in 4 experiments. These data fit the idea that the presence of hydroxylamine prevents the Na^+-stimulated phosphorylation of the enzyme by cleaving the phosphoprotein, $E\sim P$ as soon as it is generated with the resultant formation of an enzyme-hydroxamate and inorganic phosphate.

The next set of experiments was designed to show the effect of 0.8 M hydroxylamine on the ^{32}P-labelled phosphoprotein after acid precipitation. The technique used was that described in METHODS, except that the microsomes were incubated with approx. 0.01 mM ATP (instead of 1.0 mM). This change in substrate concentration was used for the purpose of increasing the specific activity of the [γ-^{32}P]ATP. 0.8 M hydroxylamine was then added to one aliquot of the precipitate and water to the other, after which both aliquots were incubated for 10 min at 38°. Then the amount of ^{32}P-binding to the microsomal precipitates was determined in the manner described previously[3].

Table V presents values for ^{32}P-labelling of the acid-precipitated microsomes (which had been incubated with [^{32}P]ATP at 0° for 50 sec under the ionic conditions designated in the first column) after their reaction for 10 min at 38° with water; and after their reaction for 10 min at 38° with hydroxylamine.

The values for ^{32}P-binding to native (water-treated) microsomal precipitates, similar to those shown in Table IV, were not different from those found in the conventional ^{32}P-binding experiments under the designated ionic conditions. The amount of ^{32}P-labelling of the precipitate formed from the Mg^{2+}-dependent phosphorylation was changed little if any after incubation of that precipitate with hydroxylamine (compare the values in the first and third rows). In contrast, the amount of ^{32}P-labelling of the precipitate formed from the (Mg^{2+} + Na^+)-induced phosphorylation was

TABLE V

^{32}P-Binding (in counts/min) of native microsomes at 0°. The procedure is that described in METHODS. The final precipitate (microsomal pellet-^{32}P) was obtained, two batches, one to which 0.8 M hydroxylamine was added, the other, to which water was added. Both batches were incubated at 38° for 10 min, after which centrifugation and washing was performed four times, then the precipitate was removed for counting. Each experiment was performed in four replicates. *P*-values shown denote probabilities that the mean differences designated [(a−b) for ionic conditions and (N−H) for native *vs.* treated enzyme] are zero.

Expt. No.	*Ionic conditions*	*Amount of ^{32}P-binding (raw counts/min)*		*P (N−H)*
		Native (N)	*Hydroxylamine (H)*	
1. (a)	Mg^{2+}	8623 ± 883	6234 ± 842	>0.1
(b)	Mg^{2+} + Na^+	12980 ± 866	6677 ± 72	<0.01
	P (a−b)	<0.05	>0.6	—
2. (a)	Mg^{2+}	13305 ± 763	14509 ± 723	>0.3
(b)	Mg^{2+} + Na^+	25126 ± 1683	12641 ± 126	<0.01
	P (a−b)	<0.01	>0.1	—

reduced by about 50 % after incubation of that precipitate with hydroxylamine (compare the values in the second and fourth rows of the table). The data show clearly that practically all of the Na^+-induced increment of ^{32}P-labelling was stripped from these microsomal precipitates after their exposure to hydroxylamine.

The data suggest that the Mg^{2+}-dependent ^{32}P-binding cannot be identified with a hydroxylamine-sensitive acyl bond. This contention is fully consistent with the lack of effect of hydroxylamine on the overall Mg^{2+}-dependent hydrolysis previously mentioned. However, it does not appear to agree with the strong inhibitory effect of hydroxylamine on the Mg^{2+}-dependent exchange reaction. Either the Mg^{2+}-dependent exchange reaction is totally independent of Mg^{2+}-dependent ATPase activity as well as of the Mg^{2+}-dependent phosphorylation; or the Mg^{2+}-dependent exchange reaction is independent of any reaction step in the overall ATPase reaction.

The data obtained after addition of Na^+ or $Na^+ + K^+$ suggest that the Na^+-induced increment of ^{32}P-binding, the Mg^{2+} and the ($Mg^{2+} + Na^+$)-dependent exchange reactions, and the ($Na^+ + K^+$)-stimulated increment of ATPase activity are all identifiable with a hydroxylamine-sensitive acyl bond such as the γ-glutamyl residue[11,12] in the protein.

DISCUSSION

The reaction model for ATPase activity proposed by us[6,7] differs from that proposed by others[5,25] insofar as the formation of $E \sim ATP$ prior to that of $E \sim P$ is explicitly stated and the reaction sequence diverges into two branches, Mg^{2+}-ATPase and ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase[6,7]. One branch which requires Mg^{2+} alone, had been represented by the equations:

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

$$E \sim P \rightarrow E + P_i$$

and the other branch, which represents the $(Na^+ + K^+)$-stimulated Mg^{2+}-dependent ATPase and which requires Mg^{2+}, Na^+ and K^+, had been represented by the equations

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

$$E' \sim P \xrightarrow{Mg^{2+},\ Na^+} E\text{–}P$$

$$E'\text{–}P \overset{K^+}{\rightarrow} E' + P_i$$

$$E' \rightleftharpoons E,$$

where E' denotes the Na^+-form of the enzyme and E, the Mg^{2+}-form.

In short, the model included the existence of $E \sim P$ (a high energy phosphoprotein) as an intermediary in the overall reaction for Mg^{2+}-dependent ATPase, and required that this Mg^{2+}-dependent $E \sim P$ phosphorylates ADP, thus contributing to the Mg^{2+}-dependent exchange activity.

Present data clearly indicate that some of these ideas require modification. For example, after addition of hydroxylamine, the overall Mg^{2+}-dependent ATPase activity is still present while there is no detectable Mg^{2+}-dependent exchange activity. This means that the intermediary phosphoprotein formed in the Mg^{2+}-ATPase reaction does not phosphorylate ADP, and consequently that there is no evidence (at least none in the turtle bladder) for endowing it with properties of a high energy bond—*i.e.*, high energy relative to that required for phosphorylating ADP. Therefore, a phosphoprotein other than that formed in the Mg^{2+}-ATPase sequence must be implicated as the substance which phosphorylates ADP in the Mg^{2+}-dependent exchange reaction. In this connection, the reaction model previously postulated for $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase[7] did require that the high energy phosphate formed after the first two intermediary steps ($E \sim P$) would phosphorylate ADP even in the absence of Na^+ and/or K^+. This requirement is satisfied by: previously reported data showing that sodium can completely inhibit the Mg^{2+}-dependent exchange reaction[7]; current data on hydroxylamine and sucrose; and by preliminary data showing that NEM (10^{-2} M), like hydroxylamine, completely inhibits the Mg^{2+}-dependent exchange reaction as well as the $(Na^+ + K^+)$-stimulated moiety of ATPase activity without affecting the Mg^{2+}-ATPase activity (A. E. SHAMOO AND W. A. BRODSKY, unpublished data).

Apart from the transphosphorylating role of $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase as adduced above, it is possible that the Mg^{2+}-dependent exchange reaction is catalyzed by a di-nucleotide kinase which is independent of the $(Na^+ + K^+)$-ATPase. Along these lines, FAHN *et al.*[8,19,23], STAHL *et al.*[18,24] reported a Na^+-dependent exchange reaction in microsomes from electric organ, cerebral tissue and nerve endings. They concluded that the part of the exchange reaction which is not stimulated by Na^+ is extraneous to the $(Na^+ + K^+)$-stimulated ATPase, and could be due to an Mg^{2+}-dependent dinucleotide kinase. Although the possibility of two separate exchange enzymes cannot be rigorously excluded, there is no conclusive evidence showing that one of these enzymes is separable from the other in turtle bladder microsomes. In fact, in the case of the turtle bladder, the data suggest that the di-

nucleotide kinase (Mg^{2+}-dependent exchange enzyme) is the same entity as, or is an integral part of the ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase. For example, the Mg^{2+}-dependent exchange reaction is stimulated by ouabain[7], a known inhibitor of ($Na^+ + K^+$)-stimulated, Mg^{2+}-ATPase; and the Mg^{2+}-dependent exchange reaction is completely inhibited in the presence of hydroxylamine, sucrose, and NEM; all of which inhibit ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase and not Mg^{2+}-ATPase (see RESULTS).

Present data on phosphorylation of the enzyme show that Mg^{2+}-dependent ^{32}P-binding to the enzyme is reduced to a small extent by hydroxylamine, whereas the ($Mg^{2+} + Na^+$)-dependent ^{32}P-binding is drastically reduced. In fact, all of the Na^+-induced increment of the ^{32}P-binding is eliminated by hydroxylamine.

Present data on the pre-formed phosphoprotein precipitate show that hydroxylamine treatment of the ^{32}P-labelled precipitate (formed from Mg^{2+}-dependent phosphorylation) does not release ^{32}P into the supernatant. However, hydroxylamine treatment does release all of that ^{32}P originating from the Na^+-induced increment of ^{32}P-binding (see Table V). This suggests that the phosphorylated intermediate formed by the Mg^{2+}-dependent ATPase is not an acyl bond type. Moreover, the phosphorylated intermediate of the Mg^{2+}-ATPase cannot be a high energy bond, because in the presence of hydroxylamine, the Mg^{2+}-dependent exchange rate is nil while the Mg^{2+}-ATPase proceeds unabated. Consequently, the pathway for Mg^{2+}-dependent ATPase may be written as follows:

$$E + \text{ATP} \rightarrow E\text{–}P + \text{ADP}$$

$$E\text{–}P \rightarrow E + P_i$$

where the notation E–P instead of $E \sim P$ denotes the relatively low energy level of this phospho-protein bond.

We have previously shown that an E–ATP complex is formed when the enzyme is incubated with [^{14}C]ATP[6]. Thus the ^{32}P-labelled phosphoprotein must include E–ATP as well as $E \sim P$. It follows that hydroxylamine could remove intact ATP as well as P_i from the $E \sim P$ complex. Preliminary data indicate that hydroxylamine does indeed remove ^{14}C-labelled ATP from the ^{14}C-labelled enzyme intermediate suggesting that the bond between ATP and the enzyme is the same as that between phosphate and the enzyme—namely, an acyl phosphate (A.E. SHAMOO AND W. A. BRODSKY, unpublished data).

ACKNOWLEDGMENTS

This study was supported, in part, by the National Institutes of Health Research Grant 13037; in part, by National Science Foundation Grant GB 7764; and in part, by National Aeronautics and Space Administration Research Grant NGR 33-171-001. The authors thank Mrs. Pamela Schiro and Mary Soliman for their technical assistance.

REFERENCES

1 J. C. SKOU, *Biochim. Biophys. Acta*, 42 (1960) 42.
2 J. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
3 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
4 J. C. SKOU, *Phys. Rev.*, 45 (1965) 596.

5 R. W. ALBERS, *Ann. Rev. Biochem.*, 36 (1967) 727.
6 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 484.
7 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 495.
8 S. FAHN, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 241 (1966) 1882.
9 W. L. STAHL, *Arch. Biochem. Biophys.*, 120 (1967) 230.
10 K. NAGANO, T. KANAZAWA, N. MIZUMO, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 759.
11 L. E. HOKIN, P. S. SASTRY, P. R. GALSWORTHY AND A. YODA, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 177.
12 A. KAHLENBERG, P. R. GALSWORTHY AND L. E. HOKIN, *Arch. Biochem. Biophys.*, 126 (1968) 331.
13 C. F. CHIGNELL AND E. TITUS, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1620.
14 W. SCHONER, R. KRAMER AND W. SEUBERT, *Biochem. Biophys. Res. Commun.*, 23 (1966) 403.
15 H. BADER AND A. H. BROOM, *Biochim. Biophys. Acta*, 139 (1967) 517.
16 R. W. ALBERS, S. FAHN AND G. J. KOVAL, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 474.
17 P. D. SWANSON AND W. L. STAHL, *Biochem. J.*, 99 (1966) 396.
18 W. L. STAHL, A. SATTIN AND H. MCILWAIN, *Biochem. J.*, 99 (1966) 404.
19 S. FAHN, M. R. HURLEY, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 241 (1966) 1890.
20 Y. E. SHAMOO AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 111.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
22 J. BOURGOIGNIE, S. KLAHR, J. YATES, L. OVERRA AND N. S. BRICKER, *Am. J. Physiol.*, 217 (1969) 1496.
23 S. FAHN, R. W. ALBERS AND G. J. KOVAL, *Science*, 145 (1964) 283.
24 R. RENDI, *Biochim. Biophys. Acta*, 118 (1966) 621.
25 G. J. SIEGEL AND R. W. ALBERS, *J. Biol. Chem.*, 242 (1967) 4972.