BBA 75550

THE EFFECT OF TEMPERATURE ON Mg^{2+}- AND $(Na^+ + K^+)$-ATPases

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(Received August 14th, 1970)

SUMMARY

1. As the incubation temperature is increased from 38 to 80°, the ATPase activity of native and/or ouabain-treated microsomes of turtle bladder epithelial cells increases, reaches maximal levels, and decreases.

2. The maximal activity occurs at 60–63° in native or ouabain-treated microsomes incubated in the presence of Mg^{2+} alone, $Mg^{2+} + Na^+$ and $Mg^{2+} + K^+$. The maximal activity occurs at 50° in native microsomes incubated in the presence of $Mg^{2+} + Na^+ + K^+$; and at 58° in ouabain-treated microsomes in the presence of $Mg^{2+} + Na^+ + K^+$.

3. The addition of ouabain stimulates ATPase activity at temperatures above 38° in the presence of Mg^{2+}, or in the presence of $Mg^{2+} + Na^+$, or in the presence of $Mg^{2+} + K^+$, but not in the presence of all three. This stimulatory action of ouabain is elicited in aging diluted suspensions, not in freshly extracted, concentrated suspensions of microsomes.

4. Between 38 and 50°, the addition of ouabain partially inhibits the ATPase activity in the presence of $Mg^{2+} + Na^+ + K^+$ (presumably by suppression of the $Na^+ + K^+$ increment of the total activity). Between 55 and 80°, oubain has no apparent effect on ATPase activity in the presence of $Mg^{2+} + Na^+ + K^+$.

5. The addition of either Na^+ or K^+ decreases the Mg^{2+}-dependent activity of the native and ouabain-treated microsomes at temperatures above 38°. This inhibition is independent of the age or degree of dilution of the microsomal suspensions. A qualitatively similar inhibition after the simultaneous addition of Na^+ and K^+ is elicited above 55°.

6. From 38 to 50°, the simultaneous addition of Na^+ and K^+ doubles the Mg^{2+}-dependent activity in native microsomes.

7. ATPase activity in the presence of Mg^{2+} alone was significantly greater than that in the presence of $Mg^{2+} + Na^+ + K^+ +$ ouabain over a wide range of temperatures.

8. The free energy of activation, E^*, and the free energy, ΔF^*, of the ATPase-catalyzed hydrolysis were estimated.

9. An attempt was made to correlate the effects of Na^+, K^+, and ouabain on some of the properties of ATPase with hydrogen bonding.

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INTRODUCTION

The microsomal fraction of epithelial cells from the urinary bladder of fresh water turtles, *Pseudemys scripta*, contains an Mg^{2+}-dependent, ($Na^+ + K^+$)-stimulatable, ouabain-inhibitable ATPase activity[1-3], the properties of which are releated in some way to the mechanism for active Na^- transport in the intact system[1,4]. Recent work has shown the Mg^{2+}-dependency together with a sensitivity to Na^+, K^+, and ouabain in the following reactions: the binding of ^{32}P to microsomes incubated with [γ-^{32}P]ATP; the binding of ^{14}C to microsomes incubated with [^{14}C]ATP; and the rate of the exchange reaction between ADP and ATP (refs 5, 6).

The reaction model invoked to account for the aforementioned data implied that the K^+-dependent step, the final dephosphorylation, was purely exergonic and could be more sensitive to increases in temperature than the rest of the intermediary reaction steps. This postulate further implied that heat might be substituted for K^+ under the conditions of ($Mg^{2+} + Na^+$)-containing incubation mixtures. However, this postulate had to be discarded because it was found that the activity of the ($Mg^{2+} + Na^+$)-containing incubation mixture remained significantly lower than that of the Mg^{2+}-containing mixture as the incubation temperature was increased. Moreover, the addition of ouabain at higher temperatures actually stimulated the Mg^{2+}-dependent ATPase activity. These unexpected findings prompted the initiation of a systematic study of the effect of temperature on native and ouabain-treated microsomes under various ionic conditions.

Apart from the serendipitous nature of the aforementioned findings, there are straightforward reasons for determining the effect of temperature on certain parameters, including enzymatic activity of protein. These reasons, spelled out in detail by KAUZMANN[7], SCHERAGA[8] and others[9,10], pertain to the determination of short-range effects of temperature (*i.e.* effects on the secondary and tertiary structure) in a protein molecule. Because microsomal ATPase is a non-isolated molecule of unknown structure, the enzymatie activity, rather than any equilibrium function of state (specific volume, dissociation constant, *etc.*), is a useful parameter for testing temperature dependency. In short, the reaction rate as a function of temperature, even in an impure protein mixture, can give data on both activation and denaturation of the enzyme molecule; and consequently lead to inferences on the nature of hydrogen and other bonds in or near the active site of the enzyme or the enzyme–substrate complexes. As a matter of fact, the data to be reported herein do indeed show changes in fundamental and specific properties of the ATPase molecule in terms of its thermal activation and denaturation.

MATERIALS AND METHODS

Materials

Tris salt of ATP, Tris salt of *p*-nitrophenylphosphate, and ouabain were obtained from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]ATP, as the ammonium salt, was obtained from ICN (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[2] and kept refrigerated at —30°.

Assay of initial rates of hydrolysis

In the standard assay procedure, the final concentrations in the incubation mixture were as follows: 3 mM [γ-^{32}P]-ATP (specific activity $1 \cdot 10^5$ counts/min per mole), 85 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 40 mM Tris–HCl (pH 7.3 at room temperature) as a buffer, 0.1 mM EDTA–Tris, inhibitors when indicated, and 4 μg of enzyme protein in a final volume at 100 μl. Ouabain, when used, reached a concentration of $1 \cdot 10^{-3}$ M in the incubation mixture.

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

The temperature was maintained constant within ± 0.2° by means of a Dubnoff shaker. The rest of the assay for determining the rate of P_i released, carried out as had been previously described[5], was a simple modification of the BERENBLUM AND CHAIN[11] method.

Age and dilution of microsomes

The time and condition of storage of freshly extracted microsomes influenced the magnitude of certain ouabain-induced effects on enzyme activity at the higher temperatures. For example, these effects occurred reproducibly after 2 days of storage of a diluted enzyme mixture (0.5–0.7 mg/ml) at −30°, but not in the freshly extracted enzyme (incubated within 24 h), and not after storage of a concentrated enzyme mixture (1.0–3.0 mg/ml) at −30° (see RESULTS for further details).

Data to be shown herein were obtained from experiments on aged, diluted microsome mixtures. Not shown, but alluded to, were data obtained from a parallel set of experiments on freshly extracted concentrated microsomal mixtures. The format and technique of assays of activity used, identical in all sets of experiments to be reported, were those described under the previous sub-heading of this section.

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. At ordinary temperatures (25–38°), the ($Na^+ + K^+$)-stimulated part is inhibited by ouabain and the Mg^{2+}-dependent part is not. The ($Na^+ + K^+$)-stimulated ATPase is often called ouabain-sensitive ATPase, and Mg^{2+}-dependent ATPase is often called ouabain-insensitive ATPase.

RESULTS

ATPase activity at 38 and 63°

Table I presents mean values and statistical parameters for the cation-sensitive rate of hydrolysis of ATP of native and of ouabain-treated microsomal proteins. Values shown are normalized with respect to the Mg^{2+}-dependent ATPase activity, *e.g.* in this case, 12 μmoles/mg protein per h was taken as 100 %.

At 38°, in the native microsomes, addition of either Na^+ or K^+ alone resulted in no change of the Mg^{2+}-dependent ATPase activity, as has been reported previously[5] (see first row, Columns B and C). Addition of $Na^+ + K^+$ increased the mean value of the Mg^{2+}-dependent ATPase from 100 to 200 % (see first row, Columns A and D).

TABLE I

EFFECT OF INCUBATION AT 63° ON THE ATPASE ACTIVITY OF TURTLE BLADDER MICROSOMES IN THE PRESENCE OF Mg^{2+} ALONE (A) AND WITH THE IONIC ADDITION INDICATED UNDER B, C, AND D

Each value in the table is the mean value obtained from the data of four experiments, each one of which involved a separate batch of microsomes. In any given experiment, the average value of activity was obtained from four replicate determinations on the microsomal batch. Details concerning the temperature conditions during preliminary incubation and during the course of the reaction as well as those concerning concentrations of substrates, ions, buffers and ouabain can be found in MATERIALS AND METHODS.

Temperature		*State of enzyme*	*Relative activity (Mg^{2+} = 100 %)*			
Preliminary incubation	*Incubation*		*(A)* Mg^{2+}	*(B)* $Mg^{2+} + Na^+$	*(C)* $Mg^{2+} + K^+$	*(D)* $Mg^{2+} + Na^+ + K^+$
38°	38°	Native	100 ± 8	92 ± 8	103 ± 10	200 ± 14
		Ouabain	102 ± 7	90 ± 6	91 ± 5	75 ± 8
38°	63°	Native	306 ± 15	226 ± 12	230 ± 11	214 ± 12
		Ouabain	566 ± 42	340 ± 40	565 ± 24	245 ± 15

At 38° in the ouabain-treated microsomes, addition of either Na^+ or K^+ resulted in no change in the Mg^{2+}-dependent activity, while addition of $Na^+ + K^+$ produced a decrease in this activity (see second row, Columns A, B, C and D).

At 63° in the native microsomal protein, the Mg^{2+}-dependent ATPase activity was increased by 206 % over its activity at 38° (see first and third rows of Column A). Addition of Na^+ (final concentration 85 mM) decreased the Mg^{2+}-dependent ATPase activity from 306 to 226 % ($P < 0.05$). Addition of K^+ (final concentration 15 mM) decreased the Mg^{2+}-dependent ATPase activity from 306 to 230 % ($P < 0.05$). Addition of $Na^+ + K^+$ decreased the Mg^{2+}-dependent ATPase activity from 306 to 214 % ($P < 0.02$).

These results demonstrate that the introduction of heat *via* incubation of the native microsomes at 63° does increase the ATPase activity either in the presence of Mg^{2+} or in the presence of $Mg^{2+} + Na^+$. Moreover, the effect of heat on the activity of the ($Mg^{2+} + Na^+$)-containing mixture is not the same as that of addition of K^+ to the same mixture at 38°. This is because the activity of the ($Mg^{2+} + Na^+$)-containing microsomes at 63° is less than that of Mg^{2+}-containing microsomes at 63°. In fact, Na^+ or K^+ are inhibitors of ATPase at 63°.

It is important to point out that the inhibitory effects of Na^+ and/or K^+ at 63° were elicited in freshly prepared concentrated microsomes, as well as in the aged diluted microsomes from which the data in Table I were derived.

At 63° in the ouabain-treated microsomes, the Mg^{2+}-dependent ATPase activity was increased by 466 % over its native or oubain-treated activity at 38° (see fourth and first rows of Column A). Surprisingly, at 63°, the activity of the ouabain-treated microsomes in the presence of Mg^{2+} alone was greater than that of the native microsomes in the presence of Mg^{2+} alone, *e.g.* 556 *vs.* 306 % ($P < 0.02$). Addition of K^+ resulted in no change of the Mg-dependent activity of the ouabain-treated microsomes. The addition of $Na^+ + K^+$ resulted in a decrease of the Mg^{2+}-dependent ATPase activity from 566 to 245 % ($P < 0.01$).

Comparing the activity of the ouabain-treated with that of the native microsomes at 63° reveals that the addition of ouabain uniformly increased the ATPase activity under all ionic conditions tested, with the possible exception of the activity under conditions of $Mg^{2+} + Na^+ + K^+$ ($P > 0.2$); (see last column, third and fourth rows).

In contrast to the independence of cationic inhibition at 63° from aging or dilution, the stimulatory effects of ouabain at 63° were elicited only in the aged diluted microsomes, data from which are shown in Table I, and not from the freshly prepared and/or more concentrated microsomal mixtures, data from which are not shown.

Effect of aging and dilution on ouabain action

The aforementioned ouabain effects at the higher temperature could not be elicited in a freshly prepared enzyme mixture or in a concentrated enzyme preparation stored at −30° after extraction. For each batch of stored diluted microsomes wherein the ouabain effect was demonstrated (see Figs. 1–4), a parallel experiment on freshly extracted or on stored, concentrated aliquots of the same batch of microsomes failed to reveal any stimulatory effect of ouabain under any one of the corresponding ionic conditions at 63°. Thus, all of the results shown herein were those obtained from a diluted enzyme mixture (0.5–0.7 mg of protein per ml) which had been stored at −30° for two or more days after extraction.

The surprising aspect of the aforementioned results is the clear cut demonstration of a ouabain-stimulated, $(Na^+ + K^+)$-inhibited ATPase activity at 63°. This prompted an investigation into the properties of ATPase as a function of the incubation temperature.

The effect of temperature of incubation on ATPase

The following experiments were designed to test the effect of a fairly wide range of incubation temperatures on the ATPase activity of native and ouabain-treated microsomes. In all cases, the enzyme source and all constituents of the incubation mixture were added at 0°, immediately after which the reaction was initiated by immersion of the incubation flask and mixture in a water bath pre-set at the appropriate temperature.

Fig. 1 presents a plot of mean values ± S.E. of ATPase activity *vs.* temperature of incubation in three batches of microsomes.

The ATPase activity of the ouabain-treated microsomes was significantly greater than that of the paired native microsomes over a temperature range of 45–60°. The maximal increment of ouabain-treated over native enzyme activity, 145%, occurred at 60°. Thus, even in the absence of Na^+ and/or K^+, the activity of the ouabain-treated enzyme was greater than that of the native enzyme over a wide range of incubation temperatures.

The cause of the temperature-dependent, ouabain-induced stimulation is not clear on the basis of these data alone. Such as stimulation might be attributed to a 'detergent' effect or to another kind of interaction between ouabain and the enzyme which seems to mimic $(Na^+ + K^+)$-induced stimulation of the enzyme at lower temperatures.

Fig. 1 also shows steep decreases in both of the Mg^{2+}-dependent activities (native and ouabain-treated) when the incubation temperature is greater than 60°. These

decreases are consistent with the occurrence of phase transitions in the conformation of the enzyme proteins during thermal denaturation.

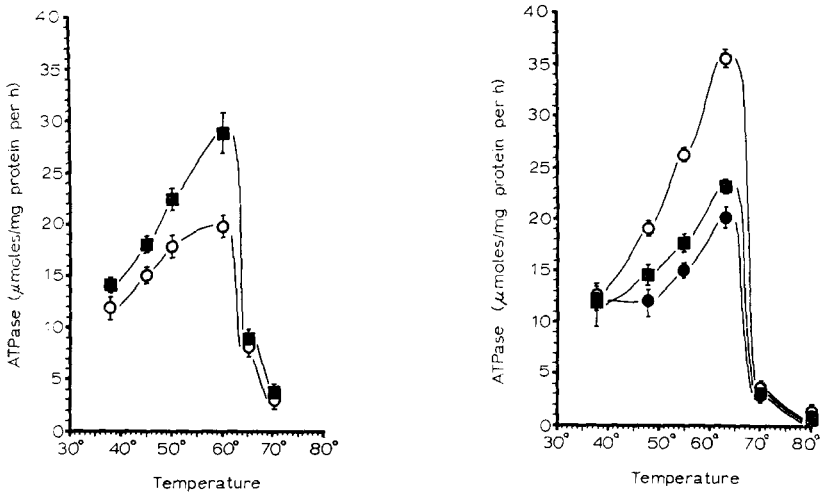

Fig. 1. The mean values ± S.E. of ATPase activity *vs.* temperature of incubation of native (○—○) and of ouabain-treated (■—■) microsomal protein in the presence of Mg^{2+}. Each point on the curve is an average from data of three experiments. Each value in one experiment was an average of four replicate determinations. The ionic composition, the substrate concentration, and other properties of the incubation mixture as well as the experimental conditions of the reaction are as described in MATERIALS AND METHODS.

Fig. 2. The mean value ± S.E. of ATPase activity *vs.* temperature of incubation of native microsomal protein in the presence of Mg^{2+} (○—○), Mg^{2+} + Na^+ (●—●), and Mg^{2+} + Na^+ + ouabain (■—■). Each point on the curve is an average from data of three experiments, each value in an experiment was an average of four replicate determinations. The ionic composition, the substrate concentration, and other properties of the incubation mixture as well as the experimental conditions of the reaction are as described in MATERIALS AND METHODS.

Effect of Na^+ on native and ouabain-treated microsomes

Fig. 2 presents a plot of mean values ± S.E. of ATPase activity in three batches of microsomes.

In the native microsomes, the Mg^{2+}-dependent ATPase activity as a function of temperature was essentially the same as that described in Fig. 1; and the activity in presence of Mg^{2+} + Na^+ was clearly less than that in the presence of Mg^{2+} alone over the range of temperature, 38–70°. The maximum Na^+-induced decrement was found at about 63°.

A stimulatory action of ouabain in the presence of Mg^{2+} and Na^+ was observed over the same temperature range insofar as the ATPase activity in the presence of Mg^{2+} + Na^+ + ouabain was significantly greater than that in the presence of Mg^{2+} + Na^+.

Effect of K^+ on native and ouabain-treated microsomes

Fig. 3, a plot of mean values ± S.E. of ATPase activity *vs.* temperature of incubation in three batches of microsomes.

In the native microsomes, the Mg^{2+}-dependent ATPase *vs.* temperature was

essentially the same as those shown in Fig. 1 and 2 and the activity in the presence of $Mg^{2+} + K^+$ was clearly less than that in the presence of Mg^{2+} alone over the range of temperature, 38–70°. The maximum decrement due to K^+ was found at about 63°.

A stimulatory effect of ouabain in the presence of $Mg^{2+} + K^+$ was observed over the same temperature range insofar as the ATPase activity in the presence of $Mg^{2+} + K^+ +$ ouabain was greater than that in the presence of $Mg^{2+} + K^+$, and furthermore, greater than that in the presence of Mg^{2+} alone. Thus, the addition of ouabain in the presence of $Mg^{2+} + K^+$ eliminated all of the K^+-induced inhibition of the ATPase activity. In contrast, the addition of ouabain in the presence of $Mg^{2+} + Na^+$ (see Fig. 2) eliminated some, but not all of the Na^+-induced inhibition of the ATPase activity.

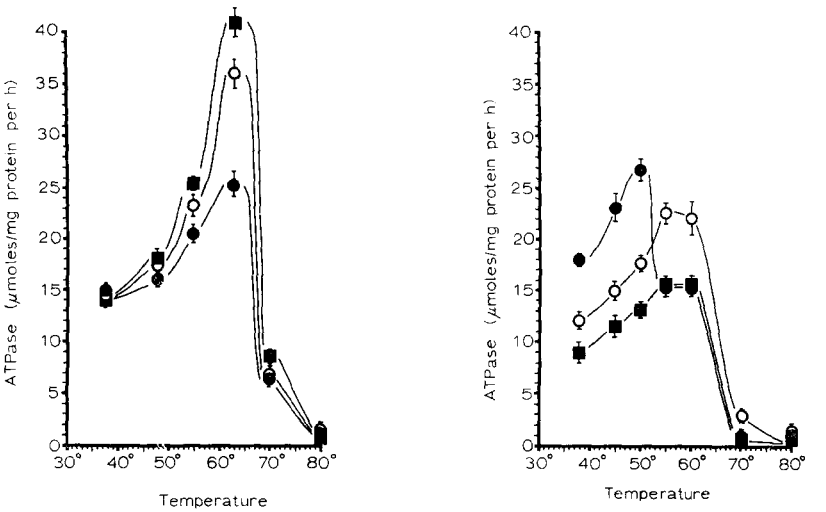

Fig. 3. The mean value ± S.E. of ATPase activity *vs.* temperature of incubation of native microsomal protein in the presence of Mg^{2+} (○—○), $Mg^{2+} + K^+$ (●—●), and $Mg^{2+} + K^+ +$ ouabain (■—■). Each point on the curve is an average from data of three experiments, each value in an experiment was an average of four replicate determinations. The ionic composition, the substrate concentration, and other properties of the incubation mixture as well as the experimental conditions of the reaction are as described in MATERIALS AND METHODS.

Fig. 4. The mean value ± S.E. of ATPase activity *vs.* temperature of incubation of native microsomal protein in the presence of Mg^{2+} (○—○), $Mg^{2+} + Na^+ + K^+$ (●—●), $Mg^{2+} + Na^+ + K^+ +$ ouabain (■—■). Each point on the curve is an average from data of three experiments, each value in an experiment was an average of four replicate determinations. The ionic composition, the substrate concentration, and other properties of the incubation mixture as well as the experimental conditions of the reaction are as described in MATERIALS AND METHODS.

Effect of $Na^+ + K^+$ on native and ouabain-treated microsomes

Fig. 4, a plot of mean values ± S.E. of ATPase activity *vs.* temperature of incubation in three batches of microsomes.

In the native microsomes, the Mg^{2+}-dependent ATPase activity as a function of temperatures was essentially the same as that described for Figs. 2 and 3. The activity in the presence of $Mg^{2+} + Na^+ + K^+$ was clearly greater than that in the presence of Mg^{2+} alone between 38 and 50°; but was clearly less than that of Mg^{2+} alone at temperatures greater than 50°. The increase in the Mg^{2+}-ATPase due to $Na^+ + K^+$ between 38 and 50° denotes the well known $(Na^+ + K^+)$-ATPase activity as a func-

tion of temperature. The maximum decrement in Mg^{2+}-ATPase activity due to $Na^+ + K^+$ was found at about 63°, which is similar to that found after the addition of either Na^+ or K^+ alone (see Figs. 2 and 3).

The well-known inhibitory effect of ouabain on the ($Mg^{2+} + Na^+ + K^+$)-ATPase was elicited between 38 and 50°; and no effect of ouabain was found at temperatures greater than 55°.

The activity of the ouabain-treated enzyme in the presence of $Mg^{2+} + Na^+ + K^+$ is usually taken to be equal of that of the native enzyme in the presence of Mg^{2+} alone. Thus, the difference between these two activities should vanish over a wide temperature range if ouabain specifically inhibits the $Na^+ + K^+$ moiety and has no effect whatsoever on the Mg^{2+}-dependent moiety of activity. Since this difference did not vanish (see Fig. 4), this commonly used assumption was not verified at any incubation temperature between 38 and 60°. The mean ± S.E. of all of the paired differences in activity ($n = 15$) between the reaction with $Mg^{2+} + Na^+ + K^+$ and that with $Mg^{2+} + Na^+ + K^+$ + ouabain, 5.02 ± 0.92 was highly significantly different from zero ($P < 0.01$). The apparent equality of the two activities between 70 and 80° is not meaningful because the activity of the native or ouabain-treated enzyme under any or all ionic conditions was reduced to zero at this temperature range.

The downsweep portions of all of the functions plotted in Figs. 1–4 were interpreted in terms of thermal denaturation of the native or ouabain-treated microsomes (see DISCUSSION for further details on such interpretations).

Effect of pre-heating at 63° on the microsomal ATPase activity at 38°

The microsomes in these experiments were pre-incubated at 63° for 10 min prior to starting the reaction at 38°. The incubation time of the hydrolysis was also 10 min. For comparative purposes, the same batch of microsomes was pre-incubated and incubated at 38°.

Table II presents mean values and statistical parameters for the cation-sensitive rate of hydrolysis of ATP of native and of ouabain-treated microsomal proteins.

TABLE II

EFFECT OF HEAT TREATMENT AT 63° ON THE ATPASE ACTIVITY AT 38° OF TURTLE BLADDER MICROSOMES IN THE PRESENCE OF Mg^{2+} ALONE (A) AND WITH THE IONIC ADDITIONS INDICATED UNDER B, C, AND D

The values in the table are determined in the manner described for Table I. Details concerning the temperature conditions during preliminary incubation and during the course of the reaction as well as those concerning concentrations of substrates, ions, buffers and ouabain can be found in MATERIALS AND METHODS.

Temperature		*State of enzyme*	*Relative activity* (Mg^{2+} = 100%)			
Preliminary incubation	*Incubation*		*(A)* Mg^{2+}	*(B)* $Mg^{2+} + Na^+$	*(C)* $Mg^{2+} + K^+$	*(D)* $Mg^{2+} + Na^+ + K^+$
38°	38°	Native	100 ± 8	95 ± 6	98 ± 8	198 ± 12
		Ouabain	105 ± 8	93 ± 5	94 ± 4	72 ± 10
63°	38°	Native	50 ± 6	37 ± 4	54 ± 2	11 ± 4
		Ouabain	51 ± 6	33 ± 3	71 ± 8	28 ± 5

Values are normalized with respect to the Mg^{2+}-dependent ATPase activity taken as 100 %.

The values of ATPase activity at 38° for native and for ouabain-treated enzyme (first and second rows) are similar to those reported in Table I at the same temperature.

After pre-incubation of the native enzyme at 63°, the Mg^{2+}-dependent ATPase activity at 38° was decreased by 50 % (compare third with first rows of Column A). Addition of Na^+ caused a slight decrease ($P < 0.1$), addition of K^+ no significant change ($P > 0.5$), and addition of $Na^+ + K^+$ caused a marked decrease in the native Mg^{2+}-dependent ATPase ($P < 0.02$). Some, but not all of these data resembled those of the previous table where the experimental protocol was the reverse (38° pre-incubation and 63° incubation) of that used in Table II (63° pre-incubation and 38° incubation).

After pre-incubation of the ouabain-treated enzyme at 63°, the changes in ATPase activity at 38° were roughly similar to those in the native enzyme under the same thermal conditions (compare third and fourth rows).

The effect of temperature of incubation on p-nitrophenylphosphatase

In a design parallel to that of Table I (pre-incubation temperature, 38°; incubation temperatures 38 and 63°; Mg^{2+}, 3 mM; K^+, 15 mM; *p*-nitrophenylphosphate, 5 mM; 0.1 mM EDTA–Tris and 40 mM Tris–HCl) *p*-nitrophenylphosphatase activity was determined by substituting *p*-nitrophenylphosphate for ATP as a substrate and by measuring the rate of P_i released by the method of BERENBLUM AND CHAIN[11]. At 38°, the ($Mg^{2+} + K^+$)-dependent *p*-nitrophenylphosphatase activity amounted to 12 μmoles/mg protein per h; and at 63° the activity reached zero.

In a design parallel to that of Table II (pre-incubation temperature, 63°; incubation temperature, 38°), the ($Mg^{2+} + K^+$)-dependent *p*-nitrophenylphosphatase activity after 10 min of exposure of the enzyme to 63°, was near zero at 38°.

The fact that ($Mg^{2+} + K^+$)-*p*-nitrophenylphosphatase and ($Na^+ + K^+$)-ATPase are inactivated at similar incubation temperatures suggests that the two activities are related to the same protein.

Thermodynamic parameters

The free energy of activation of the hydrolysis of ATP in the presence of microsomal proteins can be estimated directly from the data shown in Figs. 1–4. Such estimates, derived from a system containing several activated complexes in series, reflect only the overall activation energy and not that for any single step in the enzyme–substrate sequence. Moreover, thermal denaturation of the enzyme is probably occurring — albeit to a small extent, during the activation stage — *i.e.* during the stage showing increasing ATPase activity with increasing incubation temperatures. Therefore, the activation energy, E^*, is a lumped parameter comprising the activation energies required for all steps of the ATPase sequence.

Data of Figs. 1–4 permit the evaluation of: the activation energy, E^*; the heat (enthalpy) of activation, ΔH^*; the free energy of activation, ΔF^*; and the entropy change during activation, ΔS^* (ref. 9). These estimates were restricted to those conditions where increases in incubation temperature caused increases in the enzyme activity, *i.e.* restricted to the upsweep portions (usually between 38 and 63°) of the temperature-dependent activity curves in Figs. 1–4.

The value of E^* was determined from the slope of the plot of values of ln v *vs.* $1/T$ (Arrhenius plot), on the basis of the equation,

$$\ln v = -\frac{E^*}{R} \cdot \frac{1}{T} \tag{1}$$

where v is the measured reaction velocity, T the absolute temperature, and R the gas constant. From E^*, the value of ΔH^* can be directly determined on the basis of the equation,

$$\Delta H^* = E^* - RT \tag{2}$$

Next, the value of ΔF^* can be obtained from the equation,

$$\Delta F^* = RT \ln (K_B T/hk) \tag{3}$$

where K_B is the Boltzmann constant; h is Planck's constant; and where k, the specific rate constant of the breakdown of the intermediary complex, can be evaluated experimentally from the measured reaction velocity under conditions of saturation of the enzyme by substrate. In the reaction between the microsomal enzyme and ATP, the maximal reaction velocity is the rate of release of P_i, or

$$dP_i/dt = k(ES) = v_{max} \tag{4}$$

where ES is the lumped value for all of the intermediary complexes formed in the overall hydrolysis, and where its rate of breakdown is assumed to be the rate-limiting step. Assuming (a) that the amount of ES is equal to that of the total enzyme; (b) that the pure enzyme comprises no more than 0.1 % of the mass of measured microsomal protein; and (c) that the molecular weight of the pure enzyme is $2.5 \cdot 10^5$ (ref. 12), the specific rate constant, k, can be estimated.

Finally ΔS^* can be estimated in the conventional manner from the values of ΔH^* and ΔF^*.

The values for all of the aforementioned thermodynamic parameters were estimated for $T = 311°K$.

In the present work, the thermodynamic parameters were estimated individually for each set of ionic conditions in both the native and ouabain-treated microsomes. The average values of $E^*_{Mg^{2+}}$, determined on the basis of data from each batch of microsomes, varied from 3.68 to 11.6 kcal/mole; and the grand mean value for all batches was 7.88 kcal/mole*.

Table III shows mean values and standard errors for the activation energies of the ATP hydrolysis in the presence of native and ouabain-treated microsomes under the ionic conditions specified in the second column.

In the native enzyme system, the energy of activation of the Mg^{2+}-dependent reaction was decreased significantly in the presence of either Na^+ or K^+; but was not significantly changed in the presence of Na^+ and K^+ together.

* This value for $E^*_{Mg^{2+}}$ is in agreement with what should have been (but was not) reported previously[2] due to an error in calculation from accurately plotted values on figure of ref. 2. Thus, the values of $E^*_{Mg^{2+}}$ previously cited in the text of ref. 2 should have been 39 kcal/mole between 15 and 23° and 8.3 kcal/mole between 23 and 45° instead of 56.2 and 13.2 kcal/mole, respectively, as actually cited in the text. The value of $E^*_{Na^+ + K^+}$ (estimated from the activity in the presence of $Mg^{2+} + Na^+ + K^+ +$ ouabain) should have been 13.4 kcal/mole instead of 27.5 kcal/mole as reported in the text of ref. 2. The comparable value of $E^*_{Na^+ + K^+}$ in the present report was 7.24 kcal/mole between 38 and 45°.

TABLE III

MEAN VALUES ± STANDARD ERRORS FOR ACTIVATION ENERGY (E^* ± S.E.) OF THE OVERALL HYDROLYSIS OF ATP IN THE PRESENCE OF NATIVE AND OF OUABAIN-TREATED MICROSOMES

Each of the values shown is the mean of three slopes estimated graphically from an Arrhenius plot of the reaction rates obtained from three batches of microsomes incubated at four temperatures (usually between 38 to 63°) under the set of ionic conditions specified in the second column. At each specified temperature, four replicate rate determinations were made on aliquots of a single batch of native or of ouabain-treated microsomes; and consequently the estimate of the mean value of each E^* involved the arithmetic processing of forty-eight replicate determinations.

From data of Fig.	*Ionic conditions*	E^* ± *S.E.* (kcal/mole)	
		Native	*Ouabain*
1	Mg^{2+}	3.68 ± 0.87	6.41 ± 1.06
2	Mg^{2+}	8.96 ± 0.08	—
	Mg^{2+} + Na^+	2.95 ± 1.40	5.10 ± 1.65
3	Mg^{2+}	11.6 ± 0.20	—
	Mg^{2+} + K^+	4.72 ± 0.15	12.3 ± 0.75
4	Mg^{2+}	7.13 ± 0.89	—
	Mg^{2+} + (Na^+ + K^+)	6.79 ± 0.98	6.95 ± 0.80
	(Na^+ + K^+)	7.24 ± 1.08	—

Moreover, the activation energy of (Na^+ + K^+)-stimulated ATPase was not significantly different from that of Mg^{2+}-dependent ATPase or from that of (Mg^{2+} + Na^+ + K^+)-ATPase.

The effect of ouabain on the activation energy can be seen under each of the ionic conditions. In the presence of Mg^{2+} alone, or of Mg^{2+} + K^+, or Mg^{2+} + Na^+, the mean value of the differences in activation energies of the ouabain-treated and those of the paired native enzyme were significantly greater than zero ($P_{(Mg^{2+})} < 0.02$; $P_{(Mg^{2+} + K^+)} < 0.001$; and $P_{(Mg^{2+} + Na^+)} < 0.02$). In the presence of Mg^{2+} + Na^+ + K^+, the mean value of the differences in the activation energies of the ouabain-treated and those of the paired native enzymes were not significantly different from zero ($P > 0.7$).

Not shown are the values of the free energy of activation, ΔF^*, which amounted to 5.4 kcal/mole under all conditions except those in the presence of Mg^{2+} + Na^+ + K^+ in the native enzyme where ΔF^* was 5.0 kcal/mole and in the ouabain-treated enzyme where ΔF^* was 5.6 kcal/mole. The value of ΔF^* for (Na^+ + K^+)-stimulated ATPase was 6.5 kcal/mole.

Effect of pH

The heat-induced changes in ATPase activity of the microsomes may be correlated with denaturation or phase changes in the protein, provided that simultaneously occurring changes due to ambient pH do not complicate the picture. In the present experiments, the pH of all incubation fluids was set at 7.3 with 40 mM Tris-HCl at 25°. The pH of Tris-HCl solutions was found to decrease by as much as 0.8 unit for a 50° increase in temperature (25–75°) suggesting that some of the observed effects could be attributed to the ambient pH as well as to heat.

The relative contributions of heat and ambient pH to changes in ATPase activity, not completely known as yet, require additional work on the activity–pH profiles at several temperatures.

However, decreases of pH of this magnitude (0.3–0.8 pH unit) at a fixed temperature (38°) produce no more than 10–20 % decreases in the activity of Mg^{2+}-dependent or of $(Na^+ + K^+)$-dependent ATPase in this tissue[2]. Thus, most of the activity changes observed in the present studies are probably ascribably to the ambient temperature.

DISCUSSION

General considerations

The heat-denaturation of the enzyme, as used in the present report, is defined operationally as the temperature dependent decrease in enzymatic activity, which can be interpreted in terms of that hydrogen bonding which is essential for the form and specificity of the enzyme[7,8,10]. In this connection, a useful parameter is the so-called $T_{1/2}$, or the temperature at which the activity has reached half its maximal value during the process of thermal denaturation. The $T_{1/2}$ is a useful parameter of the denaturation and the concurrent loss of enzymatic activity are apparently a single process, as is suggested by the present data.

For example, in the case of Mg^{2+}-dependent ATPase activity of native and ouabain-treated microsomes, the maximal level of activity occurs at 60° for both proteins, but the $T_{1/2}$ occurs at 63° for the native protein and at 64° for the ouabain-treated protein (see Fig. 1). Thus, the ouabain-treated form of the protein, not only more enzymatically active than the native form, may be more stable to high temperatures than is the native form.

For example, if the breaking of hydrogen bonds occurred independently during the heating of either the native or of the ouabain-treated enzyme, then the $T_{1/2}$ would be unchanged by any ouabain-induced changes in the number of essential hydrogen bonds, and consequently the stability of the ouabain-treated enzyme would be no different than that of the native enzyme. However, the $T_{1/2}$ of the ouabain-treated enzyme was greater than that of the native enzyme. Such a stabilization of the ouabain-treated enzyme suggests that there exists a cooperativity between the hydrogen bonds of the native enzyme and the new hydrogen bonds formed in the ouabain-treated enzyme. The cooperativity would require that the breaking of one set of hydrogen bonds could occur only with the breaking of the other set.

The downsweep patterns of the plots of activity *versus* temperature (Figs. 1–4) merit additional comment. When the incubation temperature exceeds that associated with maximal activity, the activity decreases to 50 % of its maximal level for a 2–4° increase in temperature. The steepness of the slope and the possible sigmoid nature of the temperature-dependent downsweep of activity resemble the classical pattern of highly cooperative order–disorder phase transitions of crystallites or other polymers[10], a well known example of which is the pattern of denaturation of DNA. The mid-point of the sigmoidal downsweep is considered as the transition temperature which is equivalent to the melting point of a crystal. In the present work, this mid-point temperature serves as an indicator of thermal stability of the protein.

Effect of aging and dilution on ouabain action

The activity of diluted microsomes decreases faster than that of concentrated microsomes. The ouabain-induced increases of ATPase activities at 55-65° were elicited in microsomes previously refrigerated for two or more days in a dilute suspension, but not from microsomes refrigerated in a concentrated suspension, nor from freshly extracted microsomes. This suggests that ouabain reacts more readily with a decaying form than it does with a highly reactive form of the enzyme. The interaction between ouabain and the decaying form of the enzyme results in an increase in the activity of the decaying enzyme, particularly at higher temperatures. Such a ouabain-induced stimulation of enzymatic activity may be due to an increase in the hydrogen bonding which is essential for reactivity of the protein. For example, the incorporation (or association) of ouabain with the protein may facilitate the formation of hydrogen bonds between water and the ouabain–protein complex; or alternatively the hydration of the protein (by water in the dilute storage mixture) facilitates the formation of hydrogen bonds between ouabain and the hydrated protein. In either case, the additional hydrogen bonds formed impart to the protein an increase in its ATPase activity at the higher temperature. The increase in hydrogen bonding could also increase the thermal stability of the ouabain-treated protein; and such increases were verified experimentally insofar as the mid-point of thermal inactivation (between 65 and 80°) in the ouabain-treated enzyme was greater than that of the native enzyme (see Figs. 1–3).

Effect of ion additons on Mg^{2+}-ATPase

According to the previous argument relating ouabain-induced hydrogen bonding to the thermal stability of the protein, it is possible that Na^+ alone or K^+ alone may decrease the hydrogen bonding essential for enzyme activity, thus decreasing the thermal stability of the protein. This possibility is consistent with the observed decreases in enzymatic activity and thermal stability ($T_{½}$) of the Mg^{2+}-ATPase activity in the presence of either Na^+ or K^+, and in the presence of $Na^+ + K^+$ at temperatures over 55°.

However, some of the findings were not consistent with the aforementioned correlations among hydrogen bonding, activity and thermal stability. For example, between 38 and 55°, the addition of $Na^+ + K^+$ increases the Mg^{2+}-ATPase activity and the addition of ouabain inhibits the $Na^+ + K^+$ increment of this activity.

Thermodynamic considerations

During the estimation of the thermodynamic parameters, it was found that the value of the energy of activation, E^*, varied from one batch of microsomes to another. Such variations cannot be due to different proportions of inert contaminants in different batches microsomes. Inert contaminants, which do not interact with the ATPase, could not change the value of the activation energy. The fact that the value of E^* did change suggests that contaminants in the microsomes do interact with ATPase thus changing the value of E^* in going from one batch of microsomes to another.

The correlation of ATPase activity with the thermodynamic parameters is unexpected because higher activation energies (or enthalpies) are associated with higher reaction velocities. For example the E^* of the reaction in the presence of Mg^{2+} alone is

greater than that in the presence of Mg^{2+} + Na^+ implying that the activity of the Mg^{2+} enzyme would be less than that of the Mg^{2+} + Na^+ enzyme, an implication which is contradicted by the experimental findings (see Fig. 2).

Therefore, the inhibition (or acceleration) of the underlying Mg^{2+}-dependent activity under any of the present conditions, cannot be explained solely by the classically expected change in the activation energy required for the formation of the intermediary complex. It remains to be determined whether the data can be explained by invoking factors such as the presence of more than one form of the enzyme, or the degree of association of ions with proteins as temperature increases, or by other mechanisms.

These problems would be clarified somewhat if the activation energy of each consecutive complex (*e.g.* $E\sim$ATP, $E\sim$P, or E-P) formed in the overall ATPase sequence could be estimated with the appropriate experimental set-up. This may be feasible because the rate of binding of [^{14}C]ATP to the enzyme, presumably the first step in the sequence, has been determined for incubation temperatures of 0° (ref. 5) which makes possible, in principle at least, a determination of the activation energy for the first enzyme–substrate complex (*e.g.* $E\sim$ATP) formed in the overall sequence of ATP hydrolysis.

A consideration of some of the problems remaining to be solved highlights the tentative nature of the preceding discussion. Apart from the purification, isolation, and chemical identification of ATPase and its moieties (Mg^{2+}-dependent, (Na^+ + K^+)-dependent, *etc.*), an exact understanding of the process of thermal denaturation is necessary. The temperature dependency of the K_m for substrates and ionic co-factors, as well as that of the pH optima remain to be determined. Moreover, the ouabain-induced stimulation of Mg^{2+}-dependent ATPase at higher temperatures is operationally similar to the (Na^+ + K^+)-stimulation of the same activity at lower temperatures. This raises the question as to whether or not (Na^+ + K^+)-ATPase is another functional form of the same molecule as that called Mg^{2+}-dependent ATPase.

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 are grateful to Dr. George Nemethy of the Rockefeller University, New York, for his critical review of and suggestions on the manuscript.

The authors thank Mrs. Pamela Schiro and Mary Soliman for their technical assistance.

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