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STUDIES ON AN ATPase OF THERMOPHILIC BACTERIA

I. PURIFICATION AND PROPERTIES

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SUMMARY

ATPase (EC 3.6.1.3) from *Bacillus stearothermophilus* is purified to a state in which it is homogeneous, both ultracentrifugally and electrophoretically. The sedimentation constant and molecular weight are 11.9 S and 280 000, respectively, and the optimum pH is 8.0 or 8.5, in a Tris–acetate or Tris–maleate buffer. Divalent cations such as Mg^{2+}, Mn^{2+} and Cd^{2+} activate the enzyme but the activity is strongly inhibited by I_2, N_3^- and CN^-. Sulfhydryl reagents and uncouplers have no effect on the activity. ADP inhibits the activity and the mode of inhibition appears to be different from that of ATPase from mesophiles. The proline content of the ATPase is low (4.3 g/100 g protein) and the average amount of proline contained in the protein of the thermophilic bacteria is also low and similar to that of mesophilic bacteria. The enzyme contains about 20% of the α-helical conformation. The activity increases gradually with increasing temperature, but above 50° the activity increases abruptly. The enzyme has an optimum temperature of 65° and exhibits a remarkable resistance to thermal inactivation; these results may indicate that the enzyme is not only thermostable but also thermophilic. Thermodynamic quantities for the enzyme, calculated in the temperature range 30–65°, suggest that a conformational change in the enzyme protein occurs at the transition temperature, 55°.

INTRODUCTION

Temperature is one of the most important environmental factors for the growth of living organisms. The thermophilic properties and thermostability of the bacteria which prefer high temperatures for their optimum growth, therefore, have long been problems greatly attracting the interest of investigators[1,2]. Many of the early investigations on these problems were focused on the thermostability of the enzymes from thermophilic bacteria[3–12]. Almost all enzymes thus examined were found to be ther-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

mostable as compared with the corresponding enzymes from mesophilic bacteria. On the thermostability of the membrane-bound enzymes, however, discrepant results have been reported. When they are free from membrane, ATPase (EC 3.6.1.3) and pyrophosphatase (EC 3.6.1.1) are still thermostable[8–10], whereas a phenylalanyl-tRNA synthetase becomes thermolabile[13].

In 1961, MANNING AND CAMPBELL[14,15] reported an extraordinary protein structure, consisting of a high proline content and a random coiled form, for α-amylase excreted from a thermophilic bacterium. Since then, many investigations have been carried out along this line of approach[13,16–19]. However, the most extensive investigations made were those with the extracellular enzymes from thermophilic bacteria[17–19].

In order to elucidate the mechanism of the thermophilicity, as well as the thermostability of thermophilic bacteria, further and extensive studies on the intracellular enzymes are apparently required. In the present study, therefore, a membrane-bound enzyme, ATPase, was isolated and purified from *Bacillus stearothermophilus* and some properties, especially thermal, of the enzyme were examined.

MATERIALS AND METHODS

Microorganisms and cultures

The thermophilic bacteria used in the present study were *Bacillus stearothermophilus* (NCA No. 2184) kindly donated by Professor C. E. George, Nebraska University, U.S.A.. The culture medium was of the following composition; 15 g polypeptone (Daigo Co.), 1 g yeast extract (Daigo), 4 g glucose, 5 g NaCl, 2 g K_2HPO_4, 1 g $MgSO_4 \cdot 7\ H_2O$, 1 g KNO_3, a few drops of oil as an anti-foam agent and 1 l of de-ionized water; the pH was adjusted to 7.2 with NaOH. The cells from a fresh slant culture were pre-incubated aerobically in a 25-ml medium at 65° for 3 h, then transferred into a 5-l medium and cultured for 12 h. Mass cultures in a 200-l medium were kindly supplied by the courtesy of Director T. Nakamura of the Central Research Laboratories, Ajinomoto Co..

Bacillus subtilis (IAM 1069), donated from the Institute of Applied Microbiology, University of Tokyo, was cultured in a medium of the following composition; 10 g polypeptone, 10 g meat extract (Mikuni Co.), 3 g NaCl and 1 l of de-ionized water; the pH was adjusted to 7.2. The cells were aerobically cultured at 37° for 10 h.

Solubilization of ATPase

The cells (200 g, wet weight) were washed twice with 400 ml of 10 mM Tris–HCl buffer (pH 7.2) containing 0.9% NaCl and 5 mM $MgCl_2$. The cells, suspended in 200 ml of the same buffer, were incubated with 100 mg lysozyme (EC 3.2.1.17) at 37°. After 2 h of incubation 500 μg of deoxyribonuclease I (EC 3.1.4.5) were added and the mixture left to stand for 30 min. Cell debris was removed by centrifugation at 5000 $\times g$ for 10 min and the resulting supernatant was centrifuged at 25 000 $\times g$ for 15 min in order to obtain the membrane ghosts.

The membrane ghosts were washed twice with 10 mM Tris–HCl buffer (pH 7.2) containing 5 mM $MgCl_2$, twice with 0.25 M Tris–HCl buffer (pH 7.2) containing 2 M LiCl, and then with 5 mM $MgCl_2$ solution. The membrane ghosts thus obtained were suspended in 250 ml of 10 mM Tris–HCl buffer (pH 7.2). The suspension was dialyzed against 10 mM Tris–HCl buffer (pH 7.2) containing 0.1 mM EDTA for 12 h and then

against distilled water containing 0.1 mM EDTA (disodium salt) at 4°. The suspension, after adjustment of the pH to 8.5, was left to stand for 30 min. As a result of these EDTA and alkali treatments ATPase was released in soluble form. Crude extracts of ATPase were obtained by centrifugation at 100 000 × *g* for 20 min.

Aassay of ATPase activity

The reaction mixture for the determination of ATPase activity was of the following final composition; 80 mM Tris–acetate (pH 8.0), 6 mM ATP, 6 mM $MgCl_2$ and 0.2 mM EDTA. To 0.47 ml of the mixture was added 0.03 ml of the enzyme solution and after 10 min of incubation 0.5 ml of 2% $HClO_4$ was added in order to stop the reaction. P_i liberated was determined according to the method of PEEL AND LOGHMAN[20] by measuring the absorbance at 700 mμ ($A_{700\ m\mu}$). When the protein concentration was below 1.1 μg a linearity of P_i liberation with respect to time was obtained for about 12 min after addition of the enzyme.

Measurement of protein

Protein concentration was determined by means of either the biuret method[21], when the concentration was above 1 mg/ml, or the method of LOWRY *et al.*[22], using crystalline bovine serum albumin as a standard, when the concentration was below 1 mg/ml.

Ultracentrifugal analysis

Sedimentation velocity experiments were performed at 20° in a Hitachi ultracentrifuge, UCA-1, with phase-plate Schlieren optics. The molecular weight was estimated by means of the meniscus depletion method[23]. Measurement was performed at a speed of 13 310 rev./min at 20° and a regular 12-mm double-sector cell was used.

Disc-electrophoretic analysis

Polyacrylamide gel disc electrophoresis was carried out in a Tris–glycine buffer system (pH 8.5) according to the procedures of ORNSTEIN[24] and DAVIS[25]. The sample, containing 60 μg protein in 0.5 M sucrose, was layered onto the gel. The electrophoresis was carried out at 4° for 15 min at 4 mA/cm and the protein was stained with Amide black 10B.

Amino acid analysis

The hydrolysis of the protein was carried out at 110° with 6 M HCl, in a sealed tube, under N_2 and the values obtained after 24 and 48 h of hydrolysis were calibrated according to the method of SMITH AND STOCKELL[26]. Analysis was carried out in a Hitachi automatic amino acid analyzer, KLA-2.

Optical rotatory dispersion

Optical rotatory dispersion measurement was made with a Jasco ORD/UV-5 recording spectropolarimeter, at 20°, with a quartz cell of either 1.0-cm or 0.1-mm path length.

Reagents

Deoxyribonuclease I was purchased from Sigma Co., ATP (disodium salt) from Kyowa Hakko Co. and other nucleotides and lysozyme from Seikagaku Co.

RESULTS

Purification of ATPase

Step 1: DEAE-cellulose column chromatography. The protein fraction precipitated from the crude extracts of ATPase by a 60% satd. solution of $(NH_4)_2SO_4$ was dissolved in water and dialyzed against 50 mM Tris–HCl buffer (pH 8.0) for 12 h. The dialyzed protein was passed through a column of DEAE-cellulose previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column was washed with 5 times the volume of the column of 50 mM Tris–HCl buffer (pH 8.0), containing 0.1 M NaCl, and then with a column volume of 50 mM Tris–HCl buffer (pH 8.0), containing 0.15 M NaCl. The enzyme was eluted by a linear gradient system of 0.15–0.5 M NaCl in 50 mM Tris–HCl buffer (pH 8.0). The elution pattern for the system is shown in Fig. 1A.

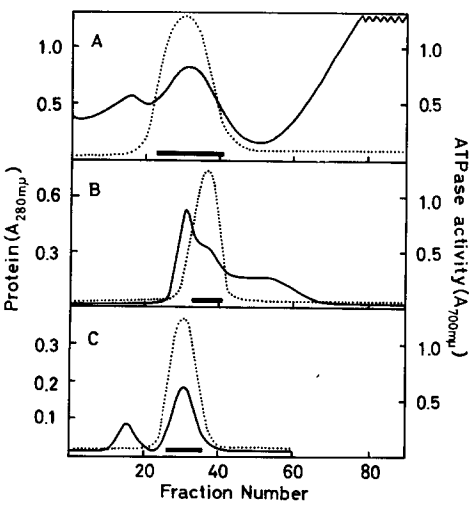


Fig. 1. Elution patterns of the ATPase on DEAE-cellulose and on Sephadex G-200. A. First chromatography on DEAE-cellulose. B. Gel filtration on Sephadex G-200. C. Second chromatography on DEAE-cellulose. Column dimensions (cm × cm): (A) 4.0 × 20, (B) 3.4 × 70 and (C) 2.0 × 30. Flow rates (ml/h): (A) 40, (B) 15 and (C) 40. Volumes collected for each tube (ml): (A) 10, (B) 5 and (C) 3. ———, protein ($A_{280\ m\mu}$); · · · · ·, ATPase activity ($\Delta A_{700\ m\mu}$/10 min).

Step 2: Hydroxyapatite column chromatography. The fractions shown covered with a black bar in Fig. 1A were collected and subjected to hydroxyapatite column chromatography. The hydroxyapatite was prepared according to the method of Tiselius *et al.*[27]. 2 mM phosphate buffer (pH 7.0) was used for equilibration and development of the column. ATPase was eluted without absorption.

Step 3: Gel filtration on Sephadex G-200. The fraction precipitated from the eluates in Step 2, during 30–60% satn. with $(NH_4)_2SO_4$, was dissolved in 50 mM Tris–HCl buffer (pH 8.0) and subjected to gel filtration on Sephadex G-200. 50 mM Tris–HCl buffer (pH 8.0) was used for equilibration and development of the column. The elution pattern is shown in Fig. 1B.

Step 4: Rechromatography on DEAE-cellulose. The fractions shown covered with a black bar in Fig. 1B were collected and brought to 80% satn. with respect to

$(NH_4)_2SO_4$. The resultant precipitate was dissolved in water, dialyzed against 50 mM Tris–HCl buffer (pH 8.0), containing 0.25 M NaCl, and subjected to DEAE-cellulose column chromatography. The column was equilibrated and developed with the same buffer as that used for dialysis and the elution pattern is shown in Fig. 1C. The fractions shown covered with a black bar in Fig. 1C were collected and brought to 80% satn. with respect to $(NH_4)_2SO_4$. The resultant precipitate was dissolved in water and dialyzed against 50 mM Tris–HCl buffer (pH 8.0) for 5 h.

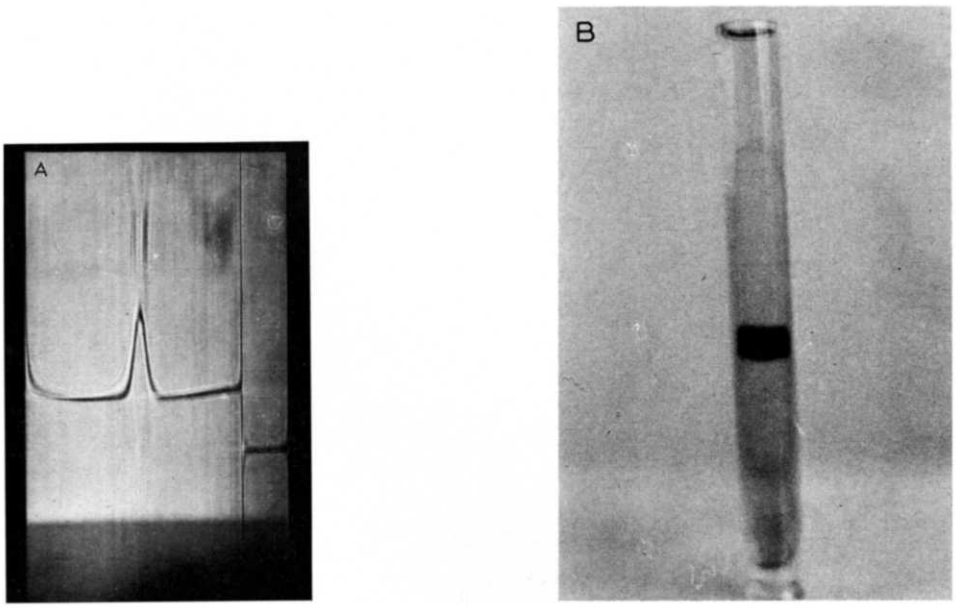


Fig. 2. Homogeneity of the ATPase. A. Sedimentation pattern of the ATPase. Protein concentration was 0.4% in 0.05 M Tris–HCl buffer (pH 8.0). The photograph was taken 27 min after reaching full speed, 60 000 rev./min. B. Disc-electrophoretic pattern of the ATPase.

Homogeneity of ATPase

As shown in Fig. 2, the ATPase thus purified was homogeneous, both ultracentrifugally and electrophoretically. The sedimentation constant ($s^0_{20,w}$) and molecular weight were estimated to be 11.9 S and 280 000, respectively. These values were almost similar to the corresponding values reported for the ATPases from mesophiles[25,28–31].

Substrate specificity of ATPase

By analyzing the reaction products according to the method of COHN AND CARTER[32] it was revealed that for each mole of ATP which disappeared 1 mole of P_i and 1 mole of ADP were formed. With ATP as substrate the ATPase exhibited the specific activity of 45.3 μmoles P_i per min per mg protein, which was 67 times the activity of the crude extracts and much higher than the activity reported by MILITZER AND TUTTLE[8]. Other nucleotide triphosphates, namely GTP, UTP and CTP, were more or less hydrolyzed by the enzyme. Relative activities with GTP, UTP and CTP represented as per cent against the activity obtained with ATP as substrate were,

TABLE I

EFFECTS OF DIVALENT CATIONS ON THE ATPase ACTIVITY

Divalent cations (final concn., 3 mM)	*Activity (μmoles P_i liberated per 10 min)*
None	0
Mg^{2+}	0.53
Mn^{2+}	0.57
Cd^{2+}	0.55
Zn^{2+}	0.35
Co^{2+}	0.34
Ca^{2+}	0.24
Hg^{2+}	0

respectively, 90, 30 and 16%. Nucleotide mono- or diphosphate such as AMP and ADP and pyrophosphate were not hydrolyzed at all.

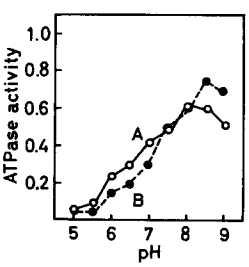
Effect of pH on the activity

As shown in Fig. 3, the enzyme exhibited a maximum activity at pH 8.0 in a 80 mM Tris–acetate buffer, while in a 80 mM Tris–maleate buffer the optimum pH was 8.5. MILITZER AND TUTTLE[8] reported that the optimum pH of the enzyme they prepared is in the region of 8.2–8.5.

Requirement of metal ions

As in the cases of the ATPases from mesophiles[24,29,33–35] the ATPase of thermophilic bacteria also required Mg^{2+} for the activity. The activity was increased on increasing the Mg^{2+} concentrations, and attained a maximum value at 2.6–3.0 mM Mg^{2+}. The activity, however, gradually decreased when the Mg^{2+} concentrations were further increased above 3 mM. As shown in Table I, Mg^{2+} was completely replaced by other divalent cations such as Mn^{2+} and Cd^{2+}, and to a lesser extent, by Zn^{2+}, Co^{2+} and Ca^{2+}.

$(Na^+\text{-}K^+)$-activated ATPase has been found in the membrane of mammalian cells and was considered to act in active transport of ions[36–37]. The activity of the ATPase from thermophilic bacteria was not significantly affected by the addition of 0.1 M Na^+, 0.1 M K^+ and 0.1 M Na^+–0.1 M K^+.



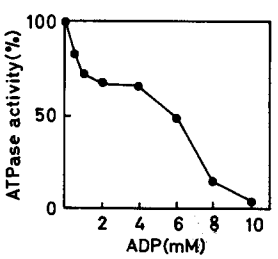


Fig. 3. Effect of pH on the ATPase activity. The activity was expressed in μmoles P_i per 10 min. A, 80 mM Tris–acetate buffer; B, 80 mM Tris–maleate buffer.

Fig. 4. Inhibition of the ATPase activity by ADP. The enzyme concentration was 0.9 μg/ml. Activities were represented as a percentage against the activity obtained without ADP.

TABLE II

EFFECTS OF VARIOUS COMPOUNDS ON THE ATPASE ACTIVITY

The compounds were added in a final concentration of 0.5 mM, except in the cases of pentachlorophenol and ouabain where 0.1 mM was used.

Compounds	*Activity (μmoles P_i liberated per 10 min)*
None	0.55
2,4-Dinitrophenol	0.54
Pentachlorophenol	0.52
Na_2SO_3	0.68
Ouabain	0.54
Monoiodoacetate	0.48
PCMB	0.55
I_2	0.07
NaF	0.46
NaN_3	0.10
NaCN	0.06

Effects of various compounds on the activity

As shown in Table II, I_2, N_3^- and CN^- strongly inhibited the activity. I_2 and N_3^- are known to be potent inhibitors on the ATPases from mitochondria[34] and mesophilic bacteria[31,38,39], while CN^- has no effect on their activities. Sulphydryl reagents such as *p*-chloromercuribenzoate (PCMB) and monoiodoacetate have been shown to inhibit the mesophile ATPases[34–35], although the ATPase of *Micrococcus lysodeikticus* is not inhibited by PCMB[31,38]. The uncouplers such as 2,4-dinitrophenol and pentachlorophenol activate mitochondrial ATPase[34]. The ATPase from thermophilic bacteria, however, was not affected by the sulphydryl reagents and uncouplers.

The ATPase from thermophilic bacteria was inhibited by ADP, as shown in Fig. 4. The inhibition curve against the ADP concentrations was different from the typical hyperbolic form observed with the ATPase from mesophiles[24,31,34]. The curve may indicate that the inhibition proceeds in two steps with increase in the ADP concentrations.

Amino acid composition

As shown in Table III, the proline content of the purified ATPase was 4.2 g/100 g protein. This value was far smaller than that reported with α-amylase produced from *B. stearothermophilus* (16.27 g/100 g protein) by MANNING AND CAMPBELL[14], and similar to those of other proteins[15,18] and α-amylase[19] from other strains of thermophilic bacteria. The amounts of proline contained in the soluble and membrane fractions of this strain of thermophilic bacteria were found to be same, 4.1 g/100 g protein, and almost similar to those of the corresponding fractions of *B. subtilis*, 3.8 and 2.6 g/100 g protein. As seen from Table III, the content of other amino acids, except alanine and methionine, of the proteins of thermophilic bacteria were also similar to those of mesophilic bacteria.

Optical rotatory dispersion

The optical rotatory dispersion profile of the ATPase exhibited a prominent

TABLE III

AMINO ACID COMPOSITIONS OF THE ATPASE AND OF THE PROTEINS OF THE SOLUBLE AND MEMBRANE FRACTIONS OF THERMOPHILIC AND MESOPHILIC BACTERIA

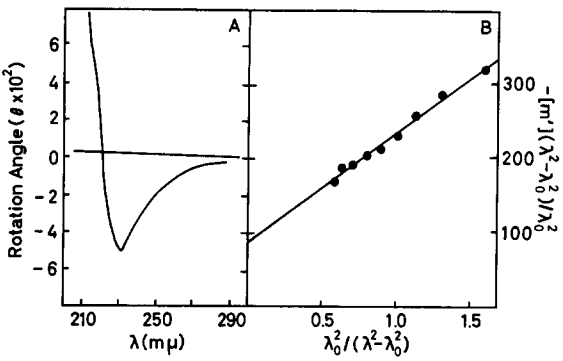
Bacterial cells (wet wt., 35 g) were suspended in a 100 ml of 10 mM Tris–HCl buffer (pH 7.2) containing 5 mM $MgCl_2$, and disrupted with a French pressure apparatus. Undisrupted cells were discarded after centrifugation at 1500 × *g* for 10 min. The resulting cell-free extracts were divided into the soluble and membrane fractions by centrifugation at 100 000 × *g* for 40 min. After removal of lipids, nucleic acids and salts from both fractions[40], the samples were subjected to amino acid analyses. Data represent g/100 g protein.

Amino acid	*ATPase*	*B. stearothermophilus*		*B. subtilis*	
		Soluble fraction	*Membrane fraction*	*Soluble fraction*	*Membrane fraction*
Asp	9.5	10.7	9.3	10.4	7.3
Thr	4.9	5.4	5.1	5.3	3.9
Ser	3.8	3.7	3.3	4.3	3.1
Glu	14.3	13.9	14.9	14.3	17.9
Pro	4.2	4.1	4.1	3.8	2.66
Gly	4.5	4.2	4.4	4.4	3.4
Ala	6.2	6.4	7.2	6.0	11.3
Val	7.3	7.3	7.2	7.0	4.9
Met	2.8	2.8	4.2	2.9	13.2
Ile	8.0	7.2	6.9	7.6	5.8
Leu	8.2	7.8	8.7	8.0	6.3
Tyr	4.0	4.2	5.1	4.5	3.2
Phe	4.9	4.2	5.2	4.1	5.4
Lys	6.3	7.6	7.6	8.7	5.8
His	2.9	2.8	2.8	2.8	1.8
Arg	7.9	7.6	5.0	5.8	4.0

Cotton effect in the ultraviolet region as shown in Fig. 5A. The curve showed a trough at 232 mμ and the specific rotation, m', at this wavelength was estimated to be −4606°. The results clearly indicate the presence of α-helical conformation in the enzyme. From the values of $m'_{232\ m\mu}$ for complete random-coiled and α-helical structures[41], the α-helix content of the ATPase was estimated to be 20.3%. From the Moffitt–Yang plot of optical rotatory dispersion of the enzyme (Fig. 5B), the values of a_0 and b_0 were, respectively, estimated to be +84 and −147.4°. The λ_0 value was assumed to be 212 mμ.

Effect of temperature on the activity

As shown in Fig. 6, the enzyme exhibited essentially no activity below 5°, and above 10° the activity increased almost in proportion to the temperature increase up to about 50°, probably indicating a general feature of a chemical reaction. Above 50°, however, the activity increased abruptly and above 65° the activity decreased, perhaps due to the heat denaturation of the protein. When the enzyme was incubated at 65° for 1 h, no loss of the activity was observed. These results may indicate that the enzyme is not only thermostable but also thermophilic. In addition, the results suggest that the thermostability of the ATPase is ascribable to the protein structure itself, and that the attachment or association of the enzyme with the membrane appears not to be an important factor in conferring thermostability. As shown by Curve B in Fig. 6, the membrane-bound ATPase also exhibited its optimum temper-



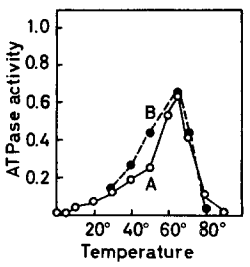


Fig. 5. Ultraviolet rotatory dispersion of the ATPase and its Moffitt–Yang plot. The sample contained 10 mg/ml enzyme and 50 mM phosphate buffer (pH 7.0). A. Optical rotatory dispersion. The light path was 10 mm or 0.1 mm, respectively, below and above 250 mμ. The curve on the upper part of the figure represents the background. B. Moffitt–Yang plot.

Fig. 6. Effect of temperature on the ATPase activity. The activity was expressed in μmoles P_i per 10 min. The concentrations of the ATPase purified and membrane-bound (membrane ghosts) were, respectively, 0.9 and 150 μg/ml. A, purified ATPase; B, membrane-bound ATPase.

ature at 65°, and no loss of the activity was observed on heating the sample at 65° for 1 h.

When the ATPase both in purified and membrane-bound states was pre-incubated at various temperatures (30–65°) for 10 min, the activities measured after cooling immediately from the incubation temperatures to 30° were all the same as the activity measured at 30° without pre-incubation. The result indicates that the enzyme does not exhibit heat activation similar to the activations reported with catalase and malate dehydrogenase from a thermophilic bacterium by NAKAMURA AND TAKAMIYA[42] and NAKAMURA *et al.*[43].

The ATPases from most mesophiles are labile to cold treatment either in the presence or absence of ADP[24,26,28], although the ATPase from *M. lysodeikticus* is very stable at 4° (ref. 31). The activity of the ATPase from thermophilic bacteria was not affected by storing the enzyme at 0–4° for 1 week, both in the presence and absence of 4 mM ADP.

Thermodynamic quantities for the ATPase

The enthalpy change (ΔH) for formation of the enzyme–substrate (ES) complex was calculated from the relationship between the Michaelis constant (K_m) and temperature (T), assuming K_m is equal to the association constant (K_s) of the ES complex[44]. As shown by Curve A in Fig. 7 the graph has a discontinuity of slope and approximates to two lines meeting at an angle, indicating a change from one value of ΔH to another at the transition temperature, 55°. The ΔH values calculated below and above 55° were, respectively, −6900 and −14 700 cal/mole. The free energy change (ΔF) calculated from K_m, also assuming that K_m is equal to K_s (ref. 44), increased continuously with increasing temperature, but an abrupt increase occurred at 55° (Table IV). The entropy change (ΔS) calculated from the equation, $\Delta F = \Delta H - T\Delta S$, was also different at temperatures below and above 55°. The results may imply that some conformational change of the enzyme molecule occurred at the transition temperature 55°.

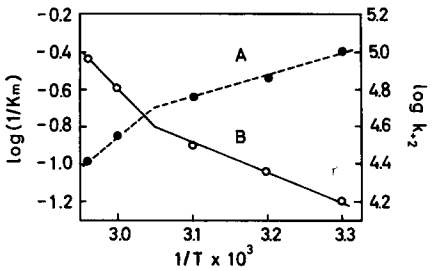


Fig. 7. Effects of temperature on K_m and k_{+2} for the ATPase. A, log $(1/K_m)$; B, log (k_{+2}).

When the reaction velocity depends on the velocity constant (k_{+2}) for the activation of the ES complex only, the absolute values of k_{+2} were obtained as the enzyme concentrations were known[44]. ΔH for activation of the ES complex, therefore, was calculated from the relation between k_{+2} and T (Fig. 7). The ΔH values were different between the temperature ranges below and above 55° (Table IV). The ΔF values calculated from k_{+2} at various temperatures exhibited an abrupt change at 55°, and the ΔS values were also different at temperatures below and above 55°.

TABLE IV

THERMODYNAMIC QUANTITIES FOR THE ATPASE

Quantity	*Temperature*				
	65°	*60°*	*50°*	*40°*	*30°*
For formation of the ES complex					
ΔH (cal/mole)		−14 700		−6900	
ΔF (cal/mole)	1550	1280	920	720	560
ΔS (cal/mole per degree)	−48	−48	−24	−24	−24
For activation of the ES complex					
ΔH (cal/mole)		18 900		8200	
ΔF (cal/mole)	9500	9600	9760	9640	9500
ΔS (cal/mole per degree)	28	28	−5	−5	−4

DISCUSSION

The investigations on the enzymes of thermophilic bacteria have so far been concerned with their thermostable properties, and none have been reported about the thermophilic properties of the enzymes. As shown in Fig. 6, the activity of the ATPase purified from *B. stearothermophilus* increased abruptly above 50° and the temperature coefficient above 50° was about 3 times greater than that below 50°. This fact may indicate that the ATPase becomes more reactive at high temperatures than at low temperatures, suggesting the thermophilic property of the enzyme. This may probably be the first observation on the thermophilicity of the enzymes from thermophiles. As the growth of the bacteria becomes rapid above this temperature, 50°, the thermophilic property of the ATPase seems to be of physiological significance. As shown by Curve B in Fig. 6, however, the membrane-bound ATPase did not

exhibit such a thermophilic property as was observed with the purified enzyme. This may indicate that conformational change of the ATPase is restricted due to the attachment or association of the enzyme with the membrane, and the enzyme appears not to exhibit the thermophilic property in intact cells.

As suggested from the thermodynamic quantities presented in Table IV, the thermophilic property of the thermophile ATPase may be due to some conformational change in the protein structure. A change from one value of ΔH to another at the transition temperature has also been reported by MASSEY[45] for mesophile fumarase (EC 4.2.1.2). This author suggested that subunit dissociation of the enzyme occurs at the transition temperature. A demonstration of a probable conformational change in the thermophile ATPase at the transition temperature (55°), therefore, is now undertaken.

MANNING AND CAMPBELL[14,15] suggested that the thermostability of α-amylase produced from *B. stearothermophilus* is ascribable to an extraordinary protein structure of a high proline content and of a random-coiled form. Protease produced from *Bacillus thermoproteolyticus* exists as a compact structure with many tyrosine and tryptophan residues buried inside the molecule, although the protein has too small a helical content[17]. Rigid structure of the protein, probably due to the characteristic high content of tyrosine[18], may account for the thermostability of the enzyme. The membrane-bound ATPase of *B. stearothermophilus*, on the other hand, was shown to contain about 20% of the α-helix form and no peculiarity in the amino acid composition can be seen in the case of this enzyme. It seems likely, therefore, that the thermostability of the thermophile ATPase is due to some factor other than an extraordinary protein structure of a peculiar amino acid composition and protein conformation, such as those of α-amylase and protease. The mode of ADP inhibition of the thermophile ATPase was found to be different from that of mesophiles. In addition, the thermophile ATPase exhibited some characteristic properties, for instance cold stability and inhibition by I_2, which are not observed with most of the mesophile ATPases. The thermostability of the thermophile ATPase, therefore, may be ascribable to some micromolecular difference in the protein structure between the thermophile and mesophile ATPases.

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