Stabilizing effect of Ca^{2+} on myosin and myofibrils of squid mantle muscle as affected by heating conditions.

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Abstract Suppressive effect of Ca^{2+} on the thermal denaturation of myosin and on myofibrils of squid mantle muscle was compared. Its stabilization effect on myosin was smaller than that on myofibrils, and was not affected by KCl concentration. The stabilizing effect of Ca^{2+} on myosin decreased with lowering the temperature for heating showing no stabilization at 20 °C, while the effect on myofibrils was evenly detected at all temperatures tested. Stabilizing effect of Ca^{2+} on myosin disappeared even at 30 °C in the presence of sorbitol showing a small inactivation rate, while the effect of Ca^{2+} was equally detected on myofibrils irrespective of the reduction of inactivation rate with sorbitol. Stabilization of myosin by Ca^{2+} again appeared even in the presence of sorbitol by raising the heating temperature to 38 °C. It was suggested that Ca^{2+} confers stabilization on myosin only when myosin is in under unstable conditions. Stabilization effect of Ca^{2+} on myosin was enhanced upon F-actin binding, namely Ca^{2+} bound myosin was more significant stabilized by F-actin binding, and the effect was no longer affected by the conditions for heating.

Keywords squid · myosin · denaturation · sorbitol · Ca^{2+}

Introduction

Thermal stability of fish myosin is deeply dependent on the environmental temperature for habitation. Consequently, the stability of myosin from cold water fish is much more labile than that from warm water fish species when compared under the identical conditions [1]. This was true for squid species [2, 3]. Squid of cold water or deep sea contained unstable myosin than one of warm water. We have reported unique properties in the thermal denaturation process of squid myofibrils and myosin subframgent-1 (S-1) that myosin in squid mantle muscle myofibrils was significantly stabilized upon addition of Ca^{2+} to the heating medium. The stabilization by Ca^{2+} was much more remarkable with myofibrils, myosin bound to F-actin, than with myosin S-1. Stabilization by Ca^{2+} with myofibrils at around 0.1 M KCl was around 100 times, while one with S-1 was as small as 5 times [4]. We studied the stabilization by Ca^{2+} by using various species of squid and cuttlefish, and concluded that the same stabilizing effect was generally observable with all species examined [3]. It is well established with fish myofibrils that high concentration of salt such as 1.5-2 M NaCl or KCl resulted in the selective denaturation of F-actin [5], and consequently myosin loses the protection by F-actin there. With squid myofibrils, salt concentration required for a complete loss of protection by F-actin occurred at about 0.75 - 0.8 M which was roughly a half concentration needed for fish myofibrils. The magnitude of the accelerated inactivation rate caused by increasing

KCl concentration or as a result of loss of protection was about 300 - 500 times, which was much greater than that obtained with fish myofibrils [2, 5]. In the present paper, we further studied the stabilizing effect of Ca^{2+} on myosin alone and on myofibrils by changing heating conditions considering the thermal inactivation rate itself to understand what factors are involved in the stabilizing action by Ca^{2+} on myosin, and how stabilizing effect of Ca^{2+} and one by F-actin binding are related to understand the significant stabilizing effect of Ca^{2+} on myofibrils.

Materials and Methods

Myofibrils were prepared from the mantle muscle of Japanese common squid *Todarodes pacificus* as described earlier [2]. Myofibrils were suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5). Myosin was prepared from the myofibrils by applying the ammonium sulfate fractionation in the presence of 2 mM $MgCl_2$-ATP, the identical procedures for isolation of myosin from carp myofibrils as has been reported [6]. In the present study, myofibrils and myosin suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) were used. For studying the stabilizing effect of Ca^{2+} on myosin and myofibrils, heating was conducted in the presence of 1 mM either $CaCl_2$ (Ca-medium) or EDTA (EDTA-medium). Free Ca^{2+} concentrations in the heating medium were set by using Ca-EGTA buffer using fixed concentration of 0.1 mM $CaCl_2$ and varied concentrations of EGTA. EGTA concentration required

was calculated by assuming the binding constant of 1 x 10^6 at pH 7.5 [7]. Heating temperature was optionally changed dependent on the purposes. In some experiments, the heating medium contained sorbitol to increase the stability or to reduce the inactivation rate at the temperature [8]. Thermal denaturation of myosin was monitored by following the Ca^{2+}-ATPase inactivation. Ca^{2+}-ATPase was assayed in a medium of 0.5 M KCl, 25 mM Tris-maletae (pH 7.0), 5 mM $CaCl_2$, and 1 mM ATP at 25°C. The inactivation rate (sec^{-1}) was calculated by assuming the inactivation as the first order reaction kinetics.

Results

Ca^{2+}-ATPase inactivation rates of squid myosin both in two media containing either Ca^{2+} or EDTA was compared with those for myofibrils by changing KCl concentrations (Fig. 1a). To estimate the inactivation rate of myosin accurately, the heating temperature was set to 25°C. The inactivation rate for myosin was slightly affected by KCl concentration both in Ca- and EDTA-media and the inactivation rate in EDTA-medium was always greater than that in Ca-medium. The stabilization by Ca^{2+} estimated from the rates was about 5 times. The inactivation rates of myofibrils in Ca- and EDTA-media above 0.8 M KCl were the same as those of myosin in respective media. It was confirmed that squid myosin lost the protection by F-actin at such high concentrations of KCl. The

rates for myofibrils below 0.4 M (Ca-medium) and 0.2 M KCl (EDTA-medium) were too small to be measured at 25 ºC. Stabilizing extent by Ca at low concentration of KCl on myofibrils was shown in Fig. 1b, in which the heating temperature was raised to 35 ºC. The result showed that strengthened F-actin binding to myosin by lowering KCl concentrations further stabilized myosin in the presence of Ca^{2+}. An apparent stabilization by Ca^{2+} at 0.05 M KCl was about 50 times, which was 10 times greater than that obtained with myosin. Moreover, the changes in the rates by increasing KCl concentration from 0.05 to 0.8 M were about 1000 times and about 40 times in Ca- and EDTA-media, respectively. The differences in the rates between two media with myofibrils decreased gradually with increasing KCl concentration due to a gradual decrease in the affinity between myosin and F-actin.

We studied whether the stabilization by Ca^{2+} on myofibrils and myosin is affected by heating temperature. Thermal inactivation rates of myosin and myofibrils at 0.1 M KCl in Ca- and EDTA-media were estimated by varying the heating temperature and the rates were analyzed by using Arrhenius plot (Fig. 2). Myofibrils gave straight lines with the identical slopes both in Ca- and EDTA-media indicating practically the same stabilizing effect of Ca^{2+} (roughly 80 times) at all temperatures studied. The estimated activation energies from the slopes were about 110 kcal/mol/degree both in Ca- and EDTA-media. However, myosin showed a complex temperature dependency giving a breaking point at around 20 ºC in Ca- and EDTA-media. At high temperature

range, the slope for EDTA-medium was steeper than that for Ca-medium. Consequently, the difference in the rates between Ca- and in EDTA-media became smaller and smaller with lowering the heating temperature. Below the breaking point, the slope was more gradual than that at high temperature range. Moreover, no stabilizing effect of Ca^{2+} was observed any more at lower temperature range. The activation energies obtained with myosin at higher temperature range in Ca- and EDTA-medium were 80 and 100 kcal/mol/degree, respectively and one at lower temperature zone was 45 kcal/mol/degree, which was roughly a half that of high temperature range. Inactivation rate of myosin at 20 ^{o}C where no stabilizing effect of Ca^{2+} on myosin was detected was 5 x 10^{-5} sec^{-1}, while the stabilizing effect by Ca^{2+} was still detectable with myofibrils with the same inactivation rates in Ca-medium. The inactivation rates of myofibrils below 30 ^{o}C were too small to measure, and the stabilizing effect of Ca^{2+} on myofibrils at such low temperatures could not be studied.

As the stabilizing effect of Ca^{2+} on myosin and on myofibrils was distinguished, we measured Ca^{2+} concentration requirement for the stabilization for both samples. Free Ca^{2+} concentration was set by using Ca-EGTA buffer system. Relative stabilization by Ca^{2+} obtained with myosin and with myofibrils was calculated by assuming the stabilization at 1 mM $CaCl_2$ and EDTA as 100 % and 0 %, respectively (Fig. 3). Myosin was fully stabilized above 10 μM with a half maximal stabilization at 12.6 nM. The concentration was 240 times lower than that required for the Ca-regulation of Mg-ATPase of squid myosin in which a half activation of Mg-ATPase was observed

at 3 μM Ca^{2+} [9]. Thus, the Ca^{2+} for regulation and for stabilization was clearly distinguished each other. Myofibrils were fully stabilized above 10 μM with half stabilization at 200 nM. The concentration required for achieving the stabilization for myofibrils was higher than that for myosin by about 16 times. The result indicated that the increased extent of stabilization by Ca^{2+} upon F-actin binding was accompanied by the lowered sensitivity to Ca^{2+}.

In Fig. 2, we found that the stabilization by Ca^{2+} disappeared at low temperatures where the inactivation rates were very small. We wondered whether stabilization by Ca^{2+} is determined by the inactivation rates themselves or heating temperatures. We tried to answer the above questions by changing inactivation rates of myosin and myofibrils artificially. Our first attempt was to study the effect of sorbitol addition to the heating medium so as to reduce the inactivation rate at the heating temperature. Heating temperatures were chosen to give similar inactivation rates for myosin and for myofibrils, 30 °C for myosin and 40 °C for myofibrils, respectively. The inactivation rates in the presence of various concentrations of sorbitol were estimated in Ca- and EDTA-media and their logarithmic values were plotted against sorbitol concentrations according to Ooizumi *et al.* [8]. As Ooizumi *et al.* have reported with fish myofibrils, the plotting gave straight lines for all cases (Fig. 4a, b). The inactivation rates for myofibrils both in Ca- and EDTA- media decreased similarly with increasing sorbitol concentration, and the same stabilization by Ca^{2+} was observed at any concentrations of sorbitol or with any inactivation rates (Fig. 4a). For example, inclusion of 1.2 M

sorbitol decreased the rate to 1/60 that without sugar, while the same stabilization by Ca^{2+} was observed. Squid myosin was also stabilized upon addition of sorbitol in a concentration dependent manner both in Ca- and EDTA-media (Fig. 4b). However, slopes for Ca- and EDTA media were clearly different from each other. The slope with Ca^{2+} (1.29/mol of sorbitol) was a little smaller than that obtained with EDTA (1.67/mol sorbitol). Consequently, the difference in the rates in Ca- and EDTA-media became smaller and smaller with increasing the concentration, and finally the rates in two media was identical at 1.8 M sorbitol. We noticed that the inactivation rate in the presence of 1.8 M sorbitol with Ca^{2+} and with EDTA was about 370 and 2170 times smaller than those without sorbitol, respectively. The rates at 30 ºC with 1.8 M sorbitol (1.42×10^{-5} sec^{-1}) corresponded to the rates at 15 ºC in the absence of sorbitol in Fig. 2. As the myofibrils with a similar inactivation rate upon addition of sorbitol still showed the stabilization effect of Ca^{2+}, the stabilized effect of Ca^{2+} was determined by the inactivation itself not by the heating temperature.

Inclusion of 1.8 M sorbitol made it possible to estimate the inactivation rates of myosin at higher temperatures, a similar temperature range for heating of myofibrils without sorbitol. Inactivation rates of myosin in Ca- and EDTA-media with 1.8 M sorbitol were estimated at various temperatures and the rates were analyzed by using Arrhenius plot (Fig. 5). The profiles without sorbitol were taken from Fig. 2 for a comparison. Stabilizing effect of Ca^{2+} on myosin appeared again even in the presence of sorbitol when the temperature was raised to 38 ºC. Heating of myosin

with sorbitol at 38 °C gave similar rates to ones without sorbitol at 30 °C in respective two media. The stabilization by Ca^{2+} obtained was 4.4 times under the conditions. A gradual decrease of stabilizing effect by Ca^{2+} with lowering the heating temperature was reproduced again. The inactivation rates with Ca^{2+} and with EDTA became the same below 31 °C in the presence of 1.8 M sorbitol. The rates at 31 °C with sorbitol were rather similar to those without sorbitol at 20 °C. A general feature of temperature dependent inactivation rates for myosin with sorbitol was similar to that without sorbitol, although the slopes were different from each other. The slopes for myosin with sorbitol were steeper than those for ones without sorbitol. The addition of sorbitol changed the activation energies for myosin denaturation both in Ca- and EDTA-media. These were 107 and 144 kcal/mol, respectively, which were larger than those without sorbitol (81 and 104 kcal/mol for Ca- and EDTA-media). At 30 °C, the inactivation rates of myofibrils were larger than those of myosin with sorbitol (see Fig. 2). Appearance of the stabilizing effect of Ca^{2+} on myosin in the presence of sorbitol at high temperature and its loss at relatively low temperature indicated that the stabilizing effect of Ca^{2+} was not determined by the temperature for heating but by the denaturation rate itself.

Discussion

As the KCl concentration for heating of myosin alone did not affect the stabilization by Ca^{2+} (about 5 times), a significantly large stabilization by Ca^{2+} observed with myofibrils (roughly

100 times) at low-salt medium was a consequence of binding with F-actin. Since F-actin binding stabilizes myosin significantly, it is certain that F-actin changes the conformation of myosin so as to be resistant to heating. We confirmed that apparent stabilization upon F-actin binding in the presence of Ca^{2+} was more than 500 times, while the one in the presence of EDTA was only about 40 times (Fig. 1b). The fact indicated that the stabilization by Ca^{2+} and by F-actin binding is not a simple sum. When we assume the stabilization extent by F-actin in the presence of EDTA is the magnitude of stabilization truly achieved by F-actin binding, the increased stabilization by F-actin was achieved with Ca^{2+} bound myosin. Protective effect of F-actin on myosin was negligible above 0.8 M KCl and small protection was found at around 0.4-0.5 M, while the apparent stabilization effect by Ca^{2+} for the sample was nearly the same as found with myosin alone (Fig. 1a). Thus the two stabilizing effects were clearly distinguished.

F-actin bound myosin, myofibrils, was different from myosin in several points in terms of stabilization by Ca^{2+}. Lowering temperature lost the stabilization by Ca^{2+} for myosin, while such change in temperature unaffected the effect by Ca^{2+} with myofibrils. Decreased inactivation rate rather than heating temperature itself was the reason for the loss of stabilization there because the inclusion of sorbitol so as to reduce the inactivation rate diminished the stabilization by Ca^{2+} at the same temperature. The conclusion was confirmed by the fact that the stabilization by Ca^{2+} appeared again even with sorbitol by raising the temperature so as to give large inactivation rate. Under these

conditions, stabilization by Ca^{2+} was equally detectable with myofibrils. These results indicated that structural change caused by Ca^{2+} binding to myosin is detectable only when myosin is placed under unstable conditions. The change might not be large because the effect was easily lost by shifting the conditions. However, myofibrils in the presence of sorbitol showing very small inactivation rates still showed the same stabilization by Ca^{2+} as observed without sorbitol. The stable structure of Ca^{2+} bound myosin seemed sensed by F-actin binding. Stabilizing effect of Ca^{2+} on myosin and on myofibrils was summarized in Table 1. With myosin, stabilization was observed only when the inactivation rates were 1-3 x 10^{-3} (sec^{-1}), and never be observed with the inactivation rate of 1-5 x 10^{-5} (sec^{-1}), 100 times smaller. However, stabilizing effect on myofibrils was equally observed irrespective of the inactivation rates. The rate with Ca^{2+} at 40 ^{o}C in the presence of 1.3 M sorbitol was al low as 1-5 x 10^{-5} (sec^{-1}). Nevertheless, the stabilizing effect was the same.

Moreover, the effect of Ca^{2+} on myosin itself and on F-actin bound myosin was also distinguished by Ca^{2+} requirement for the stabilization (Fig. 3). Although the stabilization extent by Ca^{2+} was greater with F-actin bound myosin, myofibrils required higher Ca^{2+} concentration for a full stabilization than myosin itself. As the Ca^{2+} concentration for stabilization was clearly lower than that for Ca-regulation, Ca^{2+} binding to regulatory light chain is not involved in the mechanism (Fig. 3). Different concentration requirement with myofibrils (F-actin-bound myosin) from myosin also suggested different mechanism from Ca-regulation because Ca^{2+} requirements for Mg-ATPase

activation with myosin and actin-myosin complex are the same [9]. At present we have no information on the Ca^{2+} binding region on myosin for stabilization.

As the Ca^{2+} concentration required for the stabilization was remarkably lower than that for Ca-regulation, myosin in muscle is always kept stable irrespective of contraction and relaxation. After the death of squid, ATP consumption leads to a rigor contraction resulted in a strong binding of F-actin to myosin [10]. The complex formation makes squid myosin very stable during the storage of squid muscle. Indeed squid myosin itself is not so stable, but two significant stabilizing factors, Ca^{2+} and F-actin, make squid myosin uniquely stable. The properties of squid myosin contribute the long life of squid muscle without loss of quality during the frozen storage.

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Legends of figures

Fig. 1 Effect of KCl concentration on the stabilizing effect of Ca^{2+}. (a); Squid myosin (circles) or myofibrils (triangles) were heated in the presence of 1 mM $CaCl_2$ (open symbols) or EDTA (closed symbols) at 25 ºC, and the inactivation rates were estimated. (b); The same experiments as in (a) were conducted only with myobibrils in the presence of 1 mM $CaCl_2$ (open symbols) or EDTA (closed symbols) at 35 ºC

Fig. 2 Temperature dependent stabilizing effect of Ca^{2+} on myosin and myofibrils. Myosin (circles) and myofibrils (triangles) of squid were suspended in a medium containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.5). Thermal inactivation rates for the two preparations were estimated by changing heating temperatures. Open and closed symbols denote the heating in media containing 1 mM $CaCl_2$ and EDTA, respectively.

Fig. 3 Ca^{2+} concentration dependent stabilization of squid myosin and myofibrils. Squid myosin (triangles) and myofibrils (circles) were suspended in a medium containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) and 0.1 mM $CaCl_2$ and desired concentrations of EGTA to give desired free Ca^{2+} concentrations. Heating temperatures for myosin and for myofibrils were 25 and 35 ºC, respectively.

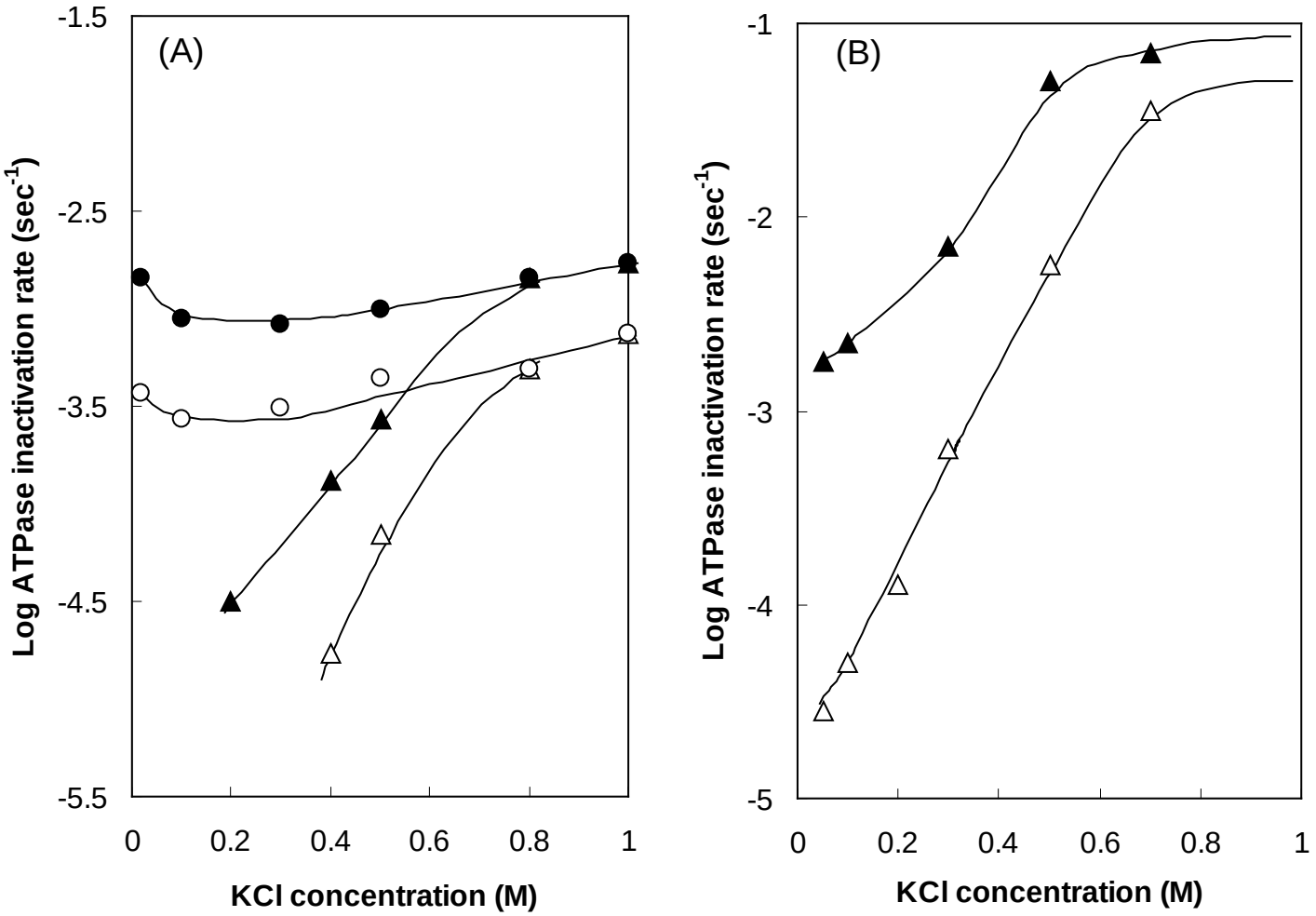
Fig. 4 Effect of sorbitol on the stabilizing effect of Ca^{2+} on myosin and myofibrils. Squid myofibrils (a) and myosin (b) were suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) in media containing 1 mM $CaCl_2$ (open) or EDTA (closed), and heated in the presence of various concentrations of sorbitol. Heating temperatures for myosin and myofibrils were 30 and 40 ºC, respectively. The inactivation rates in logarithmic values were plotted against sorbitol concentrations added.

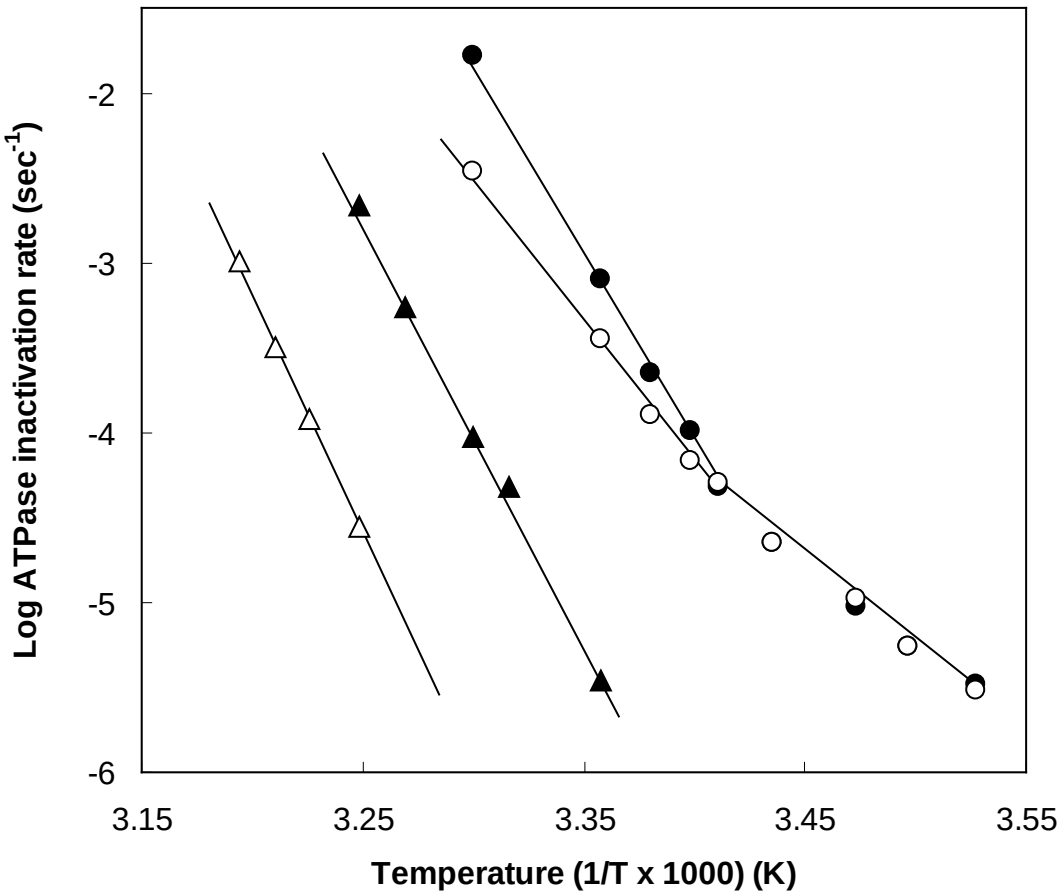
Fig. 5 Temperature dependent stabilizing effect of Ca^{2+} on myosin with and without sorbitol. Squid myosin with either 1 mm $CaCl_2$ (open) or EDTA (closed) was heated as in Fig. 2. The heating media also contained 1.8 M sorbitol (squares). The same results without sorbitol (circles) as presented in Fig. 2 were shown again.

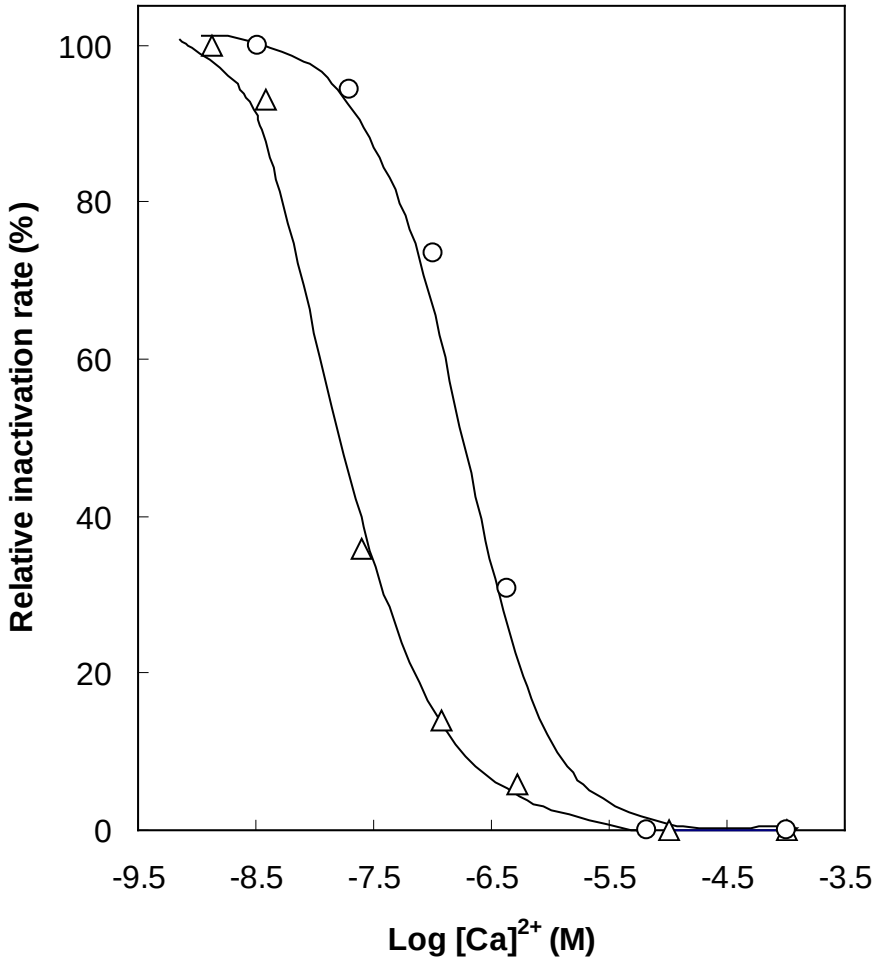
Table 1 Stabilizing effect of Ca^{2+} on myofibrils and on myosin under various heating conditions.

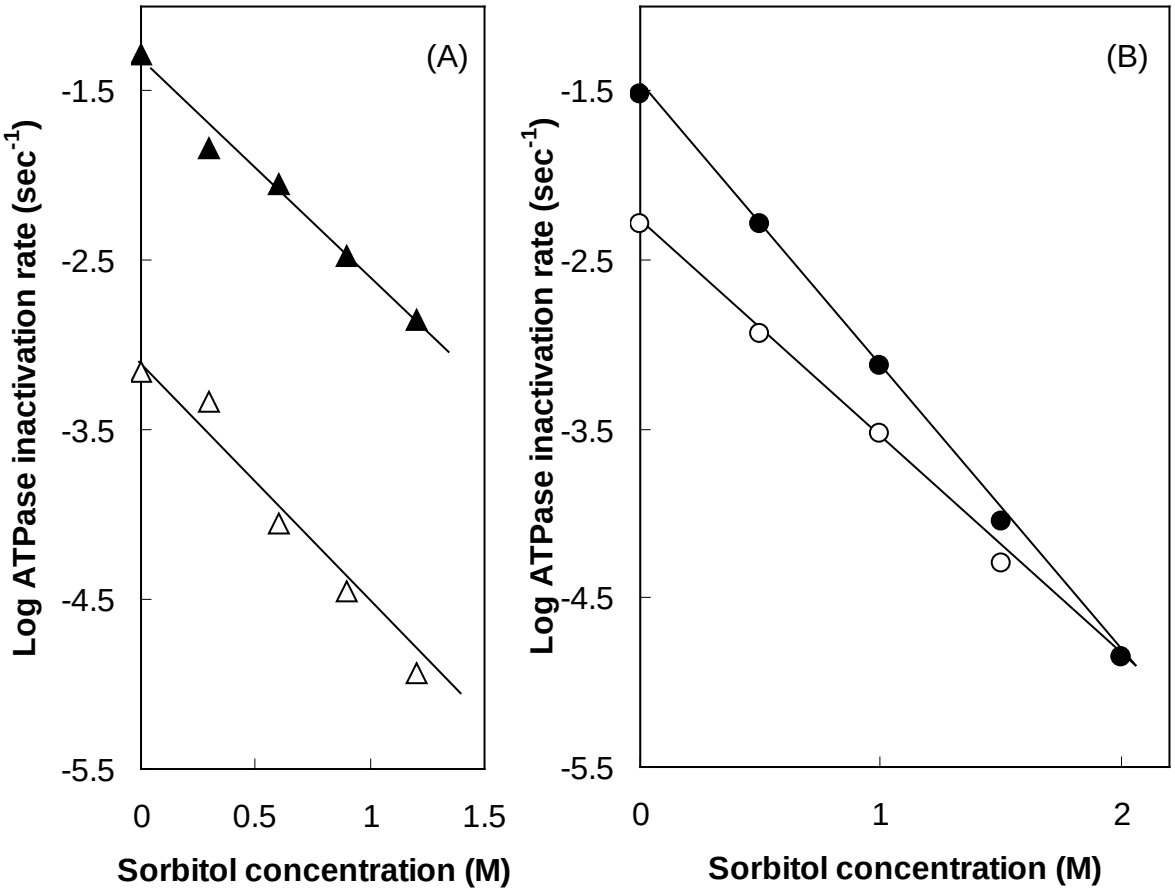
Sample	Temperature (ºC)	Sorbitol (M)	Inactivation rate in Ca-medium(sec^{-1})	Stabilization*
Myofibrils	40		2.5×10^{-5}	100
Myosin	40	1.3	1×10^{-5}	100
	30		3.5×10^{-3}	5
	20		4.5×10^{-5}	0
	30	1.8	1.2×10^{-5}	0
	40	1.8	1.4×10^{-3}	4.4

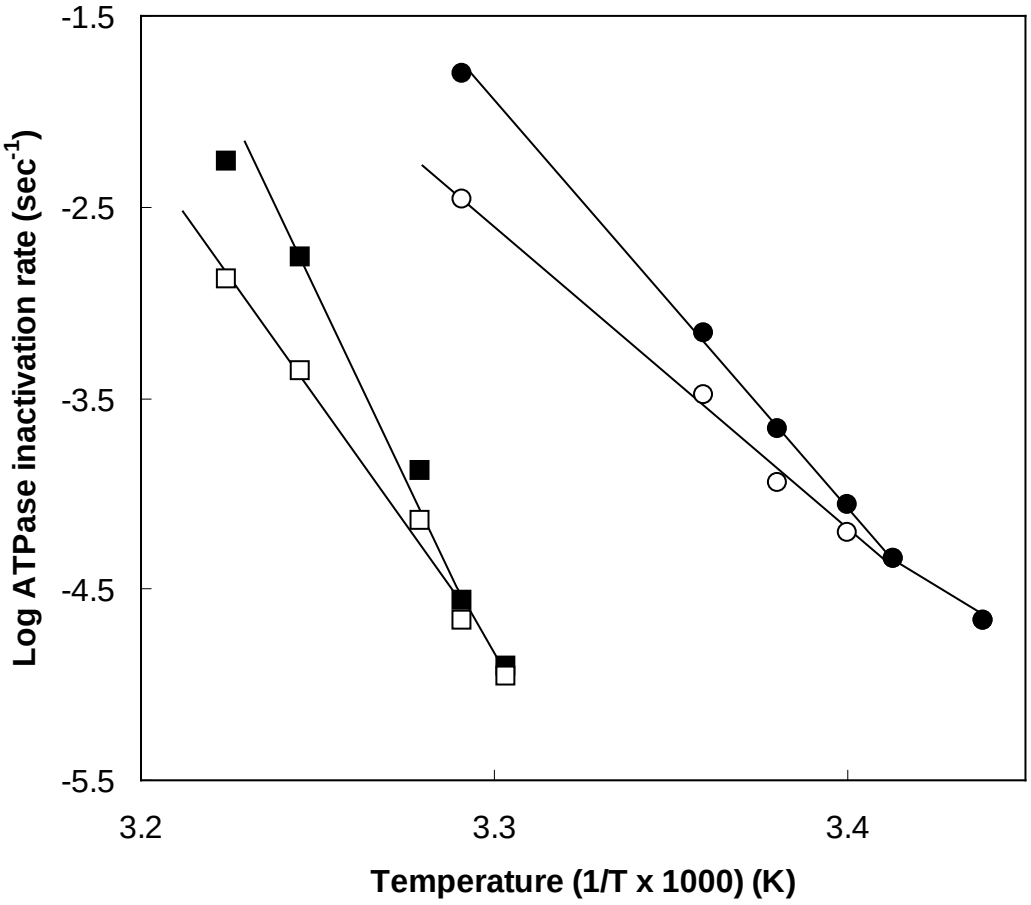
* The extent was expressed as “Rate in EDTA-medium/Rate in Ca-medium”


(A)
(B)
Log ATPase inactivation rate (sec-1)
KCl concentration (M)
-1.5
-2.5
-3.5
-4.5
-5.5
-1
-2
-3
-4
-5
0
0.2
0.4
0.6
0.8
1


Log ATPase inactivation rate (sec-1)
-2
-3
-4
-5
-6
3.15
3.25
3.35
3.45
3.55
Temperature (1/T x 1000) (K)


Relative inactivation rate (%)
100
80
60
40
20
0
-9.5
-8.5
-7.5
-6.5
-5.5
-4.5
-3.5
Log [Ca]2+ (M)


(A)
(B)
Log ATPase inactivation rate (sec-1)
-1.5
-2.5
-3.5
-4.5
-5.5
0
0.5
1
1.5
2
Sorbitol concentration (M)


-1.5
-2.5
-3.5
-4.5
-5.5
3.2
3.3
3.4
Log ATPase inactivation rate (sec-1)
Temperature (1/T x 1000) (K)