CHAPTER IV

ROLES OF ALKALI LIGHT CHAINS IN STABILIZATION OF MYOSIN

In the previous chapter, it was established that alkali light chains are directly involved in actin-myosin interaction. However, since the interaction can be recognized only at low salt concentration, it is probable that they have another significant role *in situ*. Mrakovcic-Zenic *et al*. (1981) reported that S1(A1) was more heat-labile than S1(A2) at KCl concentration as low as 5 mM. They also observed by circular dichroism measurements that KCl induced different conformational changes in S1 isozymes. Therefore, it is natural that alkali light chains are involved in maintaining the conformation of myosin head. However, differences in properties between S1 isozymes other than ATPase activity and behaviors on actin- or nucleotide-matrix have not been reported. In general, physicochemical properties have been regarded as the same for both isozymes.

Burke and Sivaramakrishnan (1981b) suggested that heavy chain-light chain interaction is not the same between S1(A1) and S1(A2), and that interaction of A2 with heavy chain is intrinsically weaker than that occurring with A1. They claimed that the folowing equilibration type exists with a very small dissociation constant,

$$HC \cdot ALC \rightleftharpoons HC + ALC,$$

where HC and ALC represent heavy chain and alkali light chains, respectively.

At 37°C or higher temperatures, there is a loss of the

normal tryptic digestion pattern of S1, as evidenced by decreased intensity of the three fragments as well as of light chains (Burke et al., 1987). The 50 K fragment seems preferentially to be unfolded during mild heat treatment (Setton and Muhlrad, 1984; Setton et al., 1984). Burke et al. (1987) revealed that thermal inactivation rate of ATPase was closely correlated with the tryptic decay of the 50 K fragment.

On the other hand, Mocz et al. (1984) reported that nucleotide stabilized S1 molecule, not only the region close to the nucleotide binding site in the 25 K fragment, but also the adjacent 50 K fragment.

As described above, there seems to be significant differences in stability between S1 isozymes, mainly due to the associated alkali light chains. In this chapter, attempts were made to compare the differences in thermostability and resistibility against denaturants between both S1 isozymes. Furthermore, protective effects of alkali light chains, actin, ATP and KCl against heat denaturation of S1 were examined.

Section 1

Thermostability of Subfragment 1 Isozymes

It has been reported that ATPase inactivation and dissociation of light chains occurred during salt and heat denaturation of myosin (Dreizen and Gershman, 1970; Dreizen and Richards, 1972; Higuchi et al., 1978;Wikman-Coffelt et al., 1979). Dreizen and Gershman (1970) also claimed that heat denaturation of myosin is accompanied by selective dissociation of light chains. However, it is not clear at present whether release of light chains is directly related with the inactivation of particular myosin head, S1(A1) or S1(A2).

Mrakovcic-Zenic et al. (1981) observed differences in heat-lability between S1 isozymes at low salt concentration, but no further report is available on the stability difference of S1 isozymes. Sivaramakrishnan and Burke (1981b) demonstrated a dynamic equilibrium in the interaction of S1 heavy chain and alkali light chains at physiological ionic strength and temperature. As demonstrated in Section 4 of Chapter III, there was a marked difference in affinity for heavy chain between A1 and A2. It follows that the above differences might affect the conformational stability or ATPase inactivation of both S1 isozymes.

In this section, thermostability of S1 isozymes was compared, taking inactivation rate of Ca-ATPase and turbidity change as parameters. In addition, heat-induced changes in tryptic digestion pattern and release of light chains were examined.

Materials and Methods

S1 isozymes from tilapia, bigeye tuna and rabbit were prepared as described in Section 1 of Chapter III.

Inactivation rate constant of Ca-ATPase

S1 in 25 mM Tris-maleate (pH 8.0) at concentration of 0.5 mg/ml was incubated in a water bath at 20 - 35°C for up to 1 h. A portion was taken out at due time intervals, immediately cooled in ice, and the remaining Ca-ATPase was measured as described in Section 1 of Chapter III.

Measurement of turbidity

S1 was incubated in 25 mM Tris-maleate (pH 8.0) at concentration of 0.7 - 1 mg/ml at 35 or 40°C for up to 1 h. At due time intervals, a portion was taken out, and the turbidity at 340 nm (T_{340}) was measured with a Shimadzu UV-160 spectrophotometer. T_{340} value was corrected for that of 1% solution incubated for t min ($T_t^{1\%}$) and used for the parameter of thermostability.

Determination of dissociation ratio of light chains from S1

S1 was incubated under the same conditions as in turbidity assay. The sampled portions were subjected to ultracentrifugation at 25,000 x g for 30 min, and the supernatants were analyzed by SDS-PAGE. The amounts of heavy and light chains remaining in the supernatant were determined densitometrically, taking the intensities of the bands of both chains in intact S1 as 100%.

Tryptic digestion pattern

Tryptic digestion was performed on heat-denatured S1 isozymes as described in Section 3 of Chapter III.

Results and Discussion

Inactivation rate of ATPase activity

Inactivation of Ca-ATPase activity of S1 isozymes proceeded as a first order reaction (data not shown). The rate constants are shown in Table IV-1-1. In case of tilapia, the constants at 30°C were 57×10^{-5} and $34 \times 10^{-5} s^{-1}$ for S1(A1) and S1(A2), respectively, and those at 35°C were 197×10^{-5} and $146 \times 10^{-5} s^{-1}$, respectively. The data indicate that S1(A2) is more thermostable than S1(A1) irrespective of incubation temperature.

Both bigeye tuna S1 isozymes were very heat labile. The inactivation rate constants at 20°C were 61×10^{-5} and $38 \times 10^{-5} s^{-1}$ for S1(A1) and S1(A2), respectively, indicating again the higher stability of S1(A2). This was also true for rabbit S1 isozymes. The rate constants at 30°C were 24×10^{-5} and 14×10^{-5} for S1(A1) and S1(A2), and those at 35°C were 43×10^{-5} and $33 \times 10^{-5} s^{-1}$, respectively. Such differences in stability between S1 isozymes have not yet been reported. If the incubation was performed at higher or lower temperatures, differences between S1 isozymes could not be recognized.

Turbidity change

Time courses of turbidity change of both isozymes during incubation are shown in Fig. IV-1-1. Throughout all the S1 isozymes examined, the rate and maximum value were clearly higher with S1(A1) than S1(A2). It is noteworthy that a marked time lag is recognized in the denaturation profile of S1(A2). In an attempt to compare the profiles of turbidity change, the turbidity of 1% S1 solution after t min incubation was calculated

Table IV-1-1. Thermal inactivation rate constants of Ca-ATPase of S1 isozymes from tilapia, bigeye tuna and rabbit

($\times 10^{-5} s^{-1}$)

	Tilapia		Bigeye tuna	Rabbit	
	30℃	35℃	20℃	30℃	35℃
S1(A1)	57	197	61	24	43
S1(A2)	34	146	38	14	33

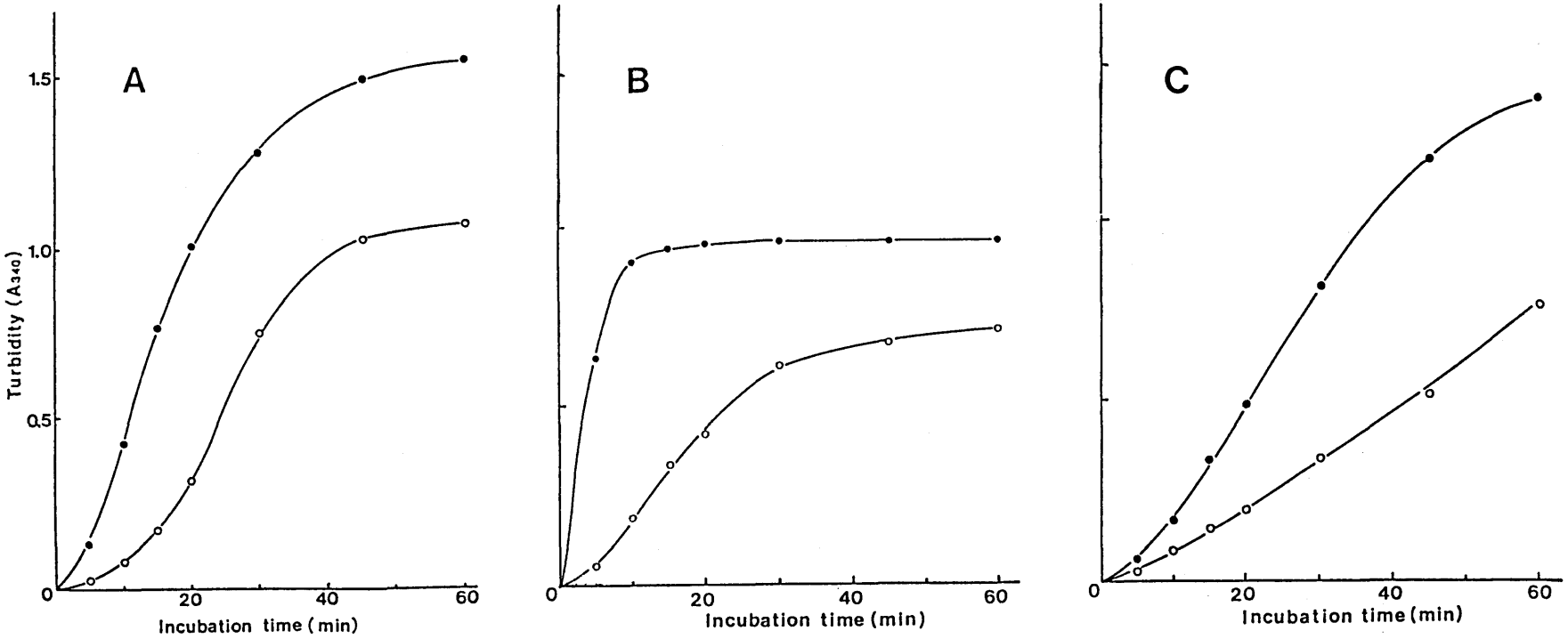

Fig. IV-1-1. Turbidity change of S1(A1) and S1(A2) from tilapia (A), bigeye tuna (B) and rabbit (C) during incubation at 35° C. The protein concentration was about 0.7 mg/ml. Closed and open circles represent S1(A1) and S1(A2), respectively.

(Table IV-1-2). In each case, $T_{10}^{1\%}$, $T_{30}^{1\%}$ and $T_{60}^{1\%}$ represent the initial rise (time lag), the denaturation rate and the maximum turbidity, respectively.

The most remarkable differences between S1 isozymes were observed with $T_{10}^{1\%}$ values for all S1's. $T_{10}^{1\%}$ values of S1(A1) were about 5, 4 and 2 times higher than S1(A2) with tilapia, bigeye tuna and rabbit, respectively. This suggests that there is at least two steps in the denaturation profiles of S1(A2). In case of rabbit, $T_{30}^{1\%}$ of S1(A1) was about 3 times of S1(A2), indicating the higher thermostability of these S1. On the contrary, turbidity of bigeye tuna S1(A1) reached a plateau at the initial stage.

When the time courses of remaining Ca-ATPase were compared to those of turbidity change (Fig. IV-1-2), the profiles were very similar with S1(A1), while the turbidity increase was retarded at the initial stage in case of S1(A2). These results suggest that subtle structural changes affecting ATPase activity precede the structural changes responsible for turbidity increase.

The S1 isozymes incubated under the same conditions were subjected to ultracentrifugation, and the supernatants were analyzed by SDS-PAGE. As a result, heavy chain content in the supernatant rapidly decreased along with the turbidity increase, whereas the light chains were partially released from the heavy chain and remained in the supernatant (Fig. IV-1-3). These data raise the possibility that release of light chains from the heavy chain, though partially, caused heavy chain denaturation.

Table IV-1-2. $T_{10}^{1\%}$, $T_{30}^{1\%}$ and $T_{60}^{1\%}$ values of S1 isozymes of tilapia, bigeye tuna and rabbit at 35°C

	Tilapia		Bigeye tuna		Rabbit	
	S1(A1)	S1(A2)	S1(A1)	S1(A2)	S1(A1)	S1(A2)
$T_{10}^{1\%}$	5.8	1.1	12.5	2.8	2.3	1.1
$T_{30}^{1\%}$	17.8	11.8	13.3	8.5	11.7	4.3
$T_{60}^{1\%}$	21.5	14.9	13.3	9.9	19.0	11.0

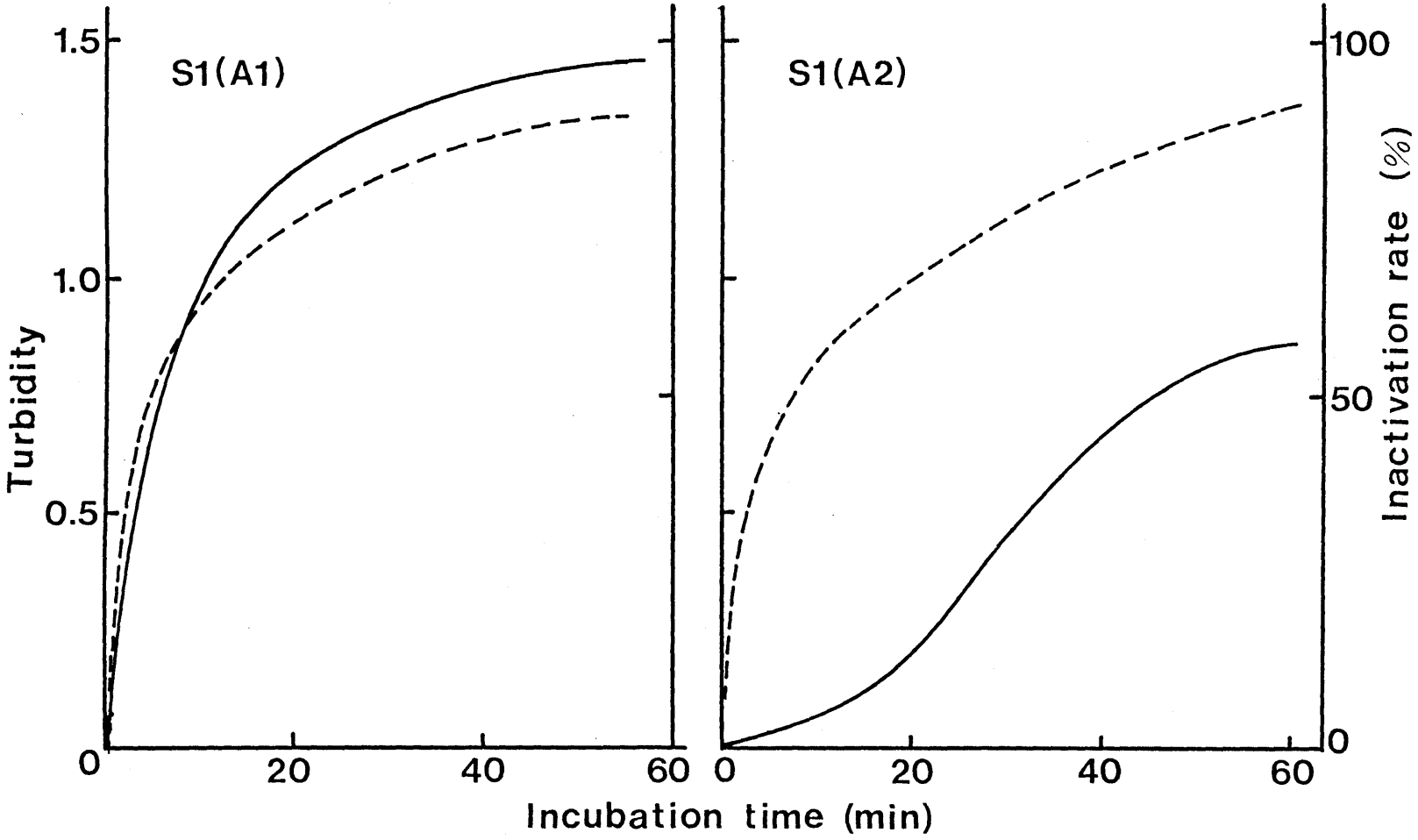

Fig. IV-1-2. Time courses of turbidity change and inactivation rate of Ca-ATPase of tilapia S1(A1) and S1(A2) during incubation at 35°C. Solid and broken lines represent turbidity and inactivation rate of Ca-ATPase, respectively.

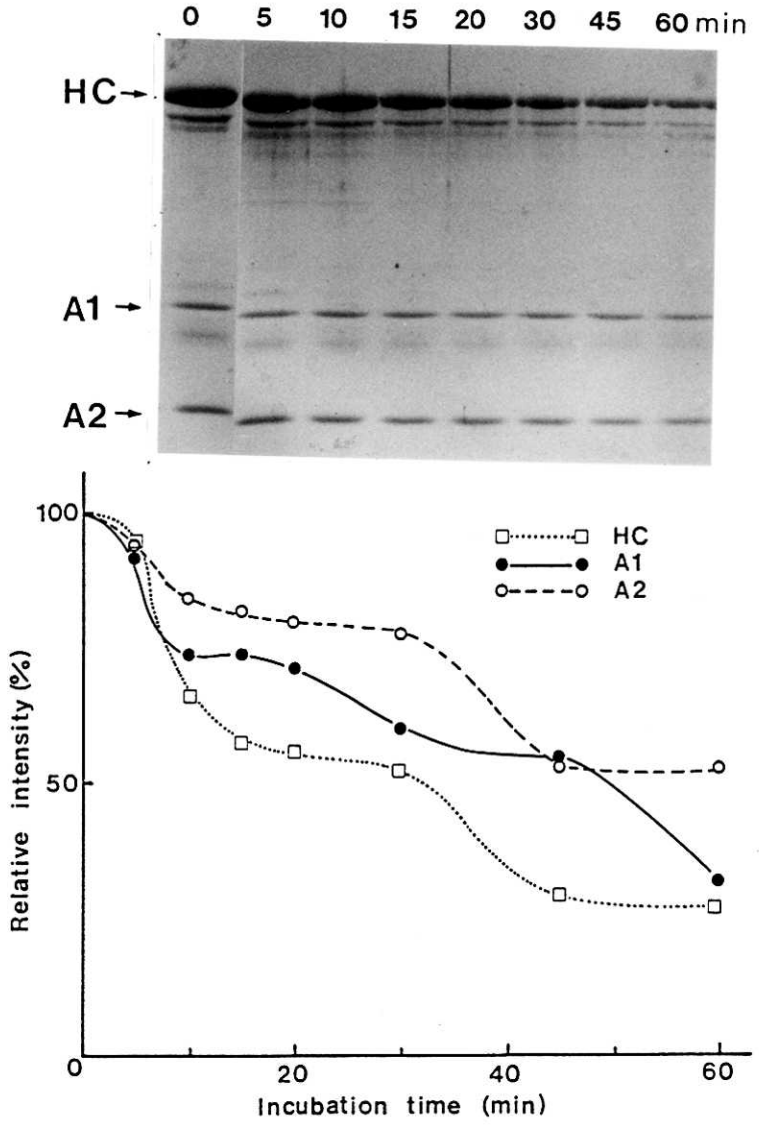

Fig. IV-1-3. Heat-induced release of light chain from rabbit S1(A1) and S1(A2). Incubation was performed at 40°C. The inserted figures represent the SDS-PAGE patterns of the supernatant obtained by ultracentrifugation of S1 solution incubated for respective times.

When heat-denatured S1 was subjected to tryptic digestion, S1(A1) became more susceptible to trypsin, while S1(A2) remained unchanged (Fig. IV-1-4). This fact supports the previous data demonstrating the higher structural stability of S1(A2). The 50 K fragment was most preferentially digested, while 25 K and 20 K fragments showed some resistance to tryptic digestion after heat treatment. Similar digestion patterns have been reported for rabbit S1. Setton and Muhlrad (1984) reported that EDTA-ATPase of S1 was decreased by 85 - 90% after 2 h incubation at 35°C. They also reported that, after mild heat treatment, trypsin digested S1 gave rise to only 25 and 20K fragments on SDS-PAGE. Burke et al. (1987) revealed that tryptic digestibility of heated S1 is markedly increased above 31°C and that 50K fragment becomes unable to refold on cooling.

Inactivation of ATPase and perturbation of heavy chain structure are considered to be due mainly to unfolding of 50 K fragment (Setton et al., 1988). Active site of ATPase has been partially localized on this fragment (Miyanishi et al., 1984; Hiratsuka, 1986), while reactive lysine residue resides on 25 K fragment (Lu et al., 1978; Hozumi and Muhlrad, 1981). Thus, interactions of A1 and A2 with 50 K fragment seem to differ from each other considerably. A1, due to presence of the difference peptide, could promote the denaturation of 50 K fragment, which in its turn, accelerates the aggregation of S1 heavy chains.

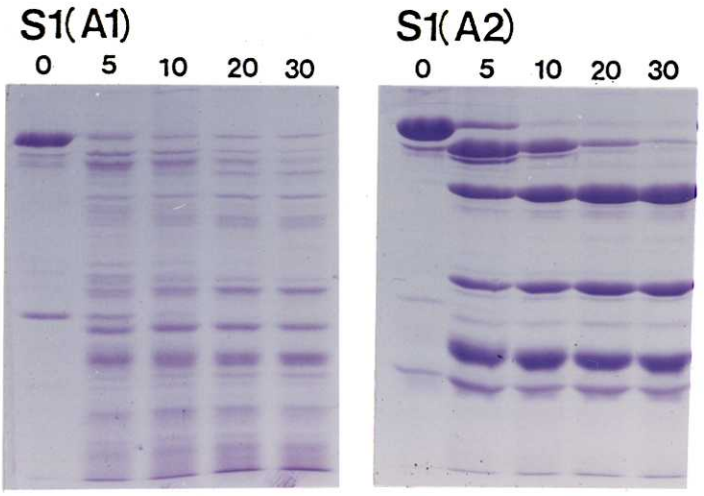

Fig. IV-1-4. Time course of tryptic digestion patterns of heat-denatured tilapia S1(A1) and S1(A2). Digestion was performed after incubation at 35°C for 10 min. 15% gels.

Section 2

Resistibility of Subfragment 1 Isozymes against Denaturants

Burke and Sivaramakrishnan (1986) examined the denaturation profile of ATPase activity of S1 in the presence of methanol and found that the inactivation rate of ATPase correlated with the loss of 50K fragment, suggesting that this fragment is essential for the ATPase function of myosin. However, such profiles have not been established in S1 isozymes.

In the previous section, it was suggested that S1(A2) is more thermostable than S1(A1). If S1(A2) possesses more rigid structure than S1(A1), S1(A2) may be more resistible against denaturants.

In this section, resistibilities against methanol and urea were compared between S1 isozymes, taking as parameters inactivation rate of ATPase and molar residue ellipticity.

Materials and Methods

S1 isozymes from tilapia and rabbit prepared in Section 1 of Chapter III were used.

Methanolic denaturation

S1 isozymes were incubated in 25 mM Tris-maleate (pH 8.0) at 20°C in the presence of 5, 10, 15 and 20% methanol. Portions were taken out at due time intervals, and measured for inactivation rate of both isozymes as described in Section 1 of this chapter.

Effect of urea concentration on molar ellipticity of S1 isozymes

S1 was incubated in 25 mM Tris-maleate (pH 8.0) at 10°C for

8 h in the presence of 0.2 - 4 M urea. Circular dichroism spectra were measured at 25° C with a JASCO J-20C spectropolarimeter equipped with a JASCO DP-501N data processor. Scanning was carried out in a range of 260 - 220 nm. Baseline was corrected by dispersion with the control buffer. The mean residue ellipticity $[\underline{\theta}]$ (degree·cm^2/dmol) at 225 nm was calculated as follows.

$$[\underline{\theta}] = \underline{\theta} / 100C \cdot \underline{l}$$

$\underline{\theta}$ = observed ellipticity in degree at 225 nm;

C = concentration in mol/liter;

$\underline{l}$ = light path length in cm.

A cell of 0.1 cm light path was used. A molecular weight of 115,000 was used for all S1 isozymes.

Results and Discussion

Inactivation of Ca-ATPase in the presence of methanol proceeded as a first order reaction (data not shown). The inactivation rate constants are shown in Table IV-2-1. In case of tilapia, the rate constants in the presence of 10% methanol were 116 x 10^{-5} and 53 x $10^{-5}s^{-1}$ for S1(A1) and S1(A2), respectively, and those in 15% methanol were 443 x 10^{-5} and 104 x $10^{-5}s^{-1}$, respectively. In both cases, the stability of S1(A2) was clearly higher than S1(A1). This was also true for rabbit S1 isozymes. The inactivation rate constants in 10% methanol were 66 x 10^{-5} and 27 x $10^{-5}s^{-1}$ for S1(A1) and S1(A2), respectively, and those in 15% methanol were 77 x 10^{-5} and 53 x $10^{-5}s^{-1}$,

Table IV-2-1. Inactivation rate constants of Ca-ATPase activity of S1 isozymes from tilapia and rabbit in the presence of 10% and 15% methanol

($\times 10^{-5} s^{-1}$)

Methanol concn.	Tilapia		Rabbit	
	S1(A1)	S1(A2)	S1(A1)	S1(A2)
10%	116	53	66	27
15%	443	104	77	53

respectively.

In 5% methanol, inactivation was hardly recognized, whereas in 20% methanol, it proceeded too rapidly to measure the rate.

Effect of methanol concentration on the tryptic digestion of S1 is shown in Fig. IV-2-1. There were no significant differences in SDS-PAGE pattern between 0 and 5% methanol incubated S1. When S1 was incubated in 10% and 15% methanol, 50 K fragment band tended to fade, due to the partial unfolding of this fragment, whereas 25 K and 20 K fragments were hardly affected irrespective of methanol concentration. These results are in good agreement with the finding by Burke and Sivaramakrishnan (1986).

Effect of urea concentration on the circular dichroism spectra of tilapia S1 is shown in Fig. IV-2-2. The ellipticity was markedly reduced in a range of 0 - 1 M urea, and at higher concentration, it decreased little by little. In this case, ellipticity due to light chain was considered to be negligeble, since the light chain content in S1 is less than one fifth of the heavy chain by weight, and the helix content in light chain is rather small (Alexis and Gratzer, 1979).

In the next place, the spectra were compared between S1 isozymes. In native state, the spectra were very similar to each other. After incubation in the absence of urea, however, the ellipticity of S1(A1) was reduced compared to S1(A2) (data not shown). As urea concentration was increased, the ellipticity was reduced faster with S1(A1) than with S1(A2). In the presence of urea higher than 1 M, the spectra were absolutely overlayed with each other (data not shown).

The mean residue ellipticity was calculated as described

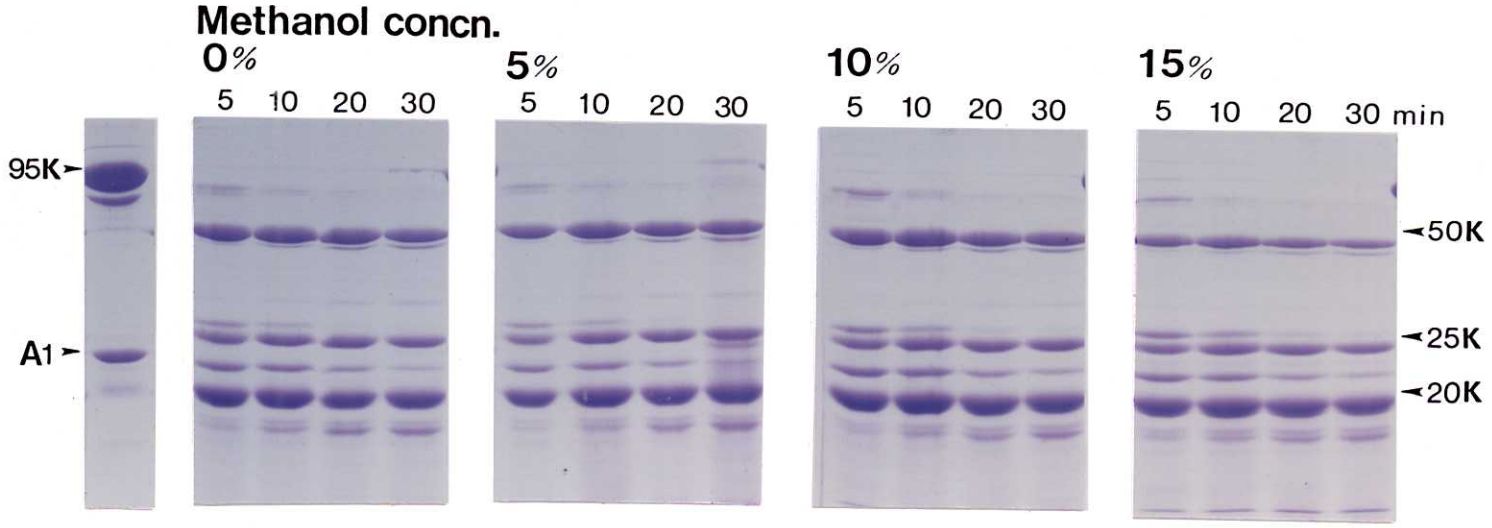

Fig. IV-2-1. Effect of methanol concentration on tryptic digestion of rabbit S1(A1), as examined by SDS-PAGE. The number at the top of each gel represents digestion time.

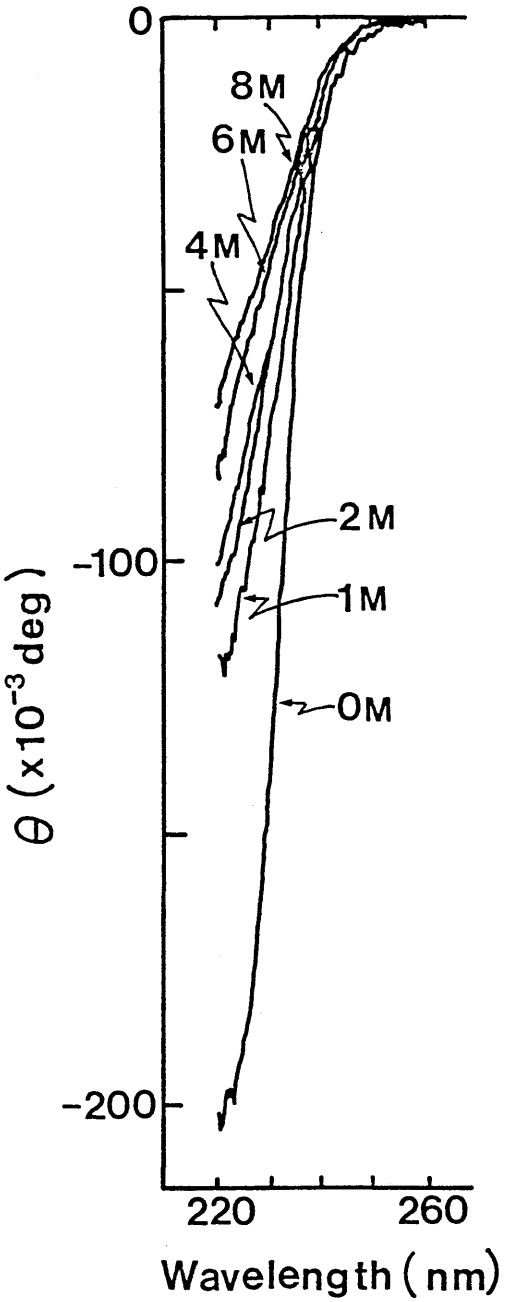

Fig. IV-2-2. Effect of urea concentration on circular dichroism spectra of tilapia S1(A2).

above, and plotted against urea concentration (Fig. IV-2-3). In the range of 0 - 1 M urea, the mean residue ellipticity was clearly higher with S1(A2) than with S1(A1), indicating that S1(A2) retains the secondary structure to more extent. These results are also in good accordance with the above data, indicating the higher stability of S1(A2).

In the presence of urea, the light chains and heavy chain might have been separated from each other, accompanying perturbation of the heavy chain. In this case as well, the differences in affinity for heavy chain between both isozymes could reflect differences in their dissociation rate. In the absence of urea, there was a significant difference in mean residue ellipticity between both isozymes. It is probable that there is a significant difference in the over-all conformation of heavy chain between both isozymes, although their heavy chains were completely or almost identical with each other (Chapter III, Section 3). It seems likely that A2 binds more tightly with the heavy chain, resulting in more rigid conformation than does A1. Such superiority of A2 can be accounted for by the absence of difference peptide.

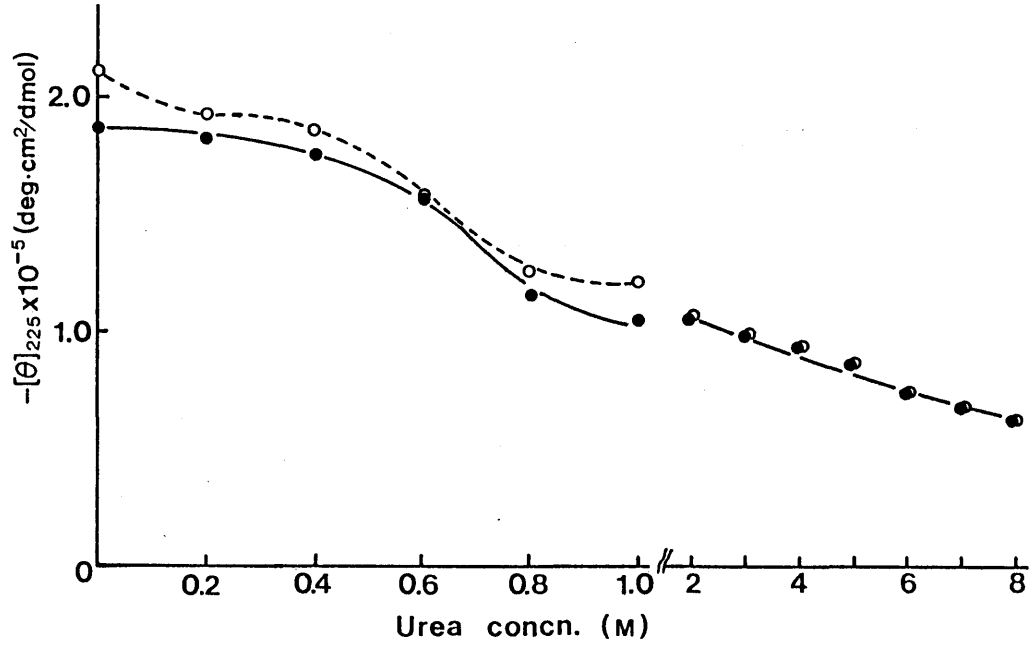

Fig. IV-2-3. Effect of urea concentration on the mean residue ellipticity of tilapia S1(A1) and S1(A2). Closed and open circles represent S1(A1) and S1(A2), respectively.

Section 3

Roles of Alkali Light Chains in Stabilization of Subfragment 1

In the previous section, it was strongly suggested that alkali light chains are involved in the stabilization of S1 heavy chain, and the denaturation was caused by the release of light chain.

Sellers and Harvey (1984) determined the binding site of both essential and regulatory light chains to be C-terminal 25 K fragment of smooth muscle S1 and 20 K fragment of skeletal S1, respectively, by overlaying radioactively labelled light chains on S1 fragments separated by SDS-PAGE. Mitchell et al. (1986) further revealed that the binding sites of both light chains were located in C-terminal 10 - 12 K region of 20 K domain. However, the most vulnerable region of S1 heavy chain is 50 K fragment. Actually, this fragment is easily unfolded, and there seemed to be differences in conformational stability of this fragment between S1 isozymes.

Burke and Sivaramakrishnan (1981b) proposed that there exists a dynamic equilibrium as to the associated and dissociated states between S1 heavy chain and alkali light chain at physiological ionic strength and temperature. Therefore, it seemed that the denaturation of S1 is inhibited, at least, to some extent, if the equilibrium is shifted to the associated state by addition of excess light chain.

In this section, S1 isozymes were incubated in the presence of excess alkali light chain, and the degree of denaturation was

examined, taking the inactivation of ATPase and turbidity change as parameters.

Materials and Methods

Preparation of light chains and S1 isozymes

A1, A2 and DTNB light chains were isolated from tilapia, bigeye tuna and rabbit myosins as described in Section 1 of Chapter II. S1 isozymes were prepared from the above preparations as described in Section 1 of Chapter III.

Incubation of S1 isozymes in the presence of excess light chains

S1 isozymes were incubated in 25 mM Tris-maleate (pH 8.0) in the presence of 0.2 - 5 fold (molar ratio) excess of A1 or A2 at 30 or 35°C. Incubation was also performed in the presence of 0.5 and 1 fold molar excess of DTNB light chain, and 0.5 and 1 mg/ml of BSA.

Analytical methods

Turbidity changes at 340 nm were measured at due time intervals as described in Section 1 of this chapter.

The inactivation rate constants of Ca-ATPase were measured as described in Section 1 of this chapter.

Ultracentrifugal analysis of the incubated samples was performed as described in Section 1 of this Chapter.

Results and Discussion

Time courses of turbidity change in tilapia S1 in the presence of excess tilapia alkali light chains are shown in Fig.

IV-3-1. Excess alkali light chains effectively inhibited the increase of heat-induced turbidity, except that in the presence of 3 - 5 molar fold excess of A1, turbidity increase was accelerated. The reason is not yet clear. However, the inhibition of turbidity change was generally dependent on the amount of light chain added, especially in case of excess A2.

The inhibitory effect was more clearly recognized with rabbit S1. As shown in Fig. IV-3-2, the turbidity change of S1(A1) was suppressed depending on the amount of alkali light chain added without any exceptions. In case of S1(A2) as well, a similar inhibitory effect was observed. When A1 or A2 was added to S1(A2), comparable inhibitory effects were noted (data not shown).

$T_{30}^{1\%}$ values of rabbit S1 in the presence of excess light chains are shown in Table IV-3-1. $T_{30}^{1\%}$ values are clearly lower in the presence of A2 (Fig. IV-3-2). This could be explained by higher affinity of A2 for heavy chain, as demonstrated in Section 4 of Chapter III.

DTNB light chain also suppressed the turbidity increase, though the degree was much less compared to alkali light chains (Fig. IV-3-3). The effect could be explained by the weak affinity of this chain to the alkali light chain binding site of S1 heavy chain, though these light chains have some sequence homology as described in Chapter II. The intrinsic binding site for DTNB light chain is missing in the present S1 heavy chain as described in Section 4 of Chapter III. In this connection, when DTNB light chain is removed from HMM or loses its 18 N-terminal

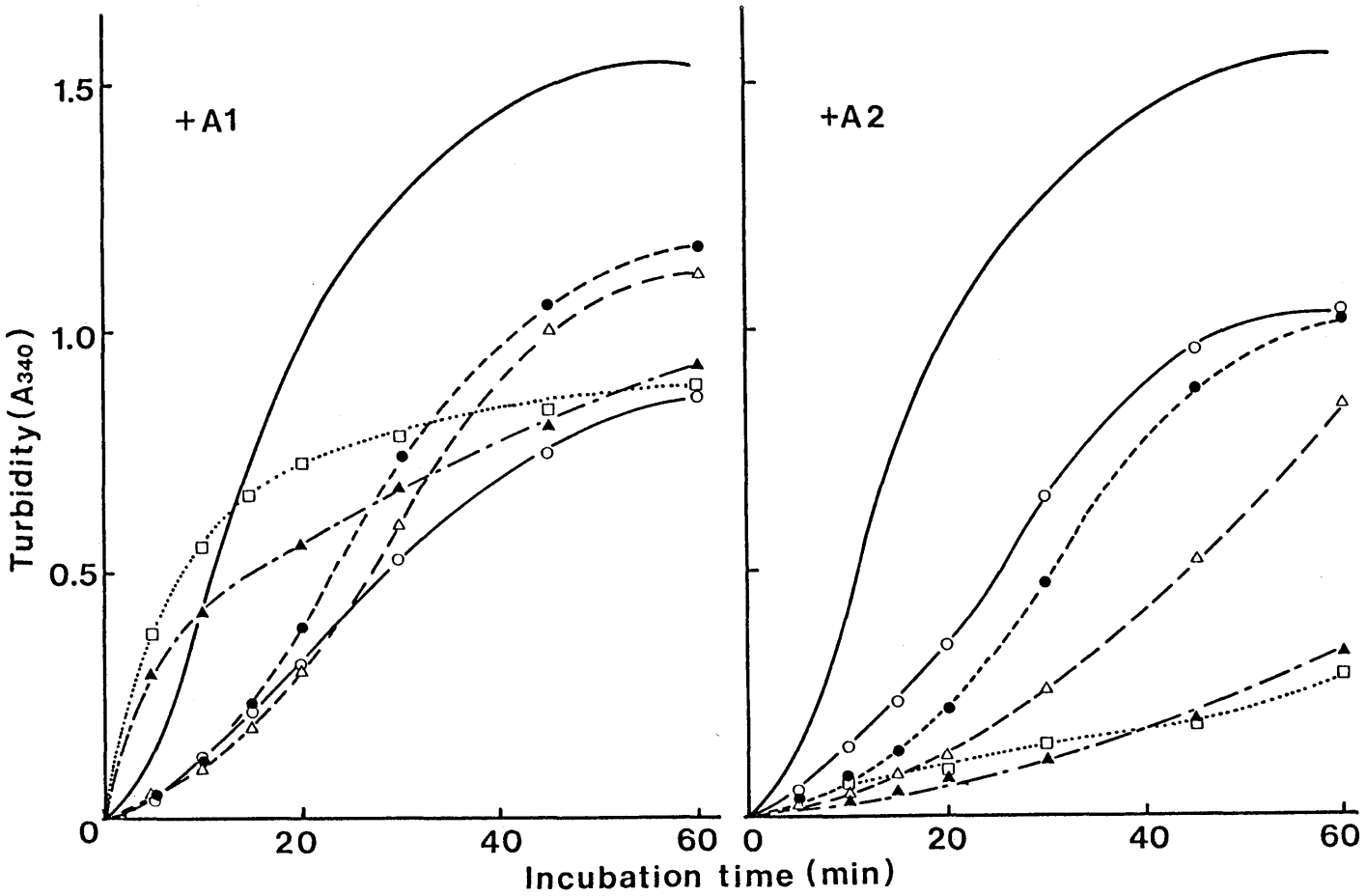

Fig. IV-3-1. Effect of alkali light chains on turbidity change of tilapia S1(A1) and S1(A2). Incubation was performed at 35° C in the presence of excess A1 or A2. Molar ratio of light chain added: ○, 0.2; ●, 0.5; △, 1; ▲, 3; □, 5. Solid line represents the data in the absence of excess light chain.

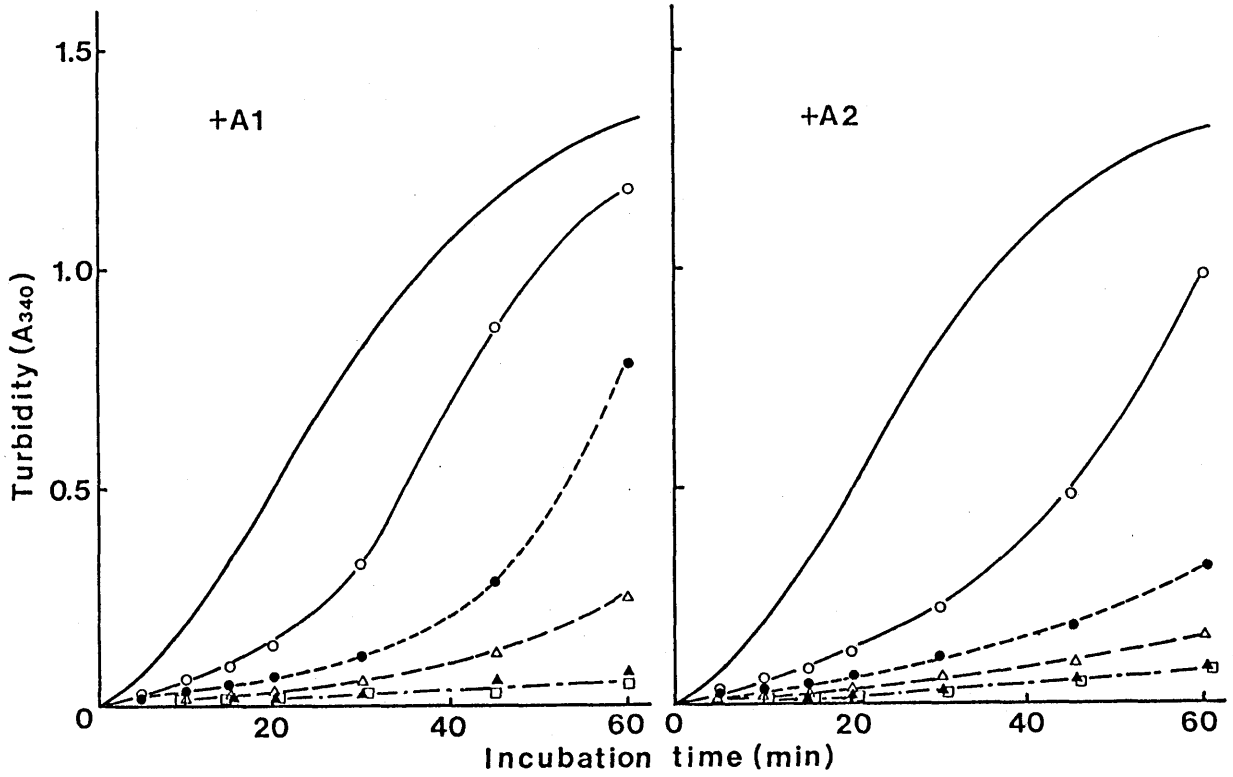

Fig. IV-3-2. Effect of alkali light chains on turbidity change of rabbit S1(A1) and S1(A2). Incubation was performed at 40° C in the presence of excess A1 or A2. Molar ratio of light chain added: ○, 0.2; ●, 0.5; △, 1; ▲, 3; □, 5. Solid line represents the data in the absence of excess light chain.

Table IV-3-1. $T_{30}^{1\%}$ values of rabbit S1 isozymes incubated at 40°C in the presence of excess light chains

Added light chain (molar ratio)		S 1 (A1)	S 1 (A2)
A1	0.2	4.48	3.06
	0.5	1.57	2.50
	1	0.91	1.66
	3	0.46	0.70
	5	0.36	0.31
A2	0.2	3.13	3.77
	0.5	1.59	2.54
	1	0.93	1.64
	3	0.56	0.84
	5	0.59	0.71
DTNB-LC	1	2.06	-
None		11.6	4.7

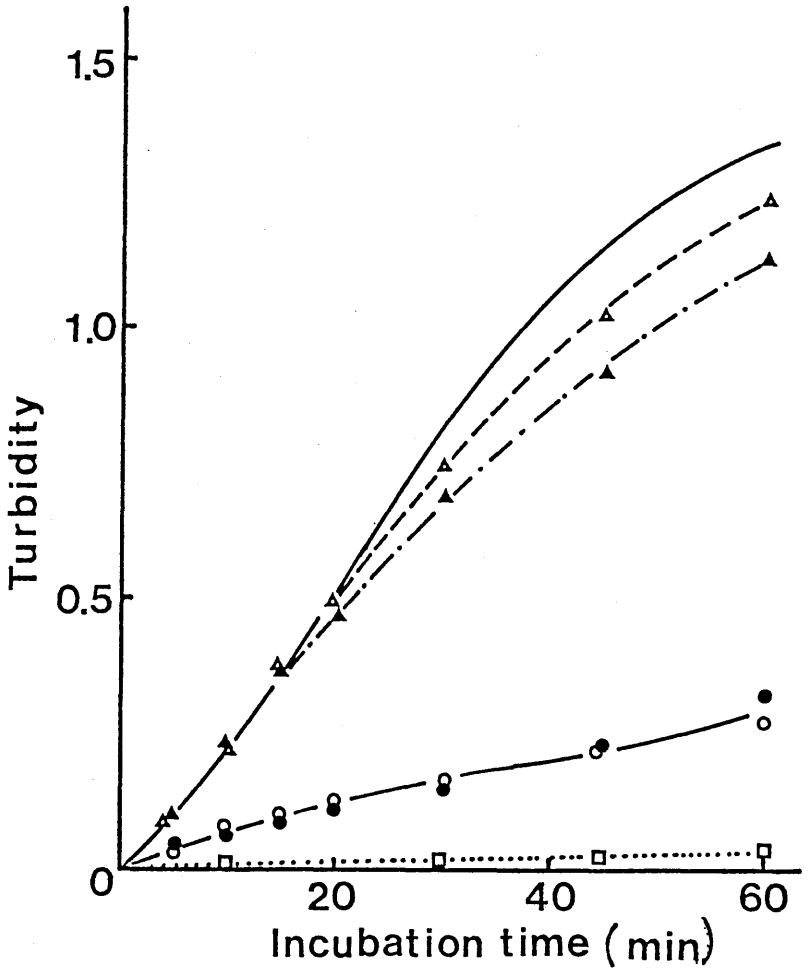

Fig. IV-3-3. Effect of DTNB light chain and bovine serum albumin (BSA) on turbidity change of rabbit S1(A1) and S1(A2). Incubation was performed at 40°C. Proteins added are as follows: ▲, 0.5 mg/ml BSA; △, 1 mg/ml BSA; o, 1 fold DTNB; ●, 3 fold DTNB. Solid line represents the data when any proteins were added. □, 1 mg/ml BSA alone (blank).

amino acids, the interaction of alkali light chain with the heavy chain is altered (Wagner and Stone, 1983). On the other hand, bovine serum albumin (BSA) showed a little effect, probably due to prevention of the aggregation of heavy chains (Fig. IV-3-3). This protein might have behaved as an obstacle for the heavy chain, but not have bound to the heavy chain.

When added to tilapia S1(A1), rabbit A1 showed little inhibitory effect (data not shown). This was also true for rabbit DTNB light chain. The light chain binding site seems to distinguish "native" light chains from foreign ones. The affinity of light chains for S1 heavy chain could correlate with the inhibitory effect.

When heat-denatured S1 was subjected to tryptic digestion, the turbidity decreased with the formation of three heavy chain fragments (Fig. IV-3-4). It follows that the turbidity increase was mainly caused by the perturbation of junction regions of the three fragments. Though the interactions among the three fragments are considerably stable than that between heavy and light chains (Burke and Kamalakannan, 1985), S1 heavy chain devoid of these junctions seems to retain its native conformation. In addtion, alkali light chains are considered to protect the junctions which are supposed to possess fragile conformations.

Alexis and Gratzer (1979) revealed by hydrodynamic measurement that alkali light chains have an elongated structure and that A1 is more asymmetric than A2. In this connection, Katoh et al. (1988) suggested that A1 combines with the heavy chain with a wide surface area of the former. Sellers and Harvey (1981) elucidated the binding site of light chains to be 20 K

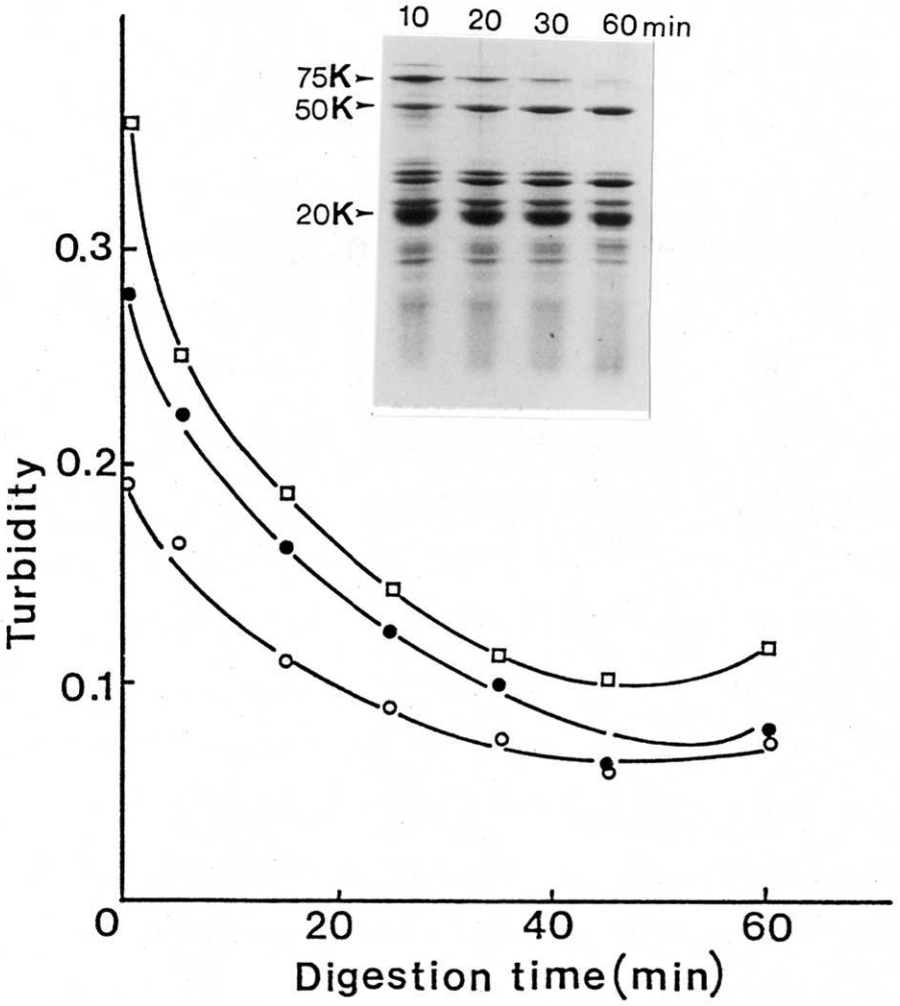

Fig. IV-3-4. Effect of tryptic digestion on the turbidity of heat-denatured rabbit S1(A1) in the presence of 0 (□), 0.2 (●) and 1 (o) fold molar excess of A2. The inserted figures represent the SDS-PAGE (15% gel) pattern at respective digestion times.

fragment, and not to be other portions or domains such as rod, light meromyosin (LMM), S2 or 75 K N-terminal of S1 heavy chain. Immunoelectron microscopy also revealed that the binding sites of light chains were located in a region near the myosin head-tail junction (Flicker et al., 1983; Winkelmann et al., 1983) and were close to each other (Waller and Lowey, 1985). Labbe et al. (1981) revealed that alkali light chains were in proximity to the 25 K domain. On the other hand, the heavy chain binding site on the alkali light chain spreads along a wide region (Burke and Wang, 1982; Katoh et al., 1988), though essential sites exist within the C-terminal 3 K region (Burke et al., 1983) and more precisely, at the C-terminal 14 residues (Ueno et al., 1985).

In the next place, the effect of light chain on the inactivation rate of S1 Ca-ATPase was examined (Fig. IV-3-5). The inactivation proceeded as a first order reaction, independently of the amount of light chain added. This implies that light chain is not involved in the active site of ATPase. In the presence of one molar fold of A1 or A2, however, the inactivation was clearly retarded at the initial stage. It is likely that light chain inhibited the initial perturbation of the active site, whose structure is subtle and thus easily perturbed by subsequent incubation.

Fig. IV-3-6 shows SDS-PAGE patterns of the supernatants which were obtained from the S1 solutions incubated at 35°C for 1 h with excess light chain and BSA, by centrifugation at 25,000 x g. It is clear that the amount of heavy chain remaining in the supernatant depended on the amount of light chain added. As

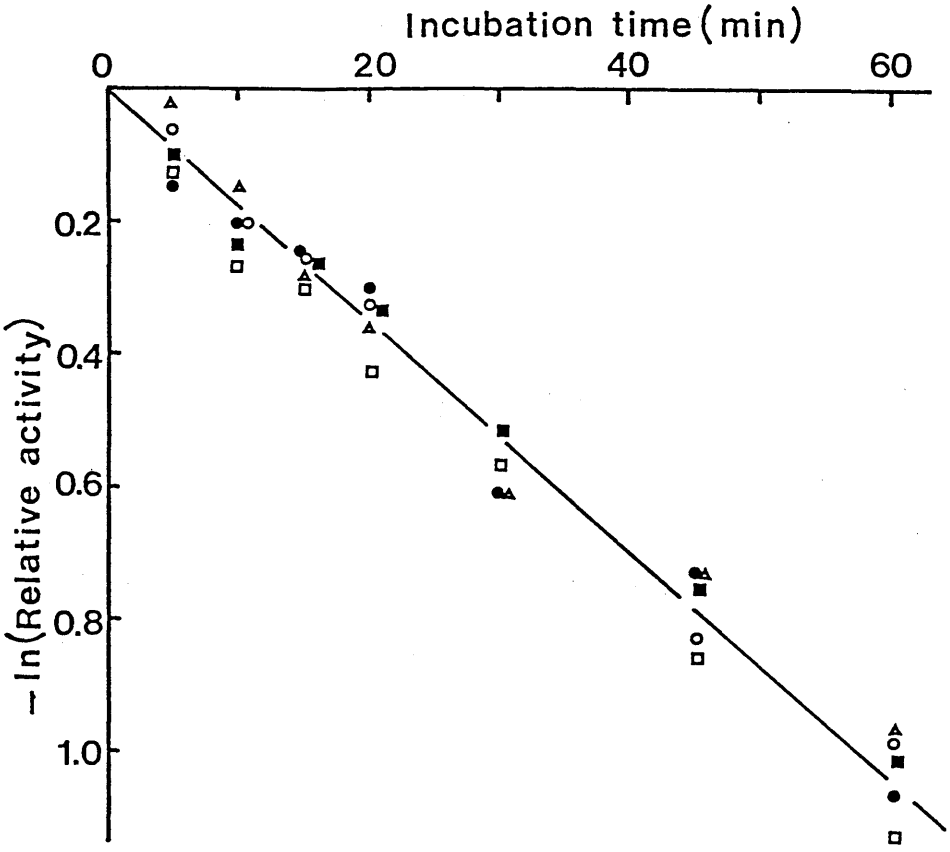

Fig. IV-3-5. Effect of alkali light chains on inactivation of Ca-ATPase of rabbit S1(A1). Excess light chains were added as follows: ● , no addition; □ , 0.2 fold A1; ■ , 0.2 fold A2; ○, 1 fold A1; ▲, 1 fold A2.

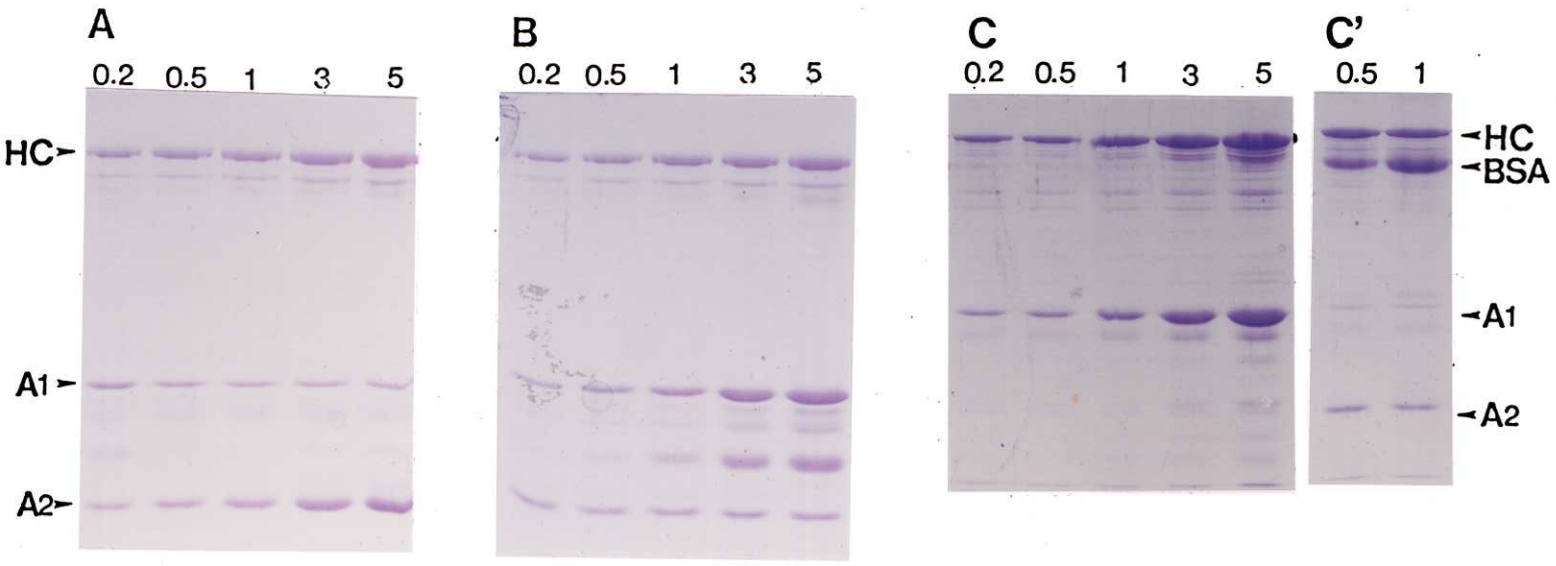

Fig. IV-3-6. SDS-PAGE patterns of the supernatants of tilapia and rabbit S1 solutions obtained by incubation at 35° C in the presence of excess light chain or bovine serum albumin (BSA) for 1 h and subsequent ultracentifugation. A, tilapia S1(A1) in the presence of tilapia A2; B, tilapia S1(A2) in the presence of tilapia A1; C and C', rabbit S1(A1) in the presence of rabbit A1 and BSA, respectively. The numbers at the top of each column represent the molar ratio of light chain added. In case of BSA, the number represents the concentration (mg/ml).

described in Section 1 of this chapter, the heavy chain in the supernatant rapidly decreased if any light chain was not added. However, excess light chain inhibited the aggregation of heavy chain. This inhibition could be due to retention of heavy chain conformation by direct binding of light chains. BSA showed a little effect, probably due to some interference with heavy chain aggregation.

The relative amounts of the remaining heavy chain were densitometrically determined, and are shown in Table IV-3-2. In case of S1(A1), the amount of heavy chain was clearly dependent on the amount of light chain added. A1 more effectively inhibited the heavy chain aggregation than A2. However, in case of S1(A2), the remaining amount of heavy chain was roughly constant irrespective of the amount of light chain added.

The above results revealed that light chains could inhibit heat-induced perturbation of the junction regions of myosin head, and that light chains could not be involved in the active site of ATPase.

Table IV-3-2. Relative amounts of the heavy chain remaining in the supernatants which were obtained from rabbit S1 solutions incubated at 35°C for 1 h in the presence of excess alkali light chains and bovine serum albumin (BSA) by ultracentrifugation

Added light chain (molar ratio)		S 1 (A1)	S 1 (A2)
A1	0.2	25.3	50.7
	0.5	27.7	40.3
	1	40.9	45.0
	3	50.6	51.3
	5	79.4	56.6
A2	0.2	20.8	56.2
	0.5	26.2	42.2
	1	38.0	59.9
	3	35.2	45.1
	5	39.4	38.6
BSA	0.5 (mg/ml)	21.1	48.6
	1.0 (〃)	19.4	43.2

Section 4

Postulated Stability Differences of Subfragment 1 Isozymes *In Vivo*

F-actin, ATP and its analogues are known to prevent the loss of S1 ATPase activity during mild heat treatment (Setton and Muhlrad, 1984). There are many other substances which bind or interact with S1, though their involvement in actin-myosin interaction *in vivo* is not fully understood.

Redowicz *et al.* (1987) revealed, using frog S1, that there are two fundamental conformational states of the myosin head in equilibrium, dependent on temperature and binding of nucleotide and actin. Okamoto and Yount (1985) examined the primary structure of ATP hydrolysis site, finding that ε-*N*-trimethyllysine and tryptophan-150 are mostly involved in the binding with triphosphate portion of ATP. The 25 K and 50 K fragments were shown to bind to nucleotide by binding of a variety of photoaffinity analogues (Mahmood and Yount, 1984; Nakamaye *et al.*, 1985). Chaussepied *et al.* (1986b) found that S1 was cleaved into two fragments, 68 K and 30 K by thrombic digestion, losing ATPase activity, and suggested the involvement of 50 K fragment in both ATPase and actin binding. Mahmood and Yount (1984) demonstrated, by photochemical modification of S1 heavy chain with ATP analogues, that portions of 50 K fragment were within 6 - 7 Å of the ATP binding site on S1 and contributed to the nucleotide binding. Differential binding of S1 isozymes to immobilized ATP occurs at a site distinct from the ATPase site (Burke *et al.*,

1987).

In this section, effects of actin, KCl, and ATP as well as chemical cross-linking, on the denaturation of S1 isozymes were examined, to clarify the *in vivo* stability differences between both isozymes.

Materials and Methods

Preparation of S1 isozymes and F-actin

S1 isozymes were prepared from tilapia and rabbit as described in Section 1 of Chapter III.

F-actin was prepared from acetone powder of rabbit fast muscle as described in Section 1 of Chapter III.

Chemical cross-linking of S1 isozymes

S1 isozymes (1 mg/ml) were incubated in 10 mM imidazole buffer (pH 7.0) containing 0.1 M NaCl, 2 mM $MgCl_2$ and 1 mg/ml of 1-ethyl-3-(3-dimethylamino)propyl carbodiimide (EDC) at 25°C for 2 h, and the reaction was terminated with 1 mM DTT. The mixture was then dialyzed against 25 mM Tris-maleate (pH 8.0) for 2 h, and used for the experiment.

Incubation of S1 isozymes in the presence of actin, KCl and ATP

S1 isozymes in 25 mM Tris-maleate (pH 8.0) were incubated at 30 - 40°C in the presence of F-actin, KCl, and ATP.

F-actin was added at an equal molar ratio to S1, in the presence of 20, 40, 60 and 100 mM KCl. Incubation with 0.04 - 0.4 mM ATP was performed in the presence or absence of 1 mM $MgCl_2$.

Analytical methods

Inactivation rate and turbidity changes were measured as described in Section 3 of this chapter.

Results and Discussion

Heat-induced turbidity increase was effectively reduced by actin, but it was strongly affected by KCl concentration (Fig. IV-4-1). The effect of KCl was enhanced with the increase of KCl concentration. The effect of KCl was more pronounced with S1(A2) than with S1(A1). These results indicated that the affinity of actin and S1 was reduced by KCl, and the affinity of actin to S1(A1) was stronger than that with S1(A2). The difference peptide of A1 might be involved in the actin-myosin interaction as seen in the kinetic data (Chapter III, Section 1). The effect of actin seems to be much stronger than alkali light chains as shown in Section 3 of this chapter. When incubation was performed at higher temperatures, the effect of KCl was more clearly discernible (Fig. IV-4-2).

In case of rabbit S1, protective effect of actin was also dependent on KCl concentration. However, the profiles were different between S1(A1) and S1(A2) (Fig. IV-4-3). In case of S1(A1), the profile was rather similar to tilapia S1, whereas turbidity increase was promoted at high KCl concentrations in case of S1(A2). In 60 and 100 mM KCl, the profiles of turbidity change were very similar between S1(A1) and S1(A2), suggesting that the interaction of light chain and actin disappeared at these KCl concentrations.

S1 heavy chain contains an actin-binding site at the C-

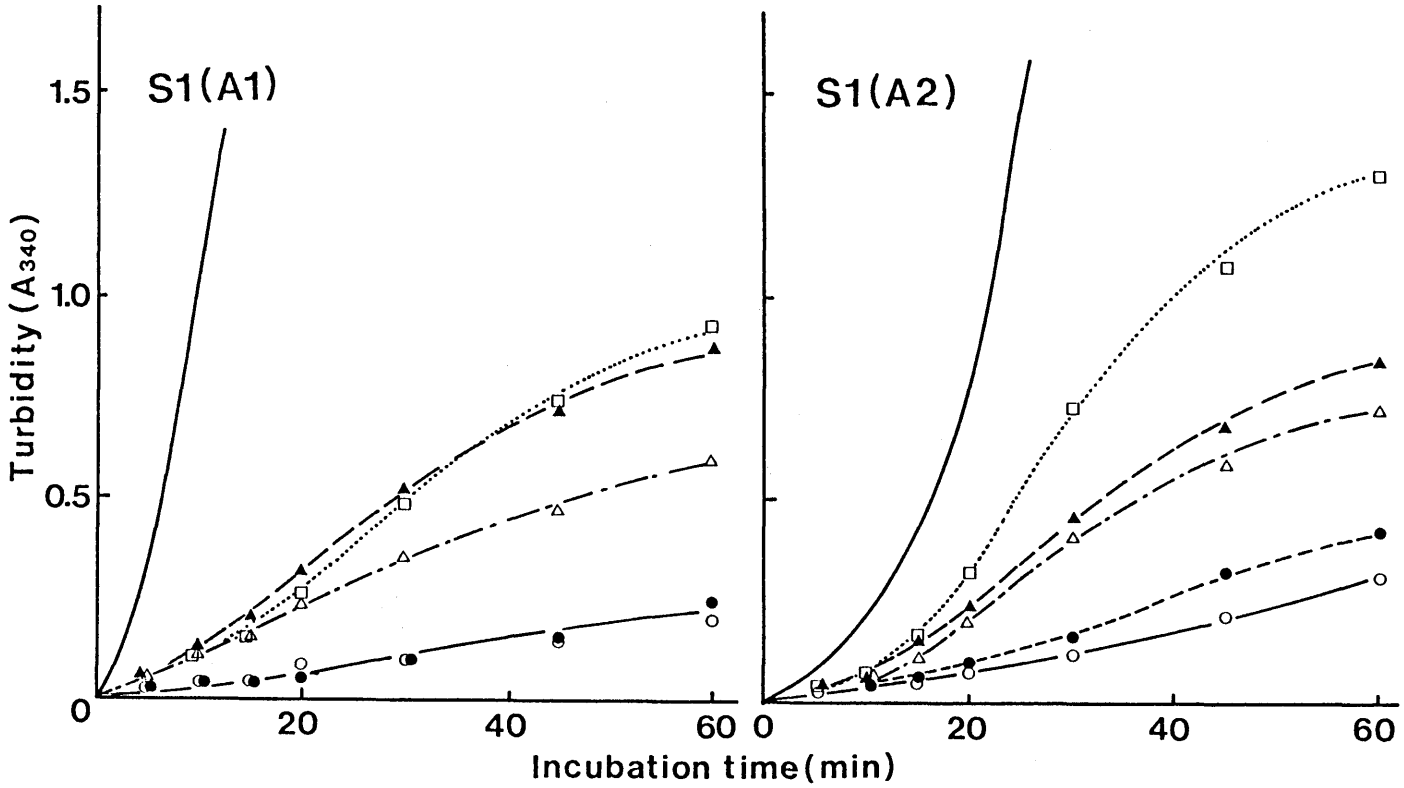

Fig. IV-4-1. Protective effect of actin on the turbidity change of tilapia S1(A1) and S1(A2) solutions at various KCl concentrations. Incubation temperature: 35 °C. KCl concentration: o, 0 mM; ●, 20 mM; △, 40 mM; ▲, 60 mM; □, 100 mM. Solid line represents the data in absence of actin and KCl. Refer to the text for further experimental details.

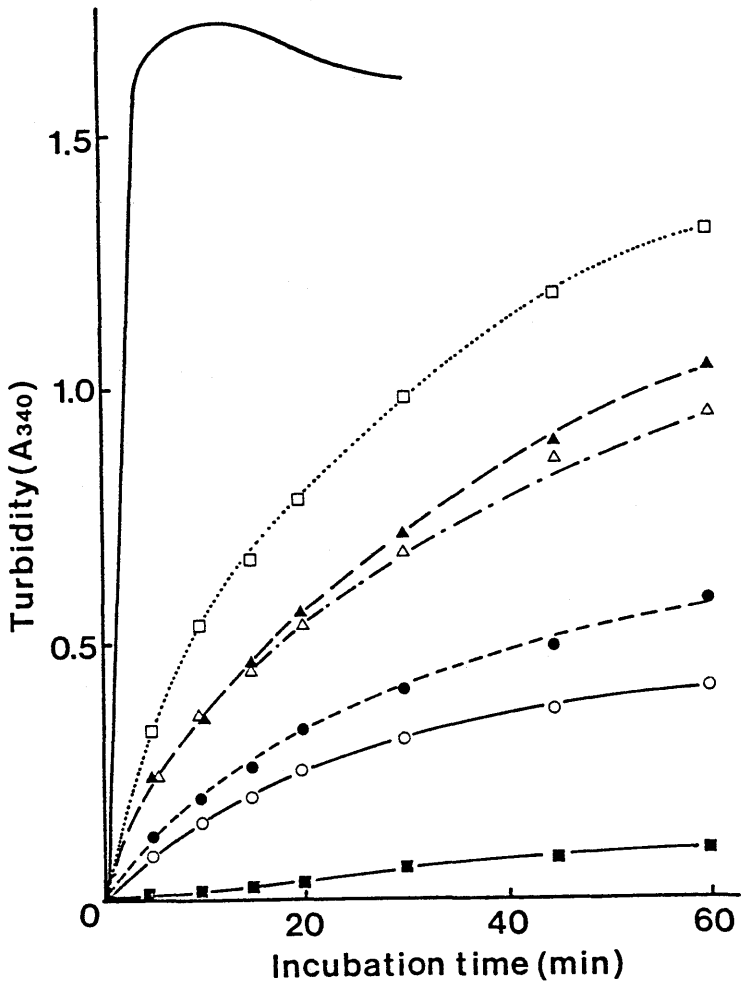

Fig. IV-4-2. Protective effect of actin on the turbidity change of tilapia S1(A1) solution at various KCl concentrations. Incubation temperature: 40°C. KCl concentration: ○, 0 mM; ●, 20 mM; △, 40 mM; ▲, 60 mM; □, 100 mM. Solid line represents the data in the absence of actin and KCl. Refer to the text for further experimental details.

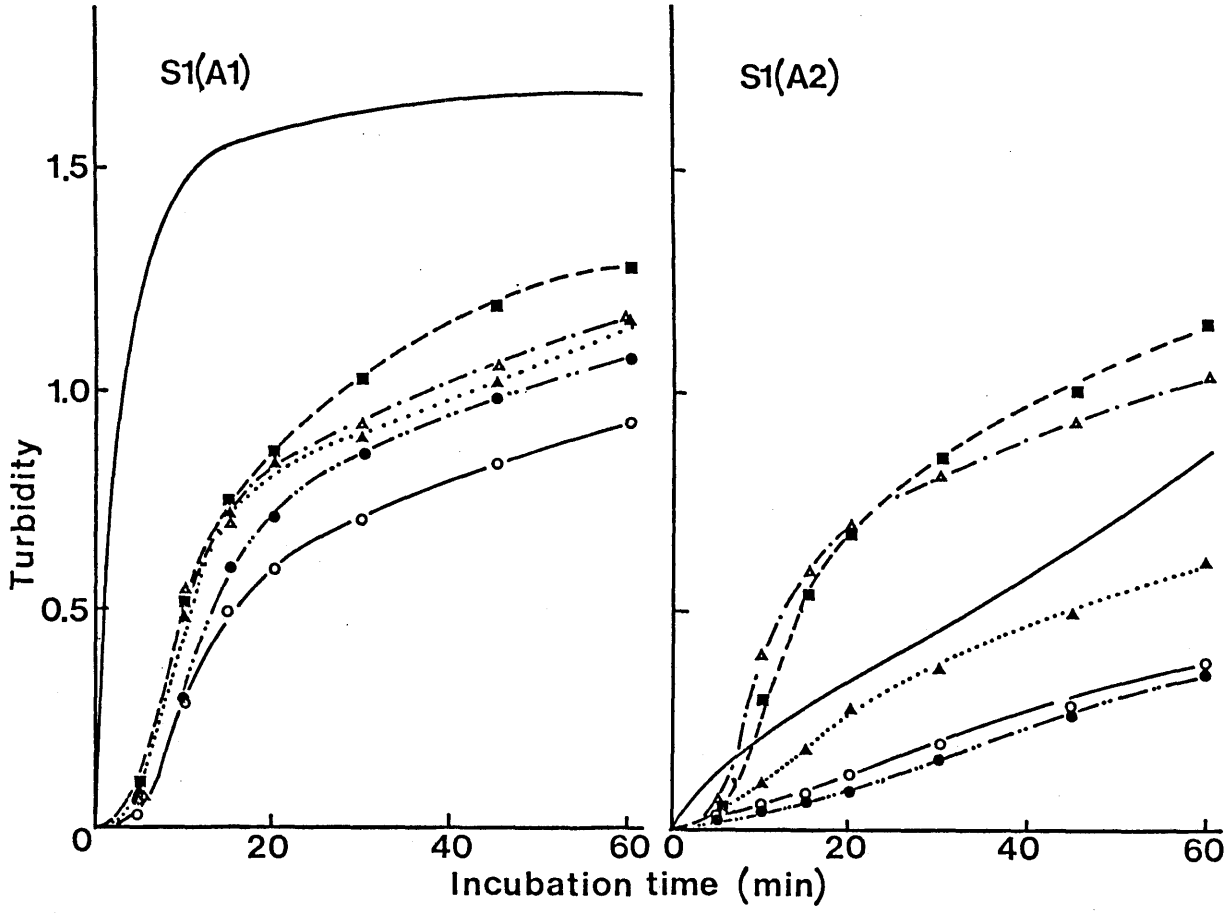

Fig. IV-4-3. Protective effect of actin on the turbidity change of rabbit S1(A1) and S1(A2) solutions at various KCl concentrations. Incubation temperature: 40°C. KCl concentration: ●, 0 mM; ○, 20 mM; ▲, 40 mM; △, 60 mM; ■, 100 mM. Solid line represents the data in the absence of actin and KCl. Refer to the text for further experimental details.

terminal, as demonstrated by its abillity to be cross-linked to actin (Mornet et al., 1981; Marianne-Pepin et al., 1982; Sutoh, 1983). The S1 heavy chain possesses "recognition sites" for actin monomer on the 20 K and 50 K fragments (Yamamoto and Sekine, 1979a,b; Mornet et al., 1981). By NMR analysis of the isolated 20 K-A2 complex, Chaussepied et al. (1986a) claimed that actin-induced conformational change could be transmitted through the 20 K fragment to the homologous C-terminal 5 K region of alkali light chains. Muhlrad et al. (1986) reported that the 20 K fragment bound to actin with higher affinity than did the 50 K fragment. However, the stoichiometry of the cross-linked complex is one S1/one F-actin monomer (Sutoh, 1983; Greene, 1984). Furthermore, the 20 K fragment has a high affinity binding site for actin in the region between SH_1 and SH_2, which are 10 residues apart in amino acid sequence (Katoh et al., 1984). In this connection, actin and the 20 K fragment showed several times higher affinity in the presence of A1 than in its absence (Katoh et al., 1988).

On the other hand, Mrakovcic-Zenic et al. (1981) reported that KCl induced conformational changes of equal magnitude with both A1 and A2, though the affinity for KCl was a little higher for A2. Therefore, the effect of KCl on the denaturation profiles of S1 isozymes was further examined. The effect of KCl on the turbidity change was quite opposite between both isozymes (Fig. IV-4-4). In case of S1(A1), the higher the KCl concentration, the lower the turbidity increase, whereas the turbidity of S1(A2) solution increased with the increase of KCl concentration.

Such an opposite effect was further supported by thermal

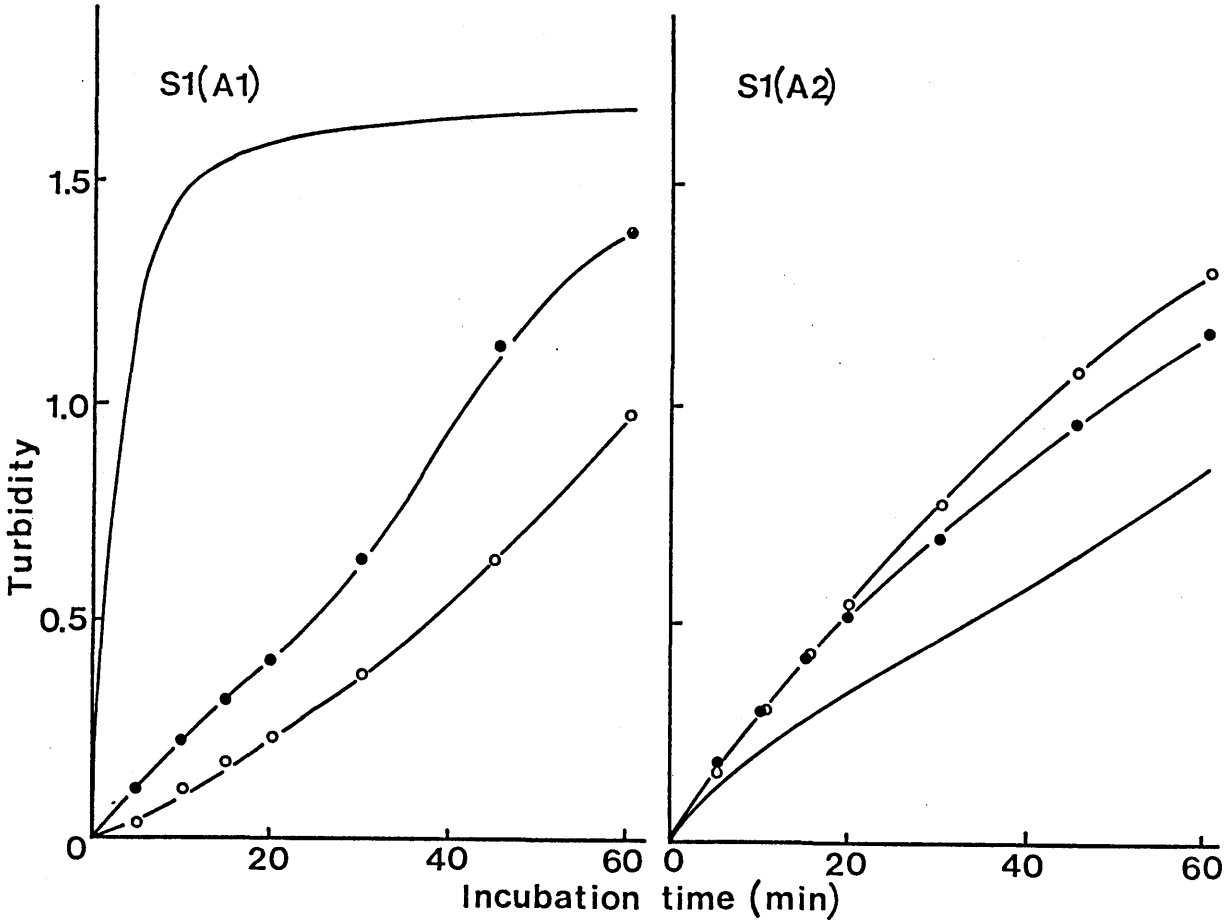

Fig. IV-4-4. Effect of KCl concentration on the turbidity change of rabbit S1(A1) and S1(A2) during incubation at 35°C. KCl concentration: ●, 40 mM; o, 100 mM. Solid line represents the data in the absence of KCl.

inactivation rate (Table IV-4-1). With increasing KCl concentration, the rate constants of S1(A1) increased only slightly, and those of S1(A2) much more. KCl did not protect the ATPase of S1(A1) as effectively as with the turbidity change. The higher affinity of KCl for S1(A2) might have caused denaturation of the heavy chain.

Effect of ATP on the denaturation profiles of S1 isozymes is shown in Fig. IV-4-5. ATP reduced the turbidity change in both isozymes, depending on its concentration. Turbidity increase of S1(A1) was markedly inhibited by ATP, but the effect was much smaller with S1(A2), although the turbidity change in the absence of ATP was much lower with S1(A2) than with S1(A1). A similar effect was observed with rabbit S1 isozymes (data not shown). These data suggest that both isozymes clearly differ from each other in the affinity for ATP.

As described previously, A1 has the difference peptide of 41 residues at the N-terminal. This peptide is abundant in lysine, alanine, and proline. Clusters of basic amino acids, especially lysine, together with alanine and proline, have been found at the terminal ends of several proteins: e.g., histone H1 from sea urchin (Strickland et al., 1980), capsid protein of Semliki forest virus (Garoff et al., 1980), and are thought to be involved in binding to nucleic acid. Thus, it is quite plausible that the difference peptide is involved in binding to nucleotides. Actually, free alkali light chains are retarded on an immobilized ATP column, suggesting that these light chains have an affinity, though low, for nucleotides (Burke et al., 1981).

Table IV-4-1. Effect of KCl concentration on the thermal inactivation rate constants of Ca-ATPase of rabbit S1(A1) and S1(A2) at 35° C

(x $10^{-5} s^{-1}$)

KCl concn.	S1(A1)	S1(A2)
20 mM	30.6	25.6
40 mM	33.3	28.9
100 mM	34.4	37.5

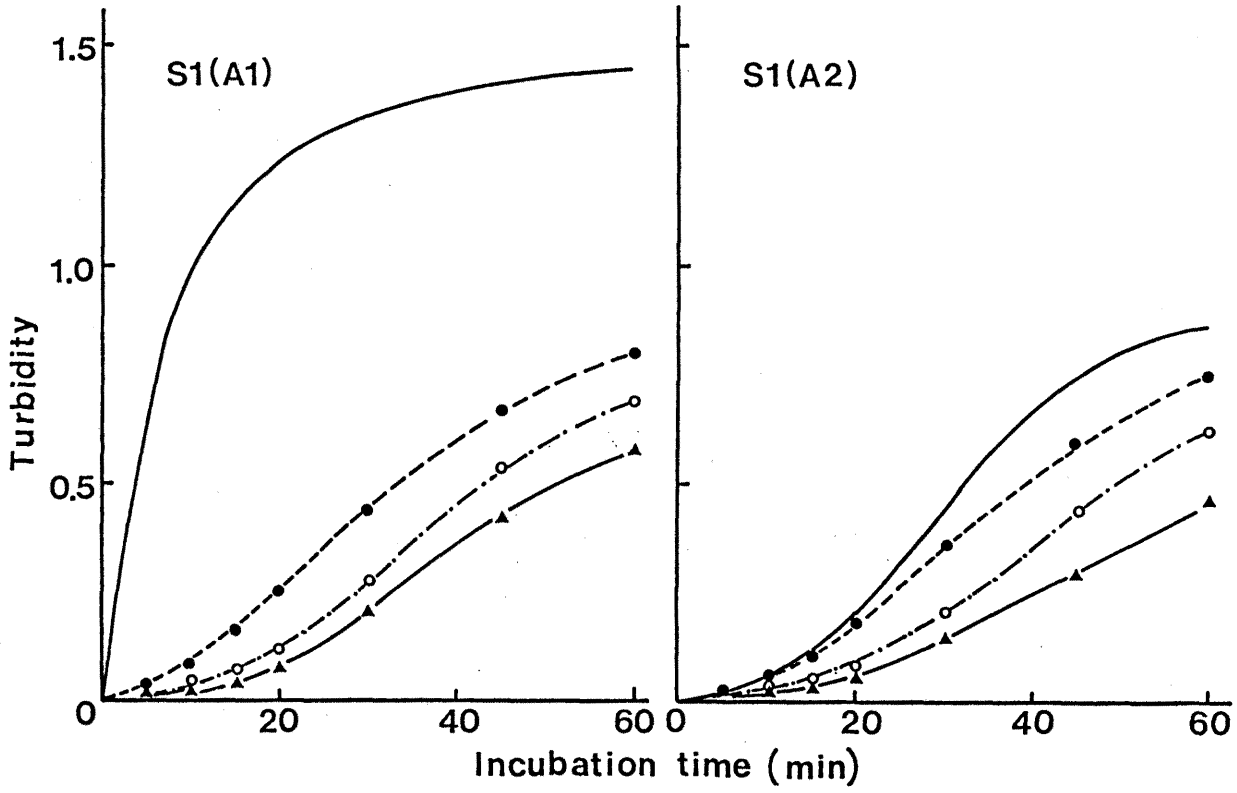

Fig. IV-4-5. Effect of ATP on the turbidity change of tilapia S1(A1) and S1(A2) during incubation at 35°C. ATP concentration: ●, 0.04 mM; ○, 0.1 mM; ▲, 0.4 mM. Solid line represents the data in the absence of ATP.

When digested with trypsin in the presence of ATP, S1 rapidly lost the ATPase activity upon mild heat treatment even if ATP or ADP was present (Pinter et al., 1986). Alkali light chains possess a weakly binding site for nucleotides with a binding constant in the order of 10^{-3} M^{-1} (Mrakovcic-Zenic et al., 1981). Unfolding of the 50 K fragment was accompanied by the loss of ATPase, whereas inactivation rate correlated with the decrease of 50 K fragment (Burke et al., 1986). Mg-nucleotides protected the perturbation of 50 K fragment during heat treatment and digestion (Setton and Muhlrad, 1984).

ATP protects the heavy chain from heat denaturation, but the difference peptide of S1(A1) takes part in the stronger binding with ATP than S1(A2), resulting in a marked protective effect of ATP on S1(A2). However, the kinetics in the absence of actin were not closely related to presence of the difference peptide. Therefore, it is unlikely that the affinity difference for ATP between both isozymes affects the turnover rate of ATP.

Effect of Mg on turbidity profiles of S1 isozymes is shown in Table IV-4-2. When $T_{10}^{1\%}$, $T_{30}^{1\%}$ and $T_{60}^{1\%}$ values were compared, the values in the presence of Mg were generally a little higher than those in its absence. The intensity was similar between the isozymes. This suggests that Mg binds to S1 isozymes with the affinity of similar magnitude, causing trivial conformational changes. Divalent metal ions such as Mn, Ca and Mg, bind to alkali light chains though their affinities are generally very low (Morita and Matsumoto, 1980, 1981). Alkali light chains, like troponin C or calmodulin, contain an 'EF-hand' structure (Collins, 1974; Weeds and MacLachlan, 1974; Kretsinger and Barry,

Table IV-4-2. Effect of Mg on turbidity change of tilapia and rabbit S1(A1) and S1(A2) at 35°C

	Tilapia				Rabbit			
	S1(A1)		S1(A2)		S1(A1)		S1(A2)	
	- Mg	+ Mg	- Mg	+ Mg	- Mg	+ Mg	- Mg	+ Mg
$T^{1\%}_{10}$	13.1	14.2	0.8	0.4	23.6	25.7	2.0	2.3
$T^{1\%}_{30}$	19.3	19.6	6.3	6.5	25.4	27.6	6.1	6.2
$T^{1\%}_{60}$	20.8	20.1	12.2	12.9	26.3	28.0	15.3	15.7

*$T^{1\%}_{t}$ represents turbidity of 1% S1 solution after t min incubation.

1975), though the affinity for divalent ions are several orders of magnitude lower than those of the so-called Ca-binding proteins (Potter and Gergely, 1975; Crouch and Klee, 1980).

Finally, the effect of chemical cross-linking with EDC on the denaturation profiles of S1 isozymes was examined. As shown in Fig. IV-4-6, cross-linking of heavy chain-light chain and heavy chain-heavy chain partially occurred as observed in the SDS-PAGE patterns (Fig. IV-4-6, c,d), but the tryptic digestion patterns of native and cross-linked S1 were very similar to each other (Fig. IV-4-6, e-g), suggesting that cross-linking took place intramolecularly, but independently of the three domains.

As the result of cross-linking, heat-induced turbidity change was remarkably suppressed (Table IV-4-3). $T_{30}^{1\%}$ values were less than one twentieth of those of native S1. It is likely that cross-linking reinforced the structure of 50 K fragment or junction regions, making it highly resistant to unfolding.

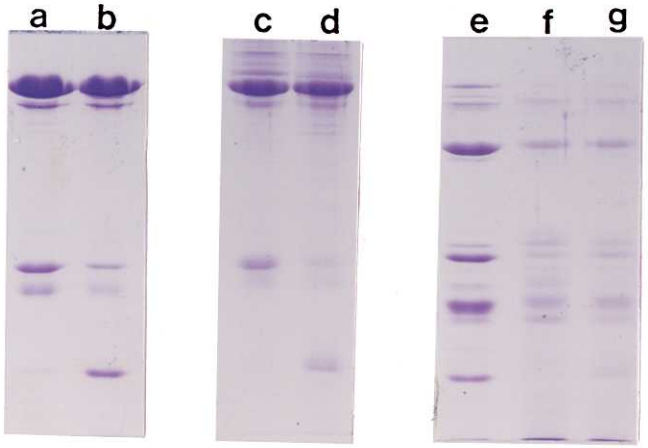

Fig. IV-4-6. SDS-PAGE patterns of native and EDC-treated S1(A1) and S1(A2) from rabbit. Tryptic digestion patterns are also shown. S1(A1) (a), S1(A2) (b), EDC-S1(A1) (c), EDC-S1(A2) (d), tryptic digestion (25°C, 30 min) patterns of S1(A1) (e), EDC-S1(A1) (f) and EDC-S1(A2) (g), respectively.

Table IV-4-3. $T_{10}^{1\%}$, $T_{30}^{1\%}$ and $T_{60}^{1\%}$ values of native and EDC-S1 isozymes of tilapia at 35°C

	S1(A1)	S1(A2)	EDC-S1(A1)	EDC-S1(A2)
$T_{10}^{1\%}$	13.1	0.8	0.5	0
$T_{30}^{1\%}$	19.3	6.3	0.8	0.2
$T_{60}^{1\%}$	20.1	12.2	0.9	0.2

*$T_t^{1\%}$ represents turbidity of 1% S1 solution after t min incubation.

Summary

S1(A1) and S1(A2) from tilapia, bigeye tuna and rabbit myosins were compared in respect of stability. S1(A1) showed higher inactivation rate constant of Ca-ATPase than S1(A2) for all species, indicating higher stability of S1(A2). S1(A1) showed faster turbidity increase and higher maximum value than S1(A2). Furthermore, the stability in the presence of methanol was higher with S1(A2) than with S1(A1) when the above inactivation rate was taken as a parameter. The mean residue ellipticity in the presence of urea decreased faster with S1(A1). All these results demonstrated that S1(A2) possesses more rigid structure than S1(A1).

On the other hand, turbidity increase was effectively inhibited by addition of excess light chain, and the effect was more enhanced with A2 than with A1. However, none of both light chains showed any protective effect on ATPase activity of S1. These results clearly indicated that alkali light chains, especially A2, could be involved in stabilization of the myosin head.

Actin and ATP also effectively inhibited the denaturation of S1, though the mode of effect differed between both isozymes. KCl protected S1(A1) from denaturation, but rather promoted denaturation of S1(A2). Cross-linking with EDC suppressed the turbidity increase, due to the formation of intramolecular binding.

CHAPTER V

GENERAL DISCUSSION

Myosins from fish ordinary muscle were demonstrated to possess three molecular species of light chains (A1, A2 and DTNB light chain) just as those from fast muscle of higher vertebrates. Fish myosin light chains were remarkably species-specific in molecular weight and isoelectric point, as examined by SDS-PAGE and two-dimensional gel electrophoresis. Among the three types of light chain, A2 light chains were found to be the most species-specific, whereas DTNB light chains resembled each other irrespective of fish species. Amino acid compositions of this class of light chain ('regulatory light chain') were quite similar to each other, suggesting their conservativeness during the molecular evolution.

On the other hand, A1 light chain has 'difference peptide' rich in lysine, proline and alanine at the N-terminal (Frank and Weeds, 1974). As for fish counterparts, the contents of these amino acids were proved to be generally higher in A1 than in A2, suggesting the presence of this peptide in fish A1 light chains as well. However, electrophoretic studies showed that A1 light chains were less species-specific than A2. The difference peptide seems to lessen the remarkable species-specificity of A2. There could be elaborated devices in the synthesis of this peptide.

Developmental changes in light chain composition in fish myosins were also demonstrated in the present study. During

development, A1 light chain increased gradually along with the decrease of A2, though the molecular weights and isoelectric points of the light chains remained unchanged. Such changes in composition were rather different from those reported for higher vertebrate myosins, in which A2 increases gradually during development. Developmental changes have also been reported for the heavy chain (Rushbrook and Stracher, 1979; Whalen et al., 1979; 1981; Bader et al., 1982; Bugaisky et al., 1984; Reiser et al., 1985). By using monoclonal antibodies against myosin head, rod and DTNB light chain, Winkelmann et al. (1983) demonstrated that the structural changes occurred in widely separated parts of the molecule during chicken muscle development. In this connection, Bandman et al. (1982) indicated that the developmental transfer was due to changes in the expression of mRNAs, and not due to post-translational modification of protein.

As demonstrated by immunochemical techniques, there existed a considerable degree of similarity between A1 and A2, and even between alkali and DTNB light chains. In addition, the structural similarity was pronounced with light chains from closely related species. Ubiquitous structures seemed to exist throughout all the light chains of myosin. Substitution rate of amino acid was as high as 15.3 and 16.4% between A1 and A2 light chains of rabbit and chicken, while 9.5% between their DTNB light chains (Matsuda et al., 1980). It has been demonstrated in rat (Periassamy et al., 1984) and in chicken (Nabeshima et al., 1984) that the two mRNAs for A1 and A2 were derived from the gene by a process of alternative promotor utilization and differential RNA splicing of the two primary transcripts.

In order to assess the physiological functions of light chains, two S1 isozymes associating different alkali light chains, S1(A1) and S1(A2), were prepared, and compared in respect of kinetics. Both isozymes differed from each other in ATPase activity, the differences being more pronounced in the presence of actin. S1(A1) showed higher affinity for actin than S1(A2) at low salt concentrations, but such affinity difference disappeared at physiological salt concentration.

There is much evidence supporting the direct interaction of actin with the difference peptide of A1. Winstanley et al. (1977) suggested that alkali light chains are involved in actin-myosin interaction, based on the differential behaviors of S1 isozymes on actin-affinity column. The difference peptide displayed a high degree of segmental mobility on S1(A1) (Prince et al., 1981), but such mobility was markedly reduced in its complex with actin. Henry et al. (1985) suggested by NMR studies that the difference peptide is highly mobile in S1(A1). It was also indicated that the difference peptide was situated on the surface of S1(A1) and interacted with actin (Dalgarno et al., 1982). A close contact of the N-terminal region of S1 to actin in the acto-S1 complex was indicated by cross-linking using a zero-length cross-linker, EDC (Sutoh, 1982; Yamamoto and Sekine, 1983). A1 was cross-linked to actin at two sites in the difference peptide, and the site closer to the N-terminal was drastically inhibited from binding to actin, when KCl concentration was increased (Yamamoto and Sekine, 1983). In this connection, the fragment containing N-terminal 76 residues of A1 was retained on an immobilized actin affinity column (Henry et al., 1985). The

N-terminal of cardiac essential light chain also showed similar properties to those of the difference peptide (Henry et al., 1985). The binding of troponin I to actin specifically weakened the interaction between the N-terminal segment of A1 on S1(A1) and actin, without affecting the heavy chain interaction sites (Grand et al., 1983). These support the direct interaction of the difference peptide with actin.

It is quite possible that A1 interacts directly with actin in vivo, but light chains seem to play other roles. Actually, Mrakovcic-Zenic et al. (1981) found significant differences in thermostability between both S1 isozymes of rabbit, taking turbidity change as a parameter. Therefore, S1 isozymes from fish were examined in this respect under mild denaturing conditions. With tilapia and bigeye tuna as well, S1(A2) showed higher thermostability than S1(A1) when either inactivation rate of Ca-ATPase or turbidity change was taken as parameters. In the presence of denaturants such as methanol and urea as well, S1(A2) showed higher resistibility than S1(A1). These results indicated that alkali light chains are involved in the stabilization of S1 heavy chain.

Heterogeneity of myosin heavy chain has been reported for rabbit and chicken fast myosin (Pope et al., 1977; Maita et al., 1987). The possibility seemed to be quite low that the differences in stability between both S1 isozymes are due to differences in the heavy chains. To make it sure, however, experiments were carried out, using peptide mapping and tryptic digestion techniques. As a result, the heavy chains in both S1 isozymes

were judged to be almost or completely identical. It follows that alkali light chains are involved in stabilization of the myosin head. Therefore, A1 could be released faster than A2 during heat treatment, resulting in lower stability of S1(A1) than S1(A2).

S1 is a molecule possessing backbone motility as well as domains of different sidechain motility (Highsmith et al., 1979; Highsmith and Jardetzky, 1980; 1981; Prince et al., 1981). S1 heavy chain is proteolytically split into three fragments 25 K, 50 K and 20 K, in the sequential order from the N-terminal. Actin-binding site resides between 20 K and 50 K fragments, while alkali light chain binds to 20 K fragment. The interaction of the three fragments are considerably stable than that between heavy and light chains (Burke and Kamalakannan, 1985). Korner et al. (1983) have shown that the 50 K fragment contained an essential carboxyl group which, when modified, causes loss of ATPase activity. Okamoto and Sekine (1987) prepared S1 lacking 20K fragment and found that this incomplete S1 retained more than 50% of the actin-activated Mg-ATPase activity and also Ca- and EDTA-ATPase activities characteristic of myosin. Based on these results, they claimed that both reactive thiols are not essential for S1 to show ATPase activity and that binding of actin to the 20K fragment is not essential to enhance Mg-ATPase activity. The communication between SH_1 and ATPase site is independent of the associated alkali light chains and it persists despite the cleavages present in free heavy chain (Burke and Kamalakannan, 1985).

In the presence of excess alkali light chains, heat denatu-

ration as observed by turbidity changes was suppressed depending on the amount of light chain added. DTNB light chain also suppressed the heat-denaturation of S1, but the extent was much smaller than with alkali light chains. This may be due to the weaker affinity of DTNB light chain for alkali light chain binding site in S1 heavy chain. However, any protective effect against inactivation of ATPase was not recognized for these light chains. On the other hand, turbidity due to heat-denatured S1 disappeared by tryptic digestion along with the cleavage of junctions of the three domains.

The active stabilization effect of alkali light chains suggests that both chains partially bind to the junction regions, which are supposed to possess fragile conformations, though light chain binding sites are known to reside on the 20 K fragment. However, light chains have an elongated asymmetric structure both in isolated state (Stafford and Szent-Györgyi, 1978; Alexis and Gratzer, 1979) and in myosin molecule (Flicker et al., 1983). The N-terminal segment of alkali light chain has been mapped to the neck region of myosin, the portion of the pear-shaped head which tapers to the rod (Elliotte and Offer, 1978). The N-terminal region of regulatory light chain is also localized to the S1/rod junction (Winkelmann et al., 1983) and that of the essential light chain to a region of the head further away from this junction (Waller and Lowey, 1985). Therefore, it is quite plausible that light chains contact other domains of S1 heavy chain than the 20 K fragment.

Protective effect against heat-denaturation of S1 was also

recognized with actin, ATP and KCl, but the effect of each of them was clearly different between S1 isozymes. Particularly, the effect of actin was weakened with the increase of KCl concentration, suggesting that the interaction of the difference peptide with actin was drastically affected by KCl. KCl also induced conformational changes of equal magnitude in A1 and A2 as has been revealed in other Ca-binding proteins, troponin C and parvalbumin (Mrakovcic-Zenic et al., 1981). It is probable that KCl affects the heavy chain-light chain binding.

In vivo, alkali light chains might strengthen physically the junction regions of myosin head, whose conformation is supposed to be distorted by the stress caused by cross-bridge cycling of actin and myosin filaments. As for the presence of two alkali light chain isoforms, it seems likely at present that A1-associating heads of myosin participates in hooking to actin filament employing the higher affinity for actin, while A2-associating heads in generating the impellent force of their own.

In the present study, possible roles of alkali light chains have partly been elucidated. However, the role of DTNB light chain remains to be solved. As its name implies, "regulatory" light chain has been thought to be involved in the regulation of actin-activated Mg-ATPase activity in some invertebrate myosin (Szent-Györgyi et al., 1973; Sellers et al., 1980) and in vertebrate smooth and other myosins (Adelstein and Conti, 1975; Chacko et al., 1977; Sobieszek and Small, 1977; Ikebe et al., 1978; Sherry et al., 1978; Yerna et al., 1978; Trotter and Adelstein, 1979; Sellers et al., 1981). The possible role of DTNB light chain in fast myosin, however, remains to be elucidated. In

fact, phosphorylation of DTNB light chain was neither necessary nor sufficient to increase cross-bridge cycling rate in skeletal muscle (Barsotti and Butler, 1984). On the other hand, DTNB light chain seemed to strengthen the binding of alkali light chains to the heavy chain (Wagner and Stone, 1983). Both alkali and DTNB light chains interacted on myosin molecule (Walliman et al., 1982). This effect can largely be accounted for by the N-terminal portion of this chain. Removal of 18 amino acids from the N-terminal by chymotryptic cleavage (Weeds and Pope, 1971) markedly enhanced the rate of alkali light chain exchange into HMM (Wagner and Stone, 1983). Such cleavage also brings about the loss of Ca sensitive binding of HMM to regulated actin. Therefore, protective effect of DTNB light chain against denaturation might be recognized more clearly on Mg·S1, HMM or myosin, which retain the binding site of this light chain.

In conclusion, fish ordinary muscle myosins possess three kinds of light chain, as do fast myosins from higher vertebrates. Fish myosin light chains are remarkably species-specific both in molecular weight and isoelectric point. In spite of this, fish myosin light chains have similar characteristics and structures. On the other hand, alkali light chains are considered to be directly involved in actin-myosin interaction, but more possibly, in the conformational stabilization of myosin head.

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