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CARDIAC MYOFIBRILLAR ATPase: A COMPARISON WITH THAT OF FAST SKELETAL ACTOMYOSIN IN ITS NATIVE AND IN AN ALTERED CONFORMATION

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SUMMARY

Some enzymatic properties of cardiac myofibrils that were low in mitochondrial contamination are compared with those of skeletal actomyosin. We have observed with skeletal actomyosin two different types of enzymatic behavior: (a) the native behavior typical for skeletal myofibrils and (b) a modified behavior, found sometimes in reconstituted skeletal actomyosin preparations. Such modified skeletal actomyosin had acquired some enzymatic properties similar to those of cardiac myofibrils. The rate of hydrolysis of ATP by modified actomyosin was greatly reduced although that of ITP remained unchanged. As a result ITPase activity was much greater than ATPase activity just as in cardiac myofibrils. However, with respect to ATPase activity as a function of MgATP concentration there was no resemblance between cardiac myofibrillar and modified reconstituted skeletal actomyosin. Whereas the ATPase activity of cardiac myofibrils shows Michaelis–Menten kinetics similar to that of skeletal myofibrils, with a K_m of about 10 μM, the modified actomyosin was inhibited by higher ATP concentrations. The Ca^{2+} concentration for half maximal ATPase activation ranged between 0.7 and $1 \cdot 10^{-6}$ M, a range similar to that reported by others for reconstituted cardiac actomyosin and similar to that of skeletal actomyosin.

INTRODUCTION

The ATPase activity of cardiac actomyosin[1] is much lower than that of native actomyosin of fast skeletal muscle. However, we have observed that the myosin of skeletal actomyosin may become spontaneously so altered that some of the enzymatic properties of its complex with actin come to resemble those of cardiac actomyosin. Such altered skeletal actomyosin showed a depression of the turnover rate of ATP whereas the turnover rate of ITP remained quite close to that of the native protein, indicating that enzyme denaturation cannot account for this change. Because of the depression of the rate of ATP hydrolysis, this altered skeletal actomyosin hydrolyzed

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ITP faster than ATP. Apparently skeletal myosin can exist in two conformations which differ in their choice of nucleoside triphosphate for preferred substrate. Knowing that cardiac actomyosin had also been observed to hydrolyze ITP faster than ATP (J. C. RUEGG, personal communication) we were intrigued by the possibility that skeletal and cardiac myosin may possess a conformation common to both. This conformation should occur only occasionally in skeletal myosin whereas it should represent the native state of cardiac myosin. We therefore compared cardiac with skeletal actomyosin in its native and in its altered form.

As this study progressed, it became obvious that optimum conditions for the study of cardiac myofibrillar ATPase activity under contracting and relaxing conditions had not been established. For this reason, a study of these properties was undertaken in order to lay the groundwork for the study of the properties of myofibrils from both normal and abnormal cardiac muscle.

EXPERIMENTAL

Preparation of cardiac myofibrils

Myofibrils were prepared from the left ventricles of dog hearts. Dog hearts rapidly chilled after removal from the animal or left at room temperature for several hours (Fig. 1) gave preparations of the same ATPase activity. Muscle that had been passed through a precooled meat grinder was homogenized for 2 min in a Waring blender with 5 vol. of ice-cold 0.1 M KCl and 5 mM imidazole buffer (pH 7.0 at 4°). The resulting homogenate was passed through cheesecloth to remove connective tissue and centrifuged at 5000 × *g* for 20 min. The supernatant was discarded and the pellet washed twice more in 5 vol. of the same solution. It was then resuspended in 5 vol. of ice-cold 40 %(w/v)sucrose, 0.1 M KCl and 5 mM imidazole (pH 7.0 at 4°) and centrifuged at 15000 × *g* for 10 min. Sucrose was added in such high concentration in order to retard the sedimentation of the mitochondria by the principle of zonal centrifugation[2]. The supernatant was carefully decanted from the loose pellet which was centrifuged twice more in the ice-cold sucrose solution. After the third sucrose treatment, the pellet was resuspended in 0.1 M KCl and 5 mM imidazole (pH 7.0 at 4°) and centrifuged at 5000 × *g* for 10 min. This stage was repeated once more. The myofibrils were finally

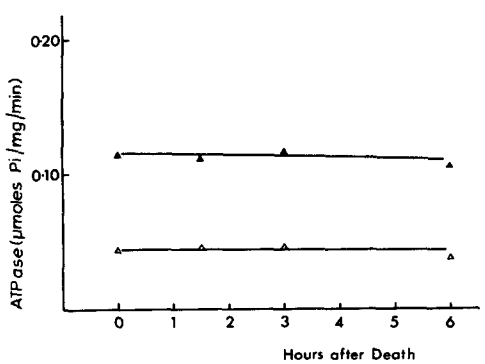

Fig. 1. The effect on myofibrillar ATPase activity of allowing heart muscle to stand at room temperature for varying lengths of time before the preparation of myofibrils in the presence (△) and absence (▲) of Ca^{2+}. Ionic strength 0.06; 5.0 mM MgATP.

resuspended in 0.1 M KCl and stored at 4°. A crystal of sodium azide was added to myofibril preparations stored for more than 36 h to prevent bacterial decomposition.

Cardiac sarcoplasmic reticulum was prepared as previously described[3].

Preparation of skeletal contractile proteins

Myofibrils[4], myosin[5] and actin[5] were prepared as previously described.

Crude troponin and tropomyosin were prepared according to the instructions given by EBASHI AND EBASHI[6].

Trypsin treatment for the removal of troponin from cardiac and skeletal muscle was performed according to EBASHI AND EBASHI[6] with one minor modification. Before incubating the myofibrils with trypsin (7 μg/mg myofibrillar protein for 3–6 min at room temperature), Mg^{2+} (5 mM) and pyrophosphate (5 mM) were added. We have consistently observed a more complete removal of troponin under these conditions, possibly due to a dissociation of actin from myosin in the presence of Mg^{2+}–pyrophosphate.

The hydrolysis of ATP, ITP and UTP was determined as previously described[4,5,7]. The standard medium for the determination of ATPase activity in the presence and absence of calcium was the following: 1–2 mg protein per ml; 10.0 mM imidazole (pH 7.0); 5.0 mM azide; KCl to adjust the ionic strength to the desired value (usually 0.06), 2.0 mM MgATP; 1.0 mM $MgCl_2$; and either 0.1 mM $CaCl_2$ or 4.0 mM EGTA (ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid). Incubation periods varied from 2 to 5 min at 25°. The assays for the determination of the hydrolysis of MgUTP and MgITP were the same except that the concentration of MgUTP was 5.0 mM and MgITP 4.0 or 6.0 mM. The concentration of $MgCl_2$ during measurements of ITPase activity was 4.0–5.0 mM. It was necessary to choose such high concentrations of MgITP because of its low affinity for actomyosin[7]. We have observed that optimal ITPase activity in addition requires a high concentration of ionized magnesium. Any changes in the composition of the assay medium for given experiments are indicated in the legends. Activation of cardiac myofibrillar ATPase activity by calcium was determined with the aid of calcium buffers as previously described[7].

Measurement of protein concentrations

The protein content of the various preparations was measured by the method of LOWRY *et al.*[8] using bovine serum albumin as a standard.

Reagents

All reagents were of analytical grade. Crystalline disodium ATP, and sodium ITP (98–100% purity), trypsin, and trypsin inhibitor were purchased from Sigma, St. Louis, and UTP and creatine phosphate from Calbiochem. Creatine phosphokinase was bought from Boehringer.

RESULTS

Purity of the myofibril preparation

Cardiac myofibrils may be separated from mitochondria and reticulum by a series of centrifugations with highly concentrated sucrose–KCl followed by manual separation of the white myofibrillar pellet from any remaining colored sediment.

Even without the last step the mitochondrial ATPase activity was reduced to about 15 % of the total by this procedure (Table I). Mitochondrial contamination was estimated in the usual way as the fraction of ATPase activity inhibited by azide[9].

The maximally possible contribution of microsomal contamination to ATP hydrolysis is given by the rate at 0.3 ionic strength. Under these conditions skeletal myofibrillar ATPase activity is lowered to about 10 % of the value observed at ionic strength of 0.1 or below, whereas the microsomal ATPase activity remains unchanged over this range of ionic strength[10] (Fig. 2). As can be seen from Table I the rate of ATP hydrolysis of cardiac myofibrils at 0.3 ionic strength was only 10–20 % of that at the low salt concentration. In addition the rate was quite similar for ATP and UTP whereas isolated reticulum hydrolyzes ATP about 5 times faster than UTP[11,12]. By contrast, skeletal actomyosin hydrolyzes UTP at a similar rate as ATP[13,7]. Therefore contamination by microsomal ATPase appears to be negligible.

Comparison between ATP hydrolysis by cardiac myofibrils and skeletal actomyosin

The ATPase activity of cardiac myofibrils was considerably more dependent on

TABLE I

PURITY OF CARDIAC MYOFIBRIL PREPARATIONS

μmoles P_i liberated/min per mg protein			
I = 0.06		*I = 0.30*	
ATP −Azide	*+Azide*	*ATP +Azide*	*UTP*
Myofibrils			
0.08	0.07	0.01	
0.10	0.09		
0.13	0.11	0.03	0.03
0.13	0.10	0.01	
0.08	0.06	0.02	
0.10	0.08	0.01	0.01
Cardiac actomyosin			
	0.17	0.02	0.02

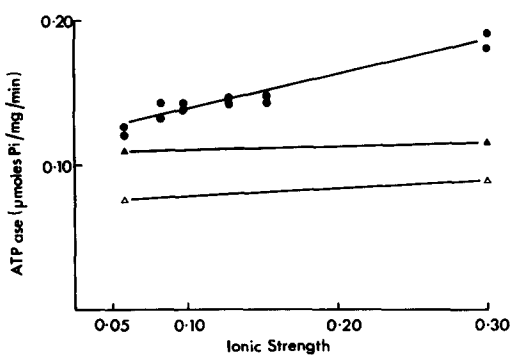

Fig. 2. The influence of ionic strength on the ATPase activity of cardiac microsomes. ●, ▲, in the presence of Ca^{2+}; △, in the absence of Ca^{2+}. 5.0 mM MgATP.

ionic strength than that of skeletal myofibrils. It declined steadily when the ionic strength of the medium was raised above 0.05 whereas that of skeletal myofibrils does not show any significant change until the ionic strength becomes greater than 0.1 (Figs. 3A and 3B). It is not as yet clear what determines the response to ionic strength.

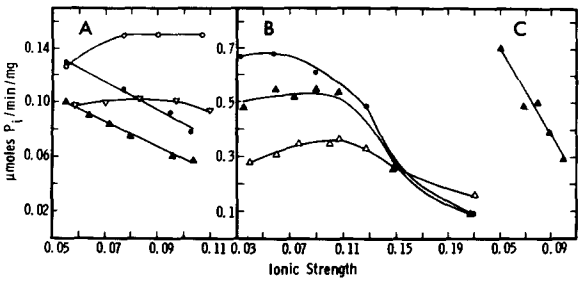

Fig. 3. Comparison between cardiac myofibrils (A) and myofibrils (B) and actomyosin (C) from fast skeletal muscle with respect to the dependence on ionic strength of nucleoside triphosphatase activity. Open symbols, MgITP; closed symbols, MgATP. $MgCl_2$: ▲, △, ○, 5.0 mM; ▽, 4.0 mM; ●, 1.0 mM.

The relative insensitivity of skeletal myofibrils is not an invariable property of skeletal actomyosin. The sensitivity of skeletal actomyosin is greatly increased when it has been reconstituted from myosin and actin (Fig. 3C) or after myofibrils have been briefly treated with trypsin as for the removal of troponin (Fig. 4). Trypsin treatment also increased the sensitivity of cardiac myofibrils to ionic strength (Fig. 4). Increasing ionic strength inhibited only the rate of hydrolysis of ATP by cardiac myofibrils whereas that of ITP was not affected (Fig. 3A).

At all ionic strengths above 0.07 cardiac myofibrils hydrolyzed ITP more rapidly than ATP. That distinguishes them from skeletal myofibrils or most skeletal actomyosin preparations whose ITPase activity is distinctly lower than their ATPase activity (Figs. 3A and 3B). However, we have observed that in some preparations of skeletal actomyosin this ratio between ITPase and ATPase is inverted so that the rate of ITP hydrolysis was 2–3 times greater than that of ATP. Thus this modification causes skeletal actomyosin to resemble cardiac myofibrils.

This alteration of the behavior of skeletal actomyosin was usually found with preparations reconstituted from actin and myosin and never with skeletal myofibrils.

TABLE II

TRANSFORMED SKELETAL ACTOMYOSIN

1 mM MgATP + 1 mM $MgCl_2$; 6 mM MgITP + 4 mM $MgCl_2$.

Myosin No.	*Actin No.*	*ATPase activity**	*ITPase activity**
421	704	0.4	0.6
421	802	0.3	0.6
400	306	0.2	0.6
201	402	0.4	0.7
Trypsin-treated myofibrils		0.75	0.3
		0.8	0.5

* μmoles P_i/min per mg.

It seems to be caused by a modification of the myosin molecule because, as shown in Table II, it persisted when the myosin was combined with different actin preparations. This modification of the myosin molecule sometimes occurred during purification procedures. We do not know the cause for it. However, modified skeletal actomyosin resembled much more closely cardiac actomyosin than does the native enzyme. Not only did this transformation cause the inversion of the ratio of ITPase activity but it produced the inversion by a considerable reduction of the rate of ATP turnover to values more similar to those of cardiac actomyosin. Therefore we were intrigued whether cardiac actomyosin possessed in the native state a conformation that could be induced in skeletal actomyosin.

To investigate this possibility we carried the comparison between cardiac myofibrillar and skeletal transformed actomyosin one step further. We measured in both preparations ATPase activity as a function of ATP concentration. We observed for transformed skeletal actomyosin that the rate of ATP hydrolysis did not increase steadily on increasing the concentration of ATP from 10^{-6} to 10^{-3} M as is true for skeletal myofibrils (Fig. 5). Instead the rate reached a maximum at 20 μM ATP and gradually declined at higher concentrations. The low ATP turnover at 1.0 mM ATP was apparently due to the binding of ATP to an inhibitory site with a rather high affinity for ATP. Therefore the effect of increasing substrate saturation was masked already at quite low ATP concentrations by the effect of increasing saturation of the inhibitory

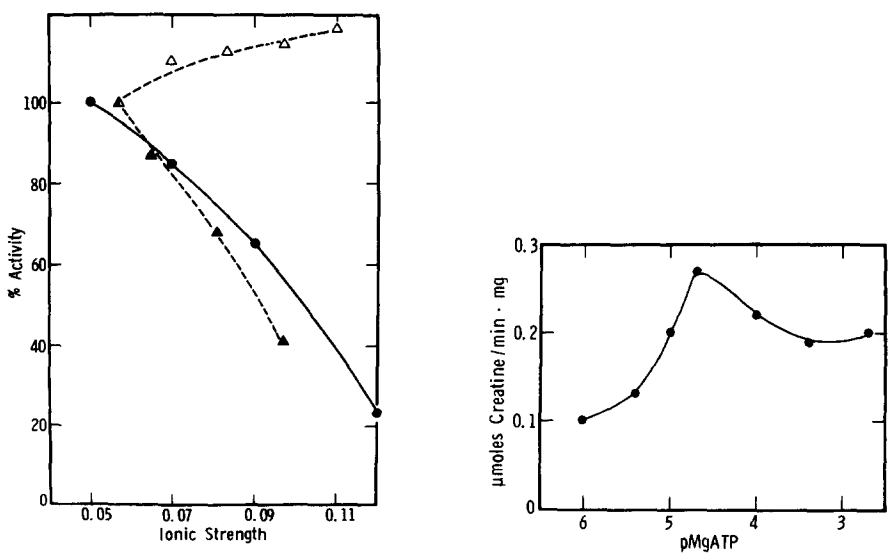

Fig. 4. The dependence of nucleoside triphosphatase activity on ionic strength after trypsin treatment of cardiac and skeletal myofibrils. ●, skeletal; ▲, △, cardiac myofibrils. Open symbols, MgITP; closed symbols, MgATP. ●, 2 mM MgATP, 1.0 mM $MgCl_2$; ▲, 5.0 mM MgATP, 1.0 mM $MgCl_2$; △, 4.0 mM MgITP, 4.0 mM $MgCl_2$.

Fig. 5. The rate of ATP hydrolysis as a function of the ATP concentration in altered skeletal reconstituted actomyosin. Actomyosin reconstituted by mixing 20 mg purified myosin with 7 mg actin nearly saturated with tropomyosin + troponin. The actomyosin was capable of 80% relaxation 0.27 mg/ml protein; 1.6 mM creatine phosphate and 400 μg/ml kinase; 0.07 ionic strength; 5.0 mM $MgCl_2$; 0.1 mM $CaCl_2$.

site. Such an action of low concentrations of ATP on isolated actomyosin has recently been proposed by WATANABE[14].

However, the low rate of ATP turnover of cardiac myofibrils was not caused by an inhibitory action of ATP. The rate increased steadily with increasing ATP and a Scatchard plot of the data gave a straight line (Figs. 6A and 6B). The value of 10 μM for the K_m is similar to that of unmodified skeletal actomyosin.

ATPase activity under relaxing conditions and the Ca^{2+} concentration required for reactivation

Cardiac myofibrils contain troponin[15] and therefore require calcium for the activation of actomyosin ATPase activity[16,10]. However, the ATPase activity of cardiac myofibrils was less depressed by Ca^{2+} removal (by only 65–75 %, Figs. 1, 8, 9; Tables III and IV) than that of skeletal myofibrils[17] (by 90–95 %). This difference may in part be explained by the loss of troponin from cardiac myofibrils because the addition of a crude extract containing troponin and tropomyosin[6] increased the effect of Ca^{2+} removal (Table III).

Just as for skeletal myofibrils, capability for relaxation was removed by short exposure to trypsin (Table IV), a treatment which has been shown to remove troponin from skeletal actomyosin[6,15] and by aging at elevated temperatures[18] (Fig. 7). Relaxation seemed also to be depressed when imidazole buffer was replaced by Tris buffer (Fig. 8).

TABLE III

EFFECT OF TROPONIN TOGETHER WITH TROPOMYOSIN ON CARDIAC MYOFIBRILLAR ATPase ACTIVITY

	ATPase activity (*μmoles P_i/min per mg protein*)			
	0.1 mM Ca^{2+}		*4.0 mM EGTA*	
	−Troponin	*+Troponin*	*−Troponin*	*+Troponin*
ATPase activity	0.150	0.139	0.052	0.029
% Maximal activity			34.7	20.8

TABLE IV

EFFECT OF TRYPSIN TREATMENT ON CARDIAC MYOFIBRILLAR ATPase ACTIVITY

5 mM MgATP + 1 mM $MgCl_2$; I = 0.06.

Treatment	*ATPase activity* (*μmoles P_i/min per mg protein*)	
	0.1 mM Ca^{2+}	*4 mM EGTA*
Control	0.11	0.04
With pyrophosphate	0.14	0.05
3 min trypsin + pyrophosphate	0.13	0.14
6 min trypsin + pyrophosphate	0.11	0.14

As regards the concentration of Ca^{2+} required to reactivate myofibrillar ATPase activity in cardiac muscle, different values have been reported (*cf.* FANBURG[19], KATZ AND REPKE[20]). The data for cardiac myofibrils presented in Fig. 9 are similar to those given by KATZ AND REPKE[20] for reconstituted cardiac actomyosin. The value of 0.6–1.0 μM for Ca^{2+} concentration for half activation is very similar to that of skeletal myofibrils[21] provided the conditions are the same with respect to the concentration of free Mg^{2+}, ionic strength, and pH.

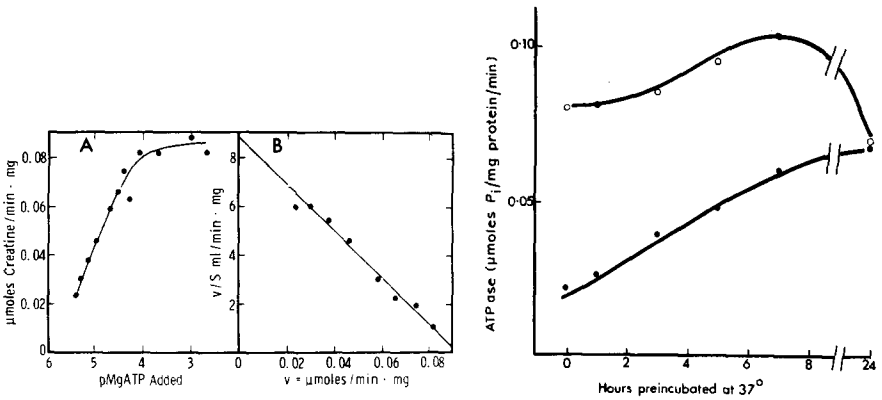

Fig. 6. Rate of ATP hydrolysis of cardiac myofibrils as a function of ATP concentration (A) and the corresponding Scatchard plot (B). Ionic strength 0.06; 4.0 mM creatine phosphate and 1.2 mg/ml kinase.

Fig. 7. Influence of aging at 37° on the Ca^{2+} dependence of cardiac myofibrillar ATPase activity, ○, 0.1 mM $CaCl_2$; ●, 4.0 mM EGTA.

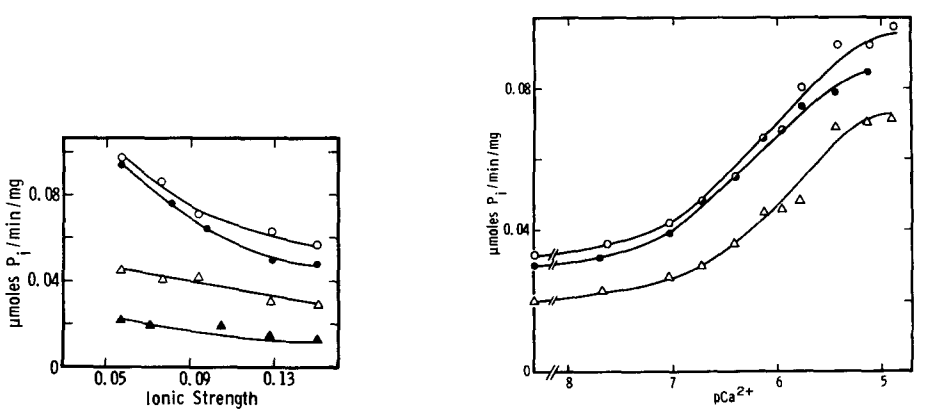

Fig. 8. Effect of Tris buffer on the Ca^{2+} dependence of cardiac myofibrillar ATPase activity. ●, ▲, imidazole buffer (pH 7.0); ○, △, Tris buffer (pH 7.0). ○, ●, in the presence of Ca^{2+}; △, ▲, in its absence. In the presence of 10 mM imidazole buffer the ionic strength was adjusted with KCl; in the presence of Tris buffer with increasing concentrations of Tris buffer; the ATP concentration was 5.0 mM MgATP.

Fig. 9. Cardiac myofibrillar ATPase activity as a function of pCa^{2+}. Three different myofibril preparations assayed under identical conditions. EGTA + CaEGTA 4.0 mM; 0.06 ionic strength.

DISCUSSION

Our studies show that the mitochondrial contamination of cardiac myofibrils was to a greater extent reduced by differential centrifugation in 40 % sucrose–KCl than with the methods previously used[19,28]. The contribution of mitochondrial ATPase activity to the total ATPase activity was decreased from about 40 % to about 15 %. We also demonstrated that in these preparations reticulum does not appear to contribute to ATPase activity to any significant extent. Furthermore, cardiac myofibrillar ATPase activity is quite stable and is not reduced if the whole muscle is allowed to stand at room temperature for several hours. Therefore cardiac myofibrillar ATPase activity may be satisfactorily studied in autopsy material[29].

Our data on the sensitivity to ionic strength differ from those of BARANY and colleagues[1]. BARANY reported that cardiac and skeletal myofibrillar ATPase respond to increasing ionic strength in a very similar manner. If, however, their preparations should have been heavily contaminated by mitochondria (they did not take any precautions to remove them nor did they attempt to estimate the extent of the contamination) the changes in myofibrillar ATPase activity may have been masked by the mitochondrial ATPase activity.

We do not understand why the extent of relaxation of cardiac myofibrils is less than that of skeletal myofibrils. Possibly troponin is more easily lost from cardiac than from skeletal myofibrils since relaxation was increased by the addition of skeletal troponin + tropomyosin. However, even after this addition the extent of relaxation did not become equal to that of skeletal preparations suggesting the possibility that cardiac myofibrils might contain some inactive, tightly bound troponin that could not be displaced by active skeletal troponin.

Our data on the calcium activation of ATPase activity of cardiac myofibrils confirm those of KATZ AND REPKE[20] on reconstituted actomyosin in spite of the great differences in the values for the maximal rates of hydrolysis. (Their maximal rates are about 1/6th of our average rate.) It is not clear why under FANBURG'S[19] conditions higher calcium concentrations were required for the activation of APTase activity. Our data do not seem to reflect the observation by EBASHI[15] and his colleagues that cardiac troponin has a lower affinity for calcium than does skeletal troponin. We found that under similar conditions the calcium concentration for half maximal activation of ATPase activity was very similar for cardiac and skeletal myofibrils (*cf.* ref. 21, Fig. 1, Curve 2). However, this observation is not in contradiction with EBASHI'S binding measurements because Ca^{2+} activation of ATPase activity is not proportional to Ca^{2+} binding[30].

Under conditions for contraction (in the presence of Ca^{2+}) cardiac myofibrillar ATPase differs from its skeletal counterpart in several aspects. In fact its resemblance to the ATPase of modified skeletal actomyosin is greater than to the native myofibrillar ATPase with regard to its lower turnover rate for ATP, the ratio of ATPase/ITPase activity and its sensitivity to ionic strength. All these properties seem to depend on the myosin moiety since they remain unchanged on combining the same modified skeletal myosin preparation with different actins (Table II) or cardiac myosin with skeletal actin[1]. Furthermore, the slow rate of ATP turnover of modified skeletal actomyosin can be returned to high values by treatment of only the myosin with low concentrations of *N*-ethylmaleimide (R. D. BREMEL, personal communication). The slow

rate of ATP turnover by cardiac actomyosin is paralleled by the slow rate by cardiac myosin alone, without actin, when activated by monovalent ions or Ca^{2+} (ref. 22). In spite of the great differences in the rate of ATP hydrolysis[1,22], in substrate preference, and in optimal conditions for hydrolysis, cardiac myosin is rather similar to the protein of fast skeletal muscle[1]. There is a close resemblance of their structures as seen by the electron microscope[23,24] (there is even evidence for two heads), of their physicochemical characteristics[23,25] and their amino acid composition[1,27,26] except for a possible 10% difference in their cysteine content[1,26,27]. In view of the close similarity of the skeletal and the cardiac protein it seemed intriguing to consider the possibility that cardiac myosin may maintain a conformation in the native state that resembled a conformation that could occur also in skeletal myosin, although only occasionally after some changes in the molecule of as yet quite unknown nature. However, this expectation was not fulfilled; the similarity between cardiac and modified skeletal myosin ended when the dependence of the rate of hydrolysis on ATP concentration was investigated. The ATPase activity of the transformed skeletal actomyosin was low because ATP exerted a dual function: in addition to being a substrate it also was bound to a second site where it acted as an inhibitor. There was no evidence for any inhibitory action of ATP in cardiac actomyosin.

We do not yet know what happens to myosin to cause the altered conformation whereby ATP becomes inhibitory in concentrations lower than those required for substrate saturation. This phenomenon is still under investigation.

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