Cryoenzymic Studies on an Organized System: Myofibrillar ATPases and Shortening[†,‡]

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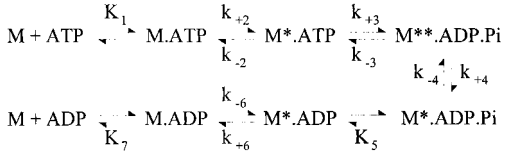
ABSTRACT: We have exploited cryoenzymology, first, to probe the product release steps of myofibrillar ATPase under relaxing conditions and, second, to define the conditions for studying the contractile process in slow motion. Cryoenzymology implies perturbation by temperature and by the antifreeze added to allow for work at subzero temperatures. Here, we studied myofibrillar shortening and ATPases by the rapid quench flow method over a wide temperature range (−15 to 30 °C) in two antifreezes, 40% ethylene glycol and 20% methanol. The choice of solvent and temperature was dictated by the purpose of the experiment. Ethylene glycol (40%) is suitable for investigating the kinetics of the products release steps which is difficult in water. In this cryosolvent, the myofibrillar ATPase is not activated by Ca^{2+} nor is there shortening, except under special conditions, i.e., Ca^{2+} plus strong rigor bridges [Stehle, R., Lionne, C., Travers, F., and Barman, T. (1998) *J. Muscl. Res. Cell Motil. 19*, 381−392]. By the use of the glycol, we show that at low Ca^{2+} the kinetics of the ADP release are much faster with myofibrils than with S1. On the other hand, the kinetics of the P_i release were very similar for the two materials. Therefore, we suggest that, upon Ca^{2+} activation, only the P_i release kinetics are accelerated. In 20% methanol, in the presence of Ca^{2+}, myofibrils shortened at temperatures above −2 °C but not below. At a given temperature above −2 °C, both the shortening and ATPase rates were reduced by the methanol. The temperature dependences of the myofibrillar ATPases ($\pm Ca^{2+}$) converged with a decrease in temperature: at 20 °C, Ca^{2+} activated 30-fold, but at −15 °C, only about 5-fold. We suggest that studies in methanol may open the way for an investigation of muscle contraction in slow motion and, further, to obtain thermodynamic information on the internal forces involved in the shortening process.

Muscle contraction depends on the cyclic interaction of actin with the myosin heads, the energy for which is supplied by the hydrolysis of ATP by the heads. The problem is to connect movement with ATP hydrolysis.

From studies with S1[1] and acto-S1, the myosin heads hydrolyze ATP by the pathway in Scheme 1 (*1*, *2*), where M represents myosin heads with or without actin interaction; K_1 and K_7 are dissociation constants, and $K_3 = k_3/k_{-3}$. The intermediates on the pathway interact in different ways with the thin filament, and it is thought that this interaction is responsible for the contractile process. Therefore, to understand fully muscle contraction, one must identify the predominant intermediates and determine their rates of interconversion in the contractile system.

The myofibril appears to be a good model for muscle contraction. Myofibrils are the functional contractile units of muscle and yet they are small enough for study by rapid transient kinetic methods. They appear to be fully regulated and unlike actomyosin can be studied at physiological ionic strengths. With them, chemical kinetic (*3*, *4* and references therein) and mechanical (*5*−*7*) studies have been carried out. The evidence available suggests strongly that in myofibrils the myosin heads hydrolyze ATP by Scheme 1. It is noteworthy that the kinetics of the initial steps of the myofibrillar and S1 ATPases (ATP binding and cleavage) are similar.

Scheme 1



The kinetics of the product release steps of myofibrillar ATPase are of special interest because they may be linked directly with movement. Thus, the P_i release step (directed by k_4), or a preceding isomerization, may be connected with the driving stroke (*8*, *9*) and the ADP release step (k_6) with shortening velocity (*10, 11*). With relaxed myofibrils (i.e., at low Ca^{2+}), k_4 is clearly rate limiting; it has been measured specifically and is similar to the k_4 with S1 (*12, 13*). Upon the addition of Ca^{2+} to relaxed myofibrils or actin to S1, k_4 is accelerated greatly.

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[1] Abbreviations: S1, myosin subfragment 1; P_i, inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Whereas the ADP release kinetics with S1 have been measured (*14* and references therein), those with relaxed myofibrils, or indeed with any structurally intact contractile system, have not. The kinetics of the release of the ADP analogue 1-N^6-etheno-2-azo-ADP from cardiac myofibrils have been measured: they are fast and similar to those from cardiac acto-S1 (*15*). However, under the conditions used (displacement of the ADP analogue bound to the myofibrils by ATP), the myofibrils were presumably rigor activated, even in the presence of EGTA. Therefore, it is not clear whether the heads having bound ADP in relaxed myofibrils are attached (in which case k_6 could be fast, as with acto-S1) or whether they are detached (ADP release kinetics slow, as with S1). The question, then, can be narrowed down to asking if with relaxed myofibrils Ca^{2+} accelerates both k_4 and k_6 or just k_4.

A powerful way to obtain information on enzyme reaction pathways is to work under cryoenzymic conditions (*16*−*18*). This technique involves two perturbants: temperature and an antifreeze, usually an organic solvent. The interest of cryoenzymology is that it may permit the accumulation of reaction intermediates that cannot be observed under normal conditions, by the slowing down of the kinetics of their formation, by a change in rate-limiting step or by shifts in equilibria. Further, it allows for Arrhenius plots over extensive temperature ranges.

Here, we applied cryoenzymology to myofibrils with two aims: first, to allow for a study of the ADP release kinetics with relaxed myofibrils and, second, to facilitate studies on the events involved in the chemo-mechanical energy transduction in the myofibril.

Because of its extensive use with S1 and acto-S1 (*14*, *19*−*23*), we began our study by the use of ethylene glycol as antifreeze. Thus, in 40% ethylene glycol, we were able to probe the coupling of Ca^{2+} and rigor activation (*24*). In this solvent, the myofibrillar ATPase was not activated by Ca^{2+} and there was no shortening, except under special conditions (presence of Ca^{2+} and strong rigor bridges).

To extend our study to conditions under which myofibrils are regulated, i.e., their ATPase rate accelerated and their shortening induced by Ca^{2+}, we then searched for another cryosolvent, and after trying several, we finally chose 20% methanol.

We show here that, in both 40% ethylene glycol and 20% methanol, the ADP release kinetics are much faster with relaxed myofibrils than with S1. This suggests that the ADP release kinetics of the myosin heads are accelerated by the myofibrillar environment, presumably because even in relaxed myofibrils the heads with bound ADP are attached to the thin filament. Further, we show that in 20% methanol, the myofibrillar shortening and ATPase rates are reduced significantly. Therefore, with the myofibrils as a model, cryoenzymology allows for the study of muscle contraction in slow motion.

MATERIALS AND METHODS

Myofibrils and Chemicals. Myofibrils were prepared from the rabbit psoas as in Herrmann et al. (*25*). They were stored at 4 °C for no longer than 3 days in 0.1 M potassium acetate, 5 mM KCl, 2 mM magnesium acetate, 50 mM Tris-acetate, 1 mM DTT, 0.5 mM sodium azide, 0.2 mM PMSF, 10 μM leupeptin, and 5 μM pepstatin adjusted to pH 7.4 with acetic acid at 20 °C. Immediately before use, aggregates were removed by filtration through a polypropylene filter with 149 μm pore openings (Spectra Mesh, Spectrum Med. Ind., Inc., California). The concentration of myosin heads was measured by absorption at 280 nm (*26*).

Ethylene glycol and methanol (analytical grade) were from Merck, Darmstadt, Germany. The sources of the other chemicals are in Herrmann et al. (*26*) and Lionne et al. (*4*).

Experimental Buffers. The basic buffer was 0.1 M potassium acetate, 5 mM KCl, and 50 mM Tris with or without 40% (v/v) ethylene glycol or 20% methanol (v/v). For experiments in the absence of Ca^{2+} (relaxing conditions) the buffer contained 2 mM EGTA and 5 mM magnesium acetate, for those in its presence (activating conditions) it contained 0.1 mM $CaCl_2$ and 2 mM magnesium acetate. For convenience sake, we term myofibrils in the buffer at low Ca^{2+} relaxed myofibrils and in the buffer at high Ca^{2+} activated myofibrils. For experiments with Mg-ADP, the buffers also contained 25 μM P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap_5A). The pH of the buffers was adjusted to 7.4, with or without added solvent, with acetic acid at 20 °C.

Chemical Kinetics Experiments. These were carried out using [γ-^{32}P]ATP and the $^{32}P[P_i]$ production measured by a filter paper method (*27*). The P_i thus determined is the sum of free and enzyme-bound P_i (see Scheme 1). *Steady-state experiments* were carried out in thermostatically controlled beakers. At suitable intervals, portions were removed and quenched in acid (22% trichloroacetic acid + 1 mM KH_2PO_4), and $^{32}P[P_i]$ was measured. We define the ratio ATPase with Ca^{2+} to that without Ca^{2+} as the activating factor. *Transient kinetic experiments* were carried out in a rapid quench flow apparatus (*28*). The procedure was to mix myofibrils or S1 with [γ-^{32}P]ATP in the apparatus, allowing the mixtures to age (0.25 s and up), quenching them in acid and then measuring the $^{32}P[P_i]$.

Measurement of Myofibrillar Shortening. Myofibrils were treated with ATP under different conditions (water, ethylene glycol, or methanol buffers, $\pm Ca^{2+}$), any shortening stopped in the rapid flow quench apparatus in 0.1 M potassium acetate adjusted to pH 4.4 with acetic acid and the sarcomere lengths measured using a Leica DMR microscope equipped with bright field optics. Images were collected by a CCD camera (Hamamatsu C5985), stored on a computer, and displayed on a high-resolution monitor. Total magnification was 2000×. Measurements were made using a grid placed on the screen of the monitor that had been calibrated with a Nikon micrometer slide. For each time point, sarcomere lengths were measured in 20−40 different myofibrils. The average sarcomere length for each myofibril was obtained by measuring the lengths of the sarcomeres in linear portions that contained at least five consecutive sarcomeres.

The pH 4.4 quench did not appear to alter the sarcomere structures. In particular, the characteristic configurations of the A and I bands remained. Thus, under relaxing conditions, the width of the A band was larger than that of the I band, whereas under rigor conditions (i.e., in the absence of ATP), the A band was compressed so the two bands had similar widths.

Analysis of Kinetic Data. For the analysis, we used Scheme 1 and the Grafit program (Erithacus Software Limited,

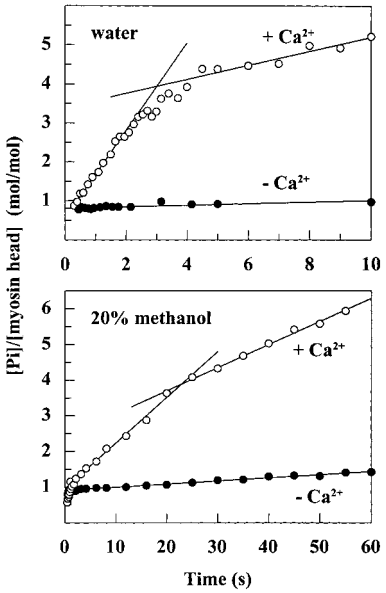

FIGURE 1: Effect of 20% methanol on the myofibrillar ATPases in the absence and presence of Ca^{2+} at 4 °C. The reaction mixtures (3 μM in myosin heads + 30 μM [γ-^{32}P]ATP) were aged for the times indicated, quenched in acid and the [^{32}P]P_i determined. The buffers are given in Materials and Methods.

Staines, U.K.). ATP sites were titrated by the cold ATP chase method (*28*). The myofibrils used here titrated 0.6−0.9 mol of ATPase site/mol of myosin head.

In our estimates of the kinetic constants, we do not give errors. The errors given by the computer analysis are small (2−3%), but these do not take account of the potentially larger experimental errors, e.g., different batches of the biological material, reactants, and solvents and inadvertent errors in the concentrations of these components. More realistically, the errors are probably in the range 10−20% but less when the data are expressed on a logaritmic scale as in Arrhenius plots.

RESULTS

Effects of Solvent Composition on the Myofibrillar ATPase and Shortening Rates at 4 °C. Typical P_i progress curves in water and 20% methanol at 4 °C are shown in Figure 1. It is noteworthy that the curves in methanol are qualitatively similar to those in water. Thus, at low Ca^{2+}, there was a transient P_i burst phase followed by a slow steady state, and at high Ca^{2+}, a P_i burst and a fast steady state (k^F) followed by a decelaration to a slow steady state (k^S). This is very different from the situation in 40% ethylene glycol where Ca^{2+} had little effect: a P_i burst followed by a slow steady state whether or not Ca^{2+} was present (*24*). In water or 20% methanol, the extrapolations of k^F and k^S intercept at a break, this is a practical parameter that we use as an indicator of myofibrillar shortening. This point of interception is subject to large errors when k^F and k^S are similar, as at low temperatures (see below). The results are summarized in Table 1.

There are several interesting features of the kinetic parameters obtained in the three solvents (Table 1). First, under relaxing conditions, the steady-state ATPase rate was little affected by the solvent. In this respect, relaxed myofibrils behave as S1 [ATPase not affected by 40% ethylene glycol (*14*)]. Second, whereas in 40% ethylene glycol the myofibrillar ATPase was hardly activated by Ca^{2+} (*24*), in 20% methanol, it was activated 13-fold. In water at 4 °C, Ca^{2+} activated the ATPase 60-fold. Third, the progress curves all have P_i bursts, whether or not organic solvent was present. This shows that, in methanol as in water (*4*), the concentration of ATP used (30 μM) was well above its K_m. As seen in Figure 1, on the time scale of these experiments, the tail end of the P_i burst kinetics were obtained in the methanol (for the sake of clarity, the early experimental points with the relaxed myofibrils were left out). We exploit this increase in the temporal resolution elsewhere.

Table 1: Myofibrillar ATPases and Shortening in Different Solvents at 30 μM ATP and 4 °C[a]

		solvent		
	parameter	water	20% methanol	40% ethylene glycol
ATPase				
$-Ca^{2+}$	k_{ss} (s^{-1})	0.019	0.010	0.015
$+Ca^{2+}$	k^F (s^{-1})	1.12	0.128	no ≪ break ≫
	k^S (s^{-1})	0.18	0.07	no ≪ break ≫
	k_{ss} (s^{-1})			0.02
	activating factor[b]	60	13	1.3
shortening	(μm hs^{-1} s^{-1})	0.06[c]	0.02[d]	no

[a] Data in water and methanol are from Figure 1, those in ethylene glycol are from Stehle et al. (*24*). [b] Ratio ATPase with Ca^{2+} to ATPase without Ca^{2+}. [c] From Lionne et al.(*4*). [d] From Figure 2.

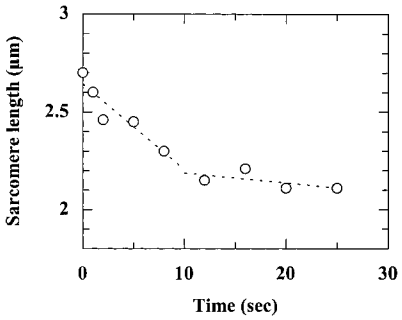

FIGURE 2: Time course for myofibrillar shortening in 20% methanol at 4 °C. Reaction mixture (3 μM in myosin heads + 30 μM ATP) were quenched at pH 4.4 at the times shown and the sarcomere lengths were measured under the microscope.

In 20% methanol, Ca^{2+}-activated myofibrils shortened, as illustrated in Figure 2. At the concentration of ATP used (30 μM), the initial shortening rate was about 0.025 ± 0.005 (μm/half sarcomere) s^{-1} which is slower than the 0.06 ± 0.01 (μm/h.s.) s^{-1} in water, also at 30 μM ATP (*4*) (Table 1). It is noteworthy that the deceleration of the shortening and ATPase rates occurred in the same time range, but since under the conditions used, k^F and k^S are similar, it was difficult to obtain a precise estimate for the transition. Further, we note that in the break region the sarcomere lengths are 2.1−2.3 μm, similar to those found in water (*4*).

In 40% ethylene glycol, myofibrils do not shorten under multiturnover conditions: shortening only occurred in the presence of Ca^{2+} and strong interactions, e.g., at low ATP concentrations (*24*).

Effect of Temperature on the Myofibrillar and S1 ATPases in Different Solvents. Typical P_i progress curves for myofibrillar ATPases in 20% methanol at 20 °C and −15 °C are illustrated in Figure 3.

At 20 °C, the ATPase at low Ca^{2+} was 0.12 s^{-1}. At high Ca^{2+}, the initial ATPase was 2.9 s^{-1} (activating factor 24×)

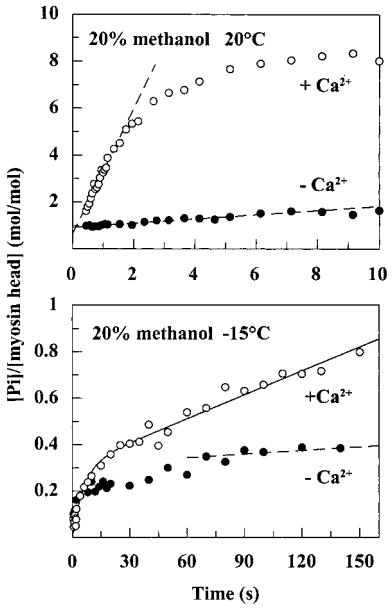

FIGURE 3: Myofibrillar ATPase progress curves in 20% methanol. The reaction mixtures (3 μM in myosin heads + 30 μM [γ-^{32}P]-ATP) were quenched in acid at the times indicated and the [^{32}P]P_i determined. At 20 °C, for Ca^{2+}-activated ATPase $k^F = 2.70\ s^{-1}$, and for relaxed ATPase $k_{ss} = 0.089\ s^{-1}$. At −15 °C, for Ca^{2+}-activated ATPase $k_{obs} = 0.17\ s^{-1}$ and $k_{ss} = 3.4 \times 10^{-3}\ s^{-1}$, for relaxed ATPase $k_{ss} = 5 \times 10^{-4}\ s^{-1}$. For fitting, see text.

and there was a break at about 2 s to a k^S of 0.46 s^{-1}. At this temperature, the myofibrils shortened. At −15 °C and high Ca^{2+}, the time course consists of a rapid rise that fit well to an exponential of $k_{obs} = 0.18\ s^{-1}$ and amplitude 0.3 mol of P_i/mol of myosin head followed by a steady-state rate of $3.4 \times 10^{-3}\ s^{-1}$. At low Ca^{2+}, there was a rapid rise, an apparently linear phase [possibly a manifestation of rigor activation (*13*) but at 0.1 mol of P_i/mol of myosin head the amplitude was too small for an exploitation] and then a steady-state rate of $5 \times 10^{-4}\ s^{-1}$. In the figure, the steady-state phase (dashed line) is an extrapolation from data on the minutes time scale, as for the Arrhenius plots (Figure 4). At −15 °C, with or without Ca^{2+}, the myofibrils did not shorten even after a long incubation time (30 min).

The low amplitude of the transient in the burst experiments at −15 °C is noteworthy. At 4 °C, the amplitude was 0.96 mol of P_i/mol of head (Figure 1) and, at −5 and −10 °C, 0.76 and 0.54 mol of P_i/mol of head, respectively (results not illustrated). This decrease of the P_i burst amplitude with the temperature is almost certainly a reflection of the temperature sensitivity of the equilibrium constant for the cleavage step (K_3), as with S1 (e.g., ref *14*), and it suggests strongly that in methanol as in water (*12*) the P_i burst is due to the accumulation of M**.ADP.P_i (Scheme 1) rather than free P_i. Were the burst to be due to free P_i (and M*.ADP), its amplitude would be high and temperature independent because under the conditions used the P_i release step is essentially irreversible (as with S1, e.g., Figure 7). We note that in ethylene glycol, too, the P_i burst decreases with the temperature (Figure 7).

The temperature dependences of the myofibrillar and S1 ATPases in water, 20% methanol, and 40% ethylene glycol are in Figure 4 and the thermodynamic parameters obtained are summarized in Table 2.

When a composite rate constant is a function of two or more rate constants of very different energies of activation, the contributions of the constants change significantly as the temperature is changed. This is the case with S1 where the composite k_{cat} is directed by the kinetics of the product release steps, P_i by k_4 (low $\Delta H^{\ddagger}$) and ADP by k_6 (high $\Delta H^{\ddagger}$) (Scheme 1). Therefore, the temperature dependence of k_{cat} exhibits a "break" with k_4 being rate limiting above and k_6 below the break [break near 0 °C first shown by Malik and Martonosi (*29*), confirmed by Biosca et al. (*14*) who extended the temperature range down to −20 °C in 40% ethylene glycol].

The dependences in Figure 4 are interesting for three reasons. First, whereas with S1 in methanol and ethylene glycol, there were distinct breaks, with the myofibrils, the dependences appear to be linear in the temperature range studied. The lack of breaks in the temperature dependences of the myofibrillar ATPase suggests but does not prove that with these the P_i release kinetics remain rate limiting down to −15 °C. We confirmed this below.

Second, in 20% methanol, the $\Delta H^{\ddagger}$ of k^F is high (118 kJ mol^{-1} compared with 68 kJ mol^{-1} in water). It is noteworthy that the dependences of the myofibrillar ATPases are parallel in water and ethylene glycol but in methanol they converge with a decrease in temperature. Thus, the $\Delta H^{\ddagger}$ of k^F is higher than that of relaxed myofibrils (84 kJ mol^{-1}); therefore, the activation factor decreased as the temperature was decreased (Table 3). Third, the $\Delta H^{\ddagger}$ of k^S is low and similar to that in water and also to the $\Delta H^{\ddagger}$ for k_{ss} (at high or low Ca^{2+}) in 40% ethylene glycol (Table 2).

Effect of Temperature on Myofibrillar Shortening in 20% Methanol. The results in 20% methanol are intriguing. In this solvent, the shortening was temperature dependent: myofibrils shortened at 20 and 4 °C but not at −15 °C.

We note that, in the experiment at 4 °C, the shortening occurred immediately on the addition of ATP, as in water (*4*). This is different from the situation in 40% ethylene glycol in which myofibrils, in the presence of Ca^{2+}, only shortened upon the depletion of the ATP, i.e., with the aid of strong head-thin filament interactions (*24*).

From the all-or-none experiments in Table 3, Ca^{2+} activated myofibrils shorten at temperatures down to −2.5 °C but not below. Further, there were breaks in the P_i progress curves at 4 °C and above (at 4 °C, Figure 1, at 20 and −15 °C, Figure 3; progress curves at the other temperature not illustrated). Below 4 °C, it was difficult to discern breaks.

In Figure 5 are shown photomicrographs of myofibrils that had been allowed to shorten for different times in 20% methanol at 4 °C. At a short reaction time (0.2 s), no measurable shortening had occurred (sarcomere lengths about 2.7 μm; not illustrated but as in panel a) and the overall myofibrillar structure was similar to that in water. After a reaction time of 1 min (Figure 5b), i.e., well after the break zones in the ATPase (Figure 1) and shortening (Figure 2) progress curves, the sarcomeres had shortened to about 2 μm, which is less than the thin filament length. At longer times, shortening continued slowly; thus, at 15 min the sarcomeres had shortened to very short lengths (about 1.4 μm) but overcontraction did not occur (i.e., as in water).

Interaction of ADP with Relaxed Myofibrils and S1 in 40% Ethylene Glycol. The kinetics of the release of P_i and ADP

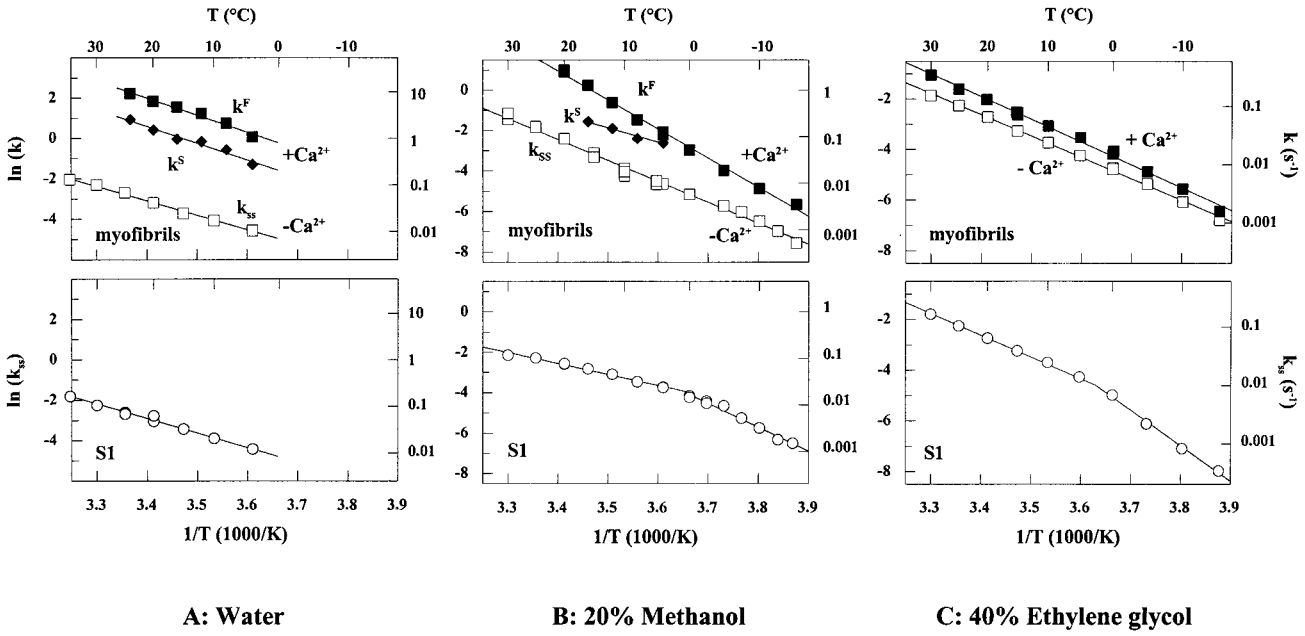

FIGURE 4: Temperature dependences of the steady-state rates of ATP hydrolysis by activated ($+Ca^{2+}$) or relaxed ($-Ca^{2+}$) myofibrils and S1 in different solvents.

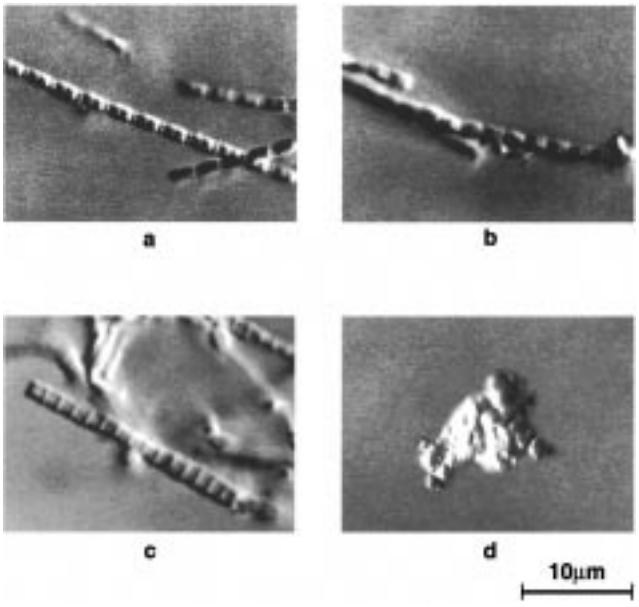

FIGURE 5: Photomicrographs of myofibrils allowed to shorten for different times in 20% methanol at 4 °C. Reaction mixtures (3 μM in myosin heads + 30 μM ATP + 0.1 mM $CaCl_2$) of different ages were quenched in pH 4.4 buffer and the myofibrils studied under the microscope. In panel a, myofibrils before addition of ATP, in panel b, following aging for 3 min and in panel c for 15 min. In panel d, the myofibrils had been allowed to shorten for 15 min in water.

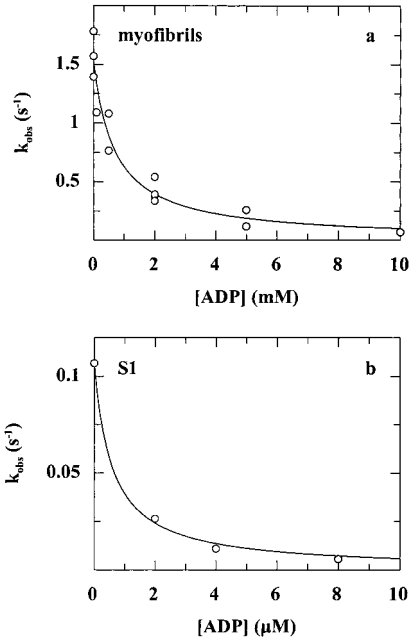

FIGURE 6: Effect of ADP upon the rate constant (k_{obs}) of P_i burst transients with relaxed myofibrils (a) and S1 (b) in 40% ethylene glycol at 4 °C. In panel a, the reaction was multiturnover (3 μM myosin heads + 30 μM [γ-^{32}P]ATP) with ADP = 0−10 mM; in panel b, it was single-turnover (1 μM S1 + 0.08 μM [γ-^{32}P]ATP) with ADP = 0−8 μM. For further details, see Sleep et al. (*34*) and the text.

with S1 ATPase are measured in single-turnover and ADP displacement experiments [k_4 and k_6, respectively, in Scheme 1 (*1*)]. However, under standard experimental conditions, these methods cannot be applied to relaxed myofibrils because of rigor activation (*24*, *30* and references therein). We approached this problem by measuring the K_d for ADP in relaxed myofibrils in the relaxant 40% ethylene glycol in which the activating effect of ADP is reduced greatly (*24*, *31*). It appears that 40% ethylene glycol inhibits Ca^{2+} activated myofibrillar ATPase by a mechanism similar to that by EGTA in water. For a further discussion, see ref *24*.

The affinity of ADP for myofibrils has been measured by a centrifugation method (*32*). Here we used a multiturnover P_i burst method that is based on the inhibitory effect (competitive) of ADP upon the kinetics of the binding of ATP to relaxed myofibrils.

As in water (*33*), in 40% ethylene glycol, progress curves of ATP hydrolysis by relaxed myofibrils consist of a rapid transient burst phase followed by the steady-state phase (not illustrated but see ref *24*). It is the transient phase (k_{obs}) that interests us. Thus, under the conditions used, k_{obs} is sensitive

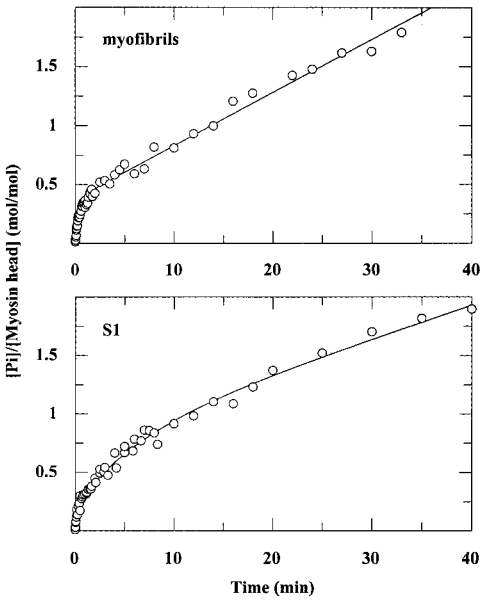

FIGURE 7: P_i progress curves in 40% ethylene glycol at −15 °C for relaxed myofibrils and S1. The reaction mixtures (3 μM in myosin heads + 30 μM [γ-^{32}P]ATP) were aged for the times indicated and quenched in acid, and the [^{32}P]P_i was determined. For relaxed myofibrils, the data were fitted to one exponential (amplitude 0.34, kinetics 0.040 s^{-1}) and a linear steady state (8.0 × 10^{-4} s^{-1}). For S1, the data were fitted to two exponentials (phase 1, amplitude 0.26, kinetics 0.04 s^{-1}; phase 2, amplitude 0.59, kinetics 1.5 × 10^{-3} s^{-1}) followed by a linear steady state (5.0 × 10^{-4} s^{-1}).

Table 2: Thermodynamic Parameters for S1 and the Myofibrillar ATPases in Different Solvents

solvent	temp range (°C)	system		break	$\Delta H^{\ddagger}$ (kJ mol^{-1})
water	4 to 40	S1[a]		none	58 ± 2
		MF − Ca^{2+} [a]		none	57 ± 2
		MF + Ca^{2+} [b]	k^F	none	68 ± 2
			k^S	none	67 ± 3
20% methanol	−15 to 30	S1		∼−5 °C	43 ± 2 (>0 °C)
					95 ± 5 (<0 °C)
		MF − Ca^{2+}		none	83 ± 2
		MF + Ca^{2+}	k^F	none	117 ± 3
			k^S	none	56 ± 4
40% glycol	−15 to 30	S1		∼0 °C	69 ± 2 (>0 °C)
					114 ± 6 (<0 °C)
		MF − Ca^{2+}		none	68 ± 1
		MF + Ca^{2+}		none	72 ± 2

[a] From Herrmann et al. (*33*). [b] From Lionne et al. (*4*). MF is myofibrils and the data in methanol and ethylene glycol (glycol) are from Figure 4.

to the ATP concentration and it is thus a reflection of the ATP binding kinetics (R.S., C.L., F.T., and T.B., unpublished results). Therefore, the dependence of k_{obs} upon the ADP concentration at a fixed ATP (30 μM) can be used to obtain K_d.

As seen in Figure 6a, the K_d of ADP to relaxed myofibrils is remarkably high at 0.7 mM. This is more than 1000× higher than the K_d to S1 (0.6 μM, Figure 6b, Table 4), which was measured by the single-turnover P_i burst method of Sleep et al. (*34*).

We also used the single-turnover method to measure the affinity of ADP to myofibrils in the absence and presence

Table 3: Effect of Temperature on Myofibrillar Shortening and ATPases in 20% Methanol

temp (°C)	shortening after incubation for 15 min[a]	break in P_i progress curve[b]	ratio ATPase rates ± Ca^{2+} [c]
20	yes	yes	28.6
10	yes	yes	17.5
4	yes	yes	12.9
2.5	yes		11.9
0	yes		10.4
−2.5	yes		9.0
−5	no		7.9
−10	no		5.9
−15	no		4.4

[a] Unless otherwise stated, reaction mixtures (3 μM in myosin heads + 30 μM ATP + 0.1 mM Ca^{2+}) were incubated at different temperatures, quenched in pH 4.4 buffer and the myofibrils examined under the microscope. [b] At T < 4 °C, breaks were difficult to detect. Only the experiments at 20, 4, and −15 °C are illustrated (Figures 3, 1 and 3, respectively). [c] Data extrapolated from the Arrhenius plots in Figure 4.

Table 4: K_d for ADP with Myofibrils and S1 in 40% Ethylene Glycol at 4 °C under Multiturnover (MTO) or Single-Turnover (STO) Conditions

system	method	K_d (μM)
S1[a]	STO[b]	0.60 ± 0.07
relaxed myofibrils[a]	MTO[c]	700 ± 140
rigor-activated myofibrils	STO[d]	840 ± 50
Ca^{2+}-activated myofibrils	STO[d]	800 ± 110

[a] From Figure 6. [b]Myosin head and ATP concentrations were, respectively, 1 and 0.08 μM. [c]Myosin head and ATP concentrations were, respectively 3 and 30 μM. [d]Myosin head and ATP concentrations were, respectively, 3 and 0.6 μM.

of Ca^{2+} [i.e., both under activating conditions (*24*)], and the values obtained are very similar to that with relaxed myofibrils in multiturnovers (Table 4).

Pi Burst Experiments at −15 °C in 40% Ethylene Glycol. Typical P_i burst experiments with S1 and relaxed myofibrils at −15 °C are shown in Figure 7.

With S1, the progress curve was triphasic: a biphasic transient phase followed by a slow steady-state rate. By Scheme 1, we interpret the initial fast transient by the kinetics of the formation of M**.ADP.P_i (a function of K_1, k_2, and $k_3 + k_{-3}$), the second transient by the P_i release kinetics ($k_o = k_4K_3/1 + K_3$), and the final steady-state phase by the rate-limiting ADP release kinetics, k_6. This interpretation is in accord with the "break" in the Arrhenius plot in Figure 4C, i.e., that at −15 °C, k_6 is clearly rate limiting ($k_6 \ll k_4$). Thus, by carrying out a single P_i burst experiment with S1 at −15 °C, one can obtain information on the release kinetics of both P_i and ADP.

However, with relaxed myofibrils, the progress curve was biphasic: a rapid transient followed by a steady-state rate. By Scheme 1, we interpret the rapid transient by the kinetics of formation of M**.ADP.P_i (as for S1 and with identical kinetics) but here the steady-state rate is governed by the rate-limiting release of P_i step. This is in accord with the absence of a break in the Arrhenius plot (Figure 4C) and with the similarity of myofibrillar steady-state rate (8 × 10^{-4} s^{-1}) with the k_o in the S1 experiments (1.5 × 10^{-3} s^{-1}, Figure 7).

From these experiments, we conclude that, whereas with S1 $k_4 \approx k_6$, with relaxed myofibrils $k_4 \ll k_6$ in the temperature range 30 to −15 °C.

DISCUSSION

Our initial objective in applying cryoenzymology to myofibrils was to increase the temporal resolution to facilitate studies on shortening and ATPases rates. First we tried 40% ethylene glycol, because of its extensive use with S1 and acto-S1, but in this solvent myofibrils do not shorten except under special conditions (*24* and references therein).

We then tried 20% methanol, and in this solvent at temperatures above 0 °C, myofibrils shorten. Further, above 0 °C, their ATPase profile have breaks reflecting the shortening process (*4*). Importantly both their shortening and ATPase rates were reduced substantially by the methanol.

The concentrations of 40% ethylene glycol and 20% methanol are 7.1 and 4.9 M, respectively. It is unlikely that the differences in the effects of the two solvents are due to the different concentrations used because myofibrils shorten in 30% methanol (7.1 M) (C.L., R.S., F.T., and T.B., unpublished results).

Does the Relaxed Myofibrillar Environment Affect the Product Release Steps? There is considerable evidence that the kinetics controlling the release of products on the myosin head ATPase pathway are involved in the contractile process (e.g., refs *8* and *9*) and it seemed important to obtain information on these kinetics with relaxed myofibrils.

The relaxed myofibrillar k_4 has been measured and shown to be very similar to the k_4 with S1; with both systems, k_4 is slow and rate limiting at temperature above 4 °C (*12*, *33*). Thus, it appears that under relaxing conditions the P_i release kinetics are little affected by the myofibrillar environment.

Next, consider k_6. By the use of Scheme 1 and making certain assumptions, we can estimate k_6 for relaxed myofibrils in 40% ethylene glycol.

First, consider S1 with which k_6 has been measured and shown to be similar to k_4, depending on the experimental conditions (*1*). Upon the addition of actin, there is a sharp increase in the overall ATPase because both k_4 and k_6 are accelerated. The second-order binding constants for ADP (k_{-6}/K_7) and ATP (k_2/K_1) are similar (*1*). Under our conditions, $k_2/K_1 = 0.15\ \mu M^{-1}\ s^{-1}$ (ATP binding experiments; R.S., C.L., F.T., and T.B., unpublished results); therefore, we assume $k_{-6}/K_7 = 0.15\ \mu M^{-1}\ s^{-1}$, too. The overall K_d for ADP is $K_6K_7/1 + K_6$, but since $K_6 \ll 1$, this reduces to $K_d = K_6K_7$ or $k_6/(k_{-6}/K_7)$. We obtained $K_d = 0.6\ \mu M$ (Figure 6, Table 4), so we estimate $k_6 = 0.09\ s^{-1}$. Taking into account the large errors involved in its calculation, this value is in reasonable agreement with the k_6 estimated from the Arrhenius plot for the k_{ss} (Figure 4C). Thus, by extrapolating the data in the low-temperature region (i.e., where the ADP release kinetics are rate limiting) to 4 °C, a k_{ss} of $0.024\ s^{-1}$ is obtained. Since the S1 used titrated 0.60 mol of site/mol of S1, this gives a k_{cat}, and therefore k_6, of $0.04\ s^{-1}$.

We now return to relaxed myofibrils. If we assume that the arguments used to obtain k_6 for S1 apply, then with a K_d of 0.7 mM (Figure 6, Table 4) and $k_2/K_1 = 0.2\ \mu M^{-1}\ s^{-1}$, (C.L., R.S., F.T., and T.B., unpublished results), k_6 is 140 s^{-1}. It has been suggested that, with acto-S1, $k_{-6}/K_7 > k_2/K_1$ (*35*) so it is not impossible that with relaxed myofibrils $k_6 > 140\ s^{-1}$. Therefore, with the caveat that a number of assumptions were made, it appears that, under our experimental conditions, the ADP release kinetics are at least 3 orders of magnitude faster with relaxed myofibrils than with S1. This difference in kinetics is supported by the linearity of the Arrhenius plots of the k_{ss} of relaxed myofibrils, both in 40% ethylene glycol and 20% methanol, whereas the plots with S1 have breaks (Figure 4, panels B and C). It is not impossible that there are slight curvatures in the myofibrillar Arrhenius plots: k_{ss} is a composite rate constant that appears to be directed by the P_i release kinetics, i.e., $k_4K_3/(1 + K_3)$ (see ref *14* for a discussion of the non linearity of the temperature dependence of the P_i release kinetics with S1). Of course, it cannot be excluded that the linearity of the myofibrillar ATPases is because, with these, k_4 and k_6 have similar $\Delta H^{\ddagger}$ (see below). The difference in the ADP release kinetics was also confirmed by the P_i burst experiments at −15 °C (Figure 7).

The kinetics of the ADP release from acto-S1 have been estimated, in the absence of ethylene glycol. By using the ATP-induced dissociation as a probe, Siemankowski et al. (*11*) suggest that $k_6 > 400\ s^{-1}$ at 15 °C. With a $\Delta H^{\ddagger}$ of about 70 kJ mol^{-1}, $k_6 > 170\ s^{-1}$ at 4 °C. This estimate is comparable with our estimate of $>140\ s^{-1}$ for relaxed myofibrils in 40% ethylene glycol.

We conclude that with relaxed myofibrils, the ADP release kinetics of the myosin heads are affected greatly by the myofibrillar environment, indeed that they are of the same order of magnitude as with acto-S1. This implies that with myofibrils, the M.ADP state is attached to the thin filament whether or not Ca^{2+} is present. However, in both cases, the concentration of M.ADP is probably low because in experiments (in water) in which the production of free P_i was followed specifically (phosphate binding protein), transient burst phases could not be detected (*12*, *13*). Therefore, it could be that in the myofibril, the ADP release kinetics are not controlled by the Ca^{2+}-regulatory system. It follows that Ca^{2+} may activate the P_i release kinetics only which would support the key role of this step (or a preceding isomerization) in the contractile process.

It has been suggested that P_i and ADP leave the ATPase site of the myosin head by different routes: P_i by a back door in the 50 kDa domain and ADP by the front door, i.e., the door by which the ATP had entered (*36*). It is possible, therefore, that in relaxed myofibrils, whereas the back door is unaffected by the myofibrillar environment, the front door is affected. In this event only the back door exit would be Ca^{2+}-regulated.

Effects of Solvent on the Relaxed Myofibrillar and S1 ATPases. At temperatures above 0 °C, the overall Mg^{2+}-ATPase of relaxed myofibrils and S1 are very similar. This is because the P_i release kinetics are both similar and rate limiting. It is noteworthy that the solvents used here had little effect on the two ATPases; this is in accord with the idea that once the ATP is bound to the active site it closes up and the ATP becomes isolated from the environment (*14* and references therein).

This protection of the bound ATP could be a mechanism to prevent waste of energy. The basal ATPase under relaxing conditions is kept under control by the regulatory system; when this is damaged, the ATPase increases, as we have observed with certain myofibrillar preparations (aging, high

temperatures, etc.). Burke and Sivaramakrishnan (*37*) found that 18% methanol destabilized specifically the 50 kDa domain of S1. We have little evidence for this effect here, presumably because ATP was present (Figure 4, panels A and B). With myofibrils, the 50 kDa domain would be protected further by the thin filament.

Effects of Solvent and Temperature on the Myofibrillar ATPase under Activating Conditions. Whereas the ATPase of relaxed myofibrils was insensitive to 40% ethylene glycol and 20% methanol, under activating conditions, it was reduced significantly. Further, in 20% methanol, the temperature dependence of the ATPase of activated myofibrils (k^F) was very different from that in water (Figure 3). These effects have almost certainly several causes, some nonspecific, others related more specifically to the myofibril.

The effect of solvent and temperature on an enzyme active site is a reflection of the thermodynamics of the system. If this is difficult to evaluate with enzymes in solution, it is even more so with systems such as the myofibril. It is likely that the effects are mainly the result of changes induced by the solvent in the solvatation environment of the biomolecules (e.g., refs *16* and *38*). The effects of different solvents on another ATP-handling enzyme, 3-phosphoglycerate kinase, are discussed in ref *39*.

The ATPase site of acto-S1 (and also that in the Ca^{2+}-ATP-system) is probably open as it is accessible to solvent perturbation (*14*, *20*). Thus, in 40% ethylene glycol, the activity of acto-S1 ATPase is about 25% that in water. This is almost certainly a nonspecific effect, because the glycol reduced the activities of other ATP handling systems by similar extends [creatine-, arginine- and 3-phosphoglycerate-kinases (*18*, *39*)].

Ethylene glycol (40%) reduces dramatically the myofibrillar ATPase at high Ca^{2+} (*24*). Here, the main effect appears to be on the regulatory apparatus which involves interactions between several proteins that are modulated by Ca^{2+} and rigor states. It is noteworthy that the effect of ethylene glycol on chemically cross-linked myofibrils, that are permanently turned on by rigor bridges, is much less and similar to that on acto-S1 (*24*).

The effect of methanol is complex. Matsuura et al. (*40*) found that 15% methanol reduces the Ca^{2+}-activated Mg^{2+}-ATPase of regulated acto-HMM to about 30% of that in water at 25 °C. Since 15% methanol had little effect on the ATPase activity of unregulated acto-HMM, Matsuura et al. (*40*) suggested that the main effect of the alcohol on regulated acto-HMM is to trap the troponin−tropomyosin−actin complex in an off state.

The myofibrillar ATPase at high Ca^{2+} was reduced to about 20% in methanol, a reduction that is in broad agreement with Matsuura et al. (*40*). We now discuss further the methanol effect with reference to the temperature dependence of the myofibrillar ATPase in 20% methanol (Table 2 and Figure 3).

The $\Delta H^{\ddagger}$ for the myofibrillar ATPase, k^F, is considerably higher in methanol than it is in water (Table 2). An obvious explanation for this is that in this solvent there is a change in rate-limiting step, i.e., a switch from the P_i release (k_4) in water to the ADP release (k_6) in methanol: with S1 ATPase in this solvent the $\Delta H^{\ddagger}$ of these steps are 43 and 119 kJ mol^{-1}, respectively (Table 2). This explanation is unlikely with the myofibrillar ATPase because first, with acto-S1, the $\Delta H^{\ddagger}$ of the ADP release kinetics is probably low [about 70 kJ mol^{-1} (*11*)]. Second, with the myofibrillar ATPase in methanol, the size of the P_i burst decreased with a decrease in temperature. This is as expected of a burst of M**.ADP.P_i (i.e., k_4 rate limiting) but not of free P_i plus M*.ADP (k_6 rate limiting; also see Results).

A more likely explanation is that the high $\Delta H^{\ddagger}$ in methanol has two causes. First, at 20%, methanol perturbs the ATPase site itself. Second—and this could be the more important effect—the methanol also perturbs the regulatory apparatus. Thus, it could be that, as the temperature is decreased, so more and more of the thin filament is turned off, leading eventually to a low activating factor and little or no shortening. The effect of temperature on myofibrillar shortening is discussed further below.

Ca^{2+}-Activated Myofibrils: ATPase and Shortening Rates in 20% Methanol. The ATPase progress curves of activated myofibrils from rabbit and frog skeletal muscle have characteristic and very similar profiles (*4*, *13*, *41*). As shown in Figure 1, this profile is retained in 20% methanol. Thus, in both water and methanol, there are three phases.

First, there is an initial P_i burst that represents the transient formation of the M**.ADP.P_i state (Scheme 1). Shortening could not be detected during this phase. Second, there is a linear phase, defined by k^F, during which rapid shortening occurs. We term the contraction during this phase as isotonic under zero external load.

Third, there is a deceleration to a slow linear phase, k^S. During this phase, the myofibrils shorten slowly, so here the contraction appears to be quasiisometric (also see ref *42*). Further, the myofibrillar structure, although remaining, appears to be perturbed (as seen under the microscope). As the shortening proceeds, in water the sarcomere structure is lost but in 20% methanol it is retained, even after the depletion of the ATP.

The above interpretation of the ATPase profile of actively shortening myofibrils is in accord with the finding that chemically cross-linked myofibrils, which maintain their sarcomere structure in the presence of Ca^{2+} and ATP, thus mimicking the isometric conditions, have only one fast steady-state ATPase rate, without a break (*25*).

Can the k^F to k^S transition be connected with some structural change in the myofibril? As a first approximation, we defined the transition by the point of intersection of the two linear phases which we called a break. This break was confirmed by a fluorescence stopped-flow method in which free P_i is measured specifically (*12*).

If we allow for the errors in our measurements, the average sarcomere lengths in the break zone (about 2.1 μm) are near the length of the thin filament. Thus, the reduction in both the ATPase and shortening rates that now occurs could be explained by an increase in the internal load.

In methanol, the myofibrils do not appear to over contract, i.e., to end up as meatballs as in water (*43*), instead the shortening stops when the sarcomere lengths are about 1.4 μm. This suggests that in this solvent shortening stops when the thick filament (1.6 μm) is up against the Z-lines. This could be because the activation factor is less than that in water, i.e., that the energy input provided by the ATP hydrolysis is lower and not sufficient to lead to the loss of the sarcomere structure of the myofibrils. It could also be that methanol stabilizes the myofibrillar structure making it

more resistant to the physical stresses generated during contraction under zero external load.

The situation at temperatures below −2 °C (Table 3, Figure 4) is interesting. Here, the contraction appears to be isometric as the myofibrillar ATPase was activated by Ca^{2+} and the myofibrils did not shorten. The activation factor was low (8 at −5°) so it could be that below −2 °C the energy for Ca^{2+}-activated ATP hydrolysis is insufficient to overcome the *internal* frictional forces in the myofibril and that this balance leads to an isometric condition as in fiber experiments where that state is obtained by *externally* imposed forces. Mechanical experiments in the subzero temperature range could provide a way of evaluating the internal forces.

Alternatively, the lack of shortening at low temperatures could be due to shifts in temperature-sensitive equilibria, in particular that of the cleavage step, K_3. Thus, K_3 and therefore the key intermediate M**ADP.P_i decrease with the temperature, and it could be that a minimum concentration of the intermediate is needed for shortening to occur. This would be in accord with the observation of Anson (*44*) that, in the in vitro motility assay, the proportion of actin filaments moving on immobilized myosin decreased with a decrease in temperature.

CONCLUDING REMARKS

In this initial study, we exploited cryoenzymology to aid toward an understanding of the kinetic aspects of energy transduction in muscle contraction. We used the myofibril as a model; despite the difficulties in interpreting the effects of solvent and temperature on such an organized system, we suggest that our results provide a base for further studies. We show that the choice of solvent and temperature is dictated by the aim of the experiment. Ethylene glycol (40%) is suitable not only for studying the regulatory process but also for investigating the kinetics of the products release steps which is difficult in water. We show that with relaxed myofibrils the kinetics of the ADP release are considerably faster than those of the P_i release. Thus, whereas the myofibrillar environment does not appear to affect the P_i release, it affects the ADP release, even in absence of Ca^{2+} and rigor activation. It could be, therefore, that upon Ca^{2+} activation, the only step that is accelerated is k_4. This implies that the concentration of M*.ADP is low, regardless of the Ca^{2+} status.

In 20% methanol, one can study isotonic and isometric contraction with increased temporal resolution, depending on the temperature. This approach could lead to information on the thermodynamics of the internal myofibrillar interactions developped during the ATPase cycle.

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