To understand mammalian skeletal myosin isoform diversity, pure myosin isoforms of the four major skeletal muscle myosin types (myosin heavy chains I, IIA, IIX, and IIB) were extracted from single rat muscle fibers. The extracted myosin (1–2 μg/15 mm length) was sufficient to define the actomyosin dissociation reaction in flash photolysis using caged-ATP (Weiss, S., Chizhov, I., and G.S. (2000) J. Muscle Res. Cell Motil. 21, 423–432). The ADP inhibition of the dissociation reaction was also studied to give the ADP affinity for actomyosin dissociation ($K_{AD}$). The apparent second order rate constant of actomyosin dissociation gets faster ($K_{diss} = 0.17 - 0.26 \text{ μm}^{-1} \text{s}^{-1}$), whereas the affinity for ADP is weakened (250–890 μm) in the isoform order I, IIA, IIX, IIB. Both sets of values correlate well with the measured maximum shortening velocity ($V_o$) of the parent fibers. If the value of $K_{AD}$ is controlled largely by the rate constant of ADP release ($k_{AD}$), then the estimated value of $k_{AD}$ is sufficiently low to limit $V_o$. In contrast, [ATP]$K_{diss}$ at a physiological concentration of 5 mM ATP would be 2.5–6 times faster than $k_{AD}$.

Muscle contraction results from a cyclical interaction of myosin cross-bridges with actin driven by ATP hydrolysis. Skeletal muscle fibers show characteristic mechanical properties including shortening velocity, power output (1–4), and ATPase activity (3, 5). It is now clear that all of these properties are to a large extent determined by the isoforms of the myosin heavy chains expressed in individual muscle cells (6, 7). Biochemical and structure-function studies have attempted to define the underlying molecular basis of these differing mechanochemical properties of myosins within the skeletal muscle myosins and in the wider family of myosins.

It is generally accepted that the sequence of events in the actomyosin cross-bridge cycle is essentially the same for all muscle myosins. The different properties can therefore be attributed to modulation of the rates and equilibrium constants (and hence free energy changes) of individual molecular events by changes in myosin sequence. Early studies suggest a correlation between the maximum shortening velocity of a muscle isoform (i.e., the velocity in the absence of any mechanical load) and the overall ATPase rate (8) for a range of muscle types, since both reflect the underlying speed of the cross-bridge cycle.

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Differing ADP Release Rates from Myosin Heavy Chain Isoforms Define the Shortening Velocity of Skeletal Muscle Fibers*

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We therefore considered that a study of the biochemical kinetic properties of the mammalian myosin isoforms may prove of interest in testing the hypothesis of Siemankowski et al. (10) on a series of closely related myosins whose contractile and energetic properties are well characterized. Although there have been many studies of the mechanical properties of mammalian muscle fibers (both intact and skinned), biochemical characterization of mammalian-striated MHC isoforms has been limited because of the difficulty of isolating pure heavy chain isoforms. There is no effective overexpression system for mammalian-striated muscle myosin isoforms. Isolation of myosin from bulk muscle (as used by Siemankowski and White (17)) results in a preparation containing mixed isoforms, since most mammalian muscles contain multiple myosin isoforms. In contrast, single isolated muscle fibers often contain a single MHC isoform (18). We recently demonstrated that the amount of myosin that can be isolated from a 2-cm-long single muscle fiber of rat is sufficient to characterize the ATP-induced dissociation reaction and the affinity of ADP for actomyosin using a flash photolysis apparatus developed for this purpose (19). We report here the first kinetic characterization of all four MHC skeletal isoforms (type I, IIA, IIB, and IIX) expressed in adult rat muscle and show that both the rate of ATP induced dissociation of actomyosin and the rate of release of ADP from actomyosin becomes faster as the maximum shortening velocity of muscle fibers expressing the MHC isoform increases.

MATERIALS AND METHODS

Preparation of Proteins—The myosin used in these measurements was extracted from 15-mm-long single muscle fibers dissected from bundles of fresh soleus and psoas rat muscle. The fibers were manually dissected, chemically skinned (according to Bottinelli et al. (4)), and cut in two segments. The smaller segment (~2 mm) was dissolved in 20 μl of standard buffer solution (20) and used for MHC isoform identification by SDS-polyacrylamide gel electrophoresis on 8% polyacrylamide gels prepared according to Talmadge and Roy (21). Electrophoresis was run for 24 h at 250 V. Gels were silver-stained, and in the region of the myosin heavy chain (molecular mass, ~220 kDa) four bands were separated corresponding to the four MHC isoforms. Fig. IA shows a gel with one example each of a fiber expressing a single MHC together with a sample containing a mixture of all four isoforms. The major part of the fiber (~13 mm) was incubated in 30 μl of myosin extraction buffer (100 mM KH2PO4, 50 mM K2HPO4, 0.3 M KCl) for 3 h on ice, after which the fiber was removed and discarded. The myosin solution was dialyzed twice in a microdialysis system developed for this purpose (20). Myosin extraction buffer (100 mM KH2PO4, 50 mM K2HPO4, 0.3 M KCl) was used without further purification.

The binding of ATP Induced Dissociation of Actomyosin—The binding of ATP to actomyosin causes fast and irreversible dissociation of the complex, and after all the ATP is hydrolyzed, the complex reforms. The rate of hydrolysis of ATP by actomyosin under these conditions is relatively slow (~10 min for 45 μM ATP under the conditions used), and to allow a faster elimination of ATP and ADP, apyrase was added to the samples. Weiss et al.
Fig. 1. Gel electrophoresis. A, electrophoretic separation of rat MHC isoforms. The area of migration of the four MHC isoforms is indicated on the left. Single muscle fibers were loaded in lanes a, b, and d–f; a sample of a mixture of slow and fast rat muscles to show all four MHC isoforms was loaded in lane c. Lane a, single muscle fiber containing MHC-IIA; lane b, single fiber containing MHC-IX; lanes d and e, single fibers containing MHC-IIB; lane f, single fiber containing MHC-I. The gel was a 10% polyacrylamide gel; silver staining was used. B, example of gels used for electrophoretic determination of the amount of myosin extracted from single fibers and of the remaining amount of myosin left in the fiber after myosin extraction. Lanes in which known amounts of myosin to be used as standards were loaded are indicated as s; from left to right, the amounts loaded were 1, 2, 2.5, 3, and 3.5 μg/lane f, myosin extracted from fiber 1; 1r, remaining amount of myosin left in the same fiber after myosin extraction and extracted by standard buffer (20). The same applies to lanes 2e and 2r for fiber 2 and to lanes 3e and 3r for fiber 3. The gel was a 10–20% gradient polyacrylamide gel; Coomassie staining was used.

(19) show that the presence of apyrase has little effect on the observed rate of the dissociation reaction. This procedure allows the same sample to be reused a number of times with different flash intensities to vary the [ATP] released.

15 μl of the extracted myosin solution in experimental buffer were mixed with 5 μl of reaction mix to give a final volume of 20 μl containing ~0.15 μm myosin, 0.5 μm phallolidin-stabilized actin and 0.5 mM cATP, 10 mM DTT, 10 μg/ml apyrase. As shown in Fig. 2A for a sample of MHC-IIB, irradiation by a series of laser pulses of different intensities released a range of ATP concentrations. The concentration of ATP liberated in each flash was estimated from the decay of the absorbance of 405 nm, which monitors the formation and decay of the acinotro photolysis intermediate (19, 25). The same sample was used for all the transients shown in Fig. 2A, and after each transient was recorded, the sample was left for 3 min for all the ATP to be hydrolyzed before the next flash. The decrease in light scattering was described by a single exponential, and the best fit to a single exponential is superimposed in each case. The observed rate constant (k_{on}) of each reaction was linearly dependent upon [ATP]. Fig. 2B shows the dependence of k_{on} on [ATP] for each of the four MHC isoforms isolated. In each case a linear relationship was observed, and the slope of the fitted line defined the apparent second order rate constant of the ATP-induced dissociation reaction (K_k_{on} Scheme 1). The plots show different slopes for each isoform except for IIB and IX, which were very similar. The pooled data from a series of measurements using at least six individual fibers for each myosin isoform are given in Table I. These show that the differences between isoforms IIX and IIB are not statistically significant, whereas all other comparisons are significant.

Determination of the ADP Affinity—The competition between ADP and ATP for the myosin nucleotide site provides a method to determine the affinity of ADP for the actomyosin complex (17). At higher concentrations, ADP will reduce the affinity of myosin for actin, and at the low protein concentra-
ADP concentrations of 0, 230, 460, 700, 1235, 1770, and 1800 μM. The best fit to a single exponential decay of the amplitude of the light-scattering changes decreased with increasing ADP concentration. The best fit to a single exponential decay of the amplitude as ADP concentration increased. The decrease in $k_{\text{obs}}$ is predicted from Equation 1 and Scheme 1. The decrease in amplitude was because of the dilution effect and the lower affinity of actin for the myosin-ADP complex. The ADP concentration in each case was calculated as the sum of the ADP added and the ADP that built up through the hydrolysis of the ATP released in each flash. This procedure has been validated using the well characterized rabbit muscle myosin subfragment 1 (S1). The values of $k_{\text{obs}}$ were corrected according to Equation 1 for the small variations in the amount of ATP released in each flash and then plotted against the ADP concentration as shown in Fig. 3B for each myosin isoform. The data was fitted to Equation 1, and the best curve was superimposed. Each MHC isoform gave a distinct value of $K_{\text{ADP}}$ and pooled data from three different experiments (Table I) show the differences to be significant.

Thermodynamic coupling between actin and ADP binding to myosin (17, 27) predicts that the weaker the affinity of actomyosin for ADP, the weaker the affinity of myosin-ADP for actin. Light-scattering measurements are not very reliable for amplitude data because of factors like micro air bubbles and dust particles. However, there was a clear trend for the MHC isoforms IIX and IIB to show a more pronounced loss of the amplitude of the light-scattering transients as ADP concentration was increased compared with isoforms I and IIA. This is consistent with weaker affinity of actin for isoforms IIX and IIB in the presence of ADP.

**DISCUSSION**

To date no satisfactory overexpression system exists for mammalian skeletal muscle myosin, and bulk preparations of myosin from tissue results in isolation of mixed isoforms. The majority of single muscle fibers do, however, contain single isoforms, and we have shown that we can extract sufficient myosin from single fibers for our measurements. Using our flash photolysis method, we were able to determine the rate of ATP-induced dissociation of and the ADP affinity for the actomyosin complex for all of the four skeletal muscle isoforms of adult rat. Measurements of this kind were previously not possible with these μg quantities of myosin.

We obtained −2 μg of pure myosin isoforms from 15-mm single fibers of the rat psoas (MHC-IIB, MHC-IIX) and soleus (MHC-I and MHC-IIA). This amount is sufficient to determine the second order rate constant of ATP-induced dissociation with the flash photolysis apparatus, whereas it would be too small an amount to obtain results from traditional stopped flow methods (24). High ADP concentrations lower the affinity of myosin for actin and decrease the dissociation signal. The amount of protein from one fiber is therefore not sufficient to measure the ADP affinity to actomyosin over a wide range of ADP concentrations. For this experiment, a higher myosin concentration is needed. Five fibers for which the same isoform type has been confirmed by electrophoresis were pooled, and the myosin was extracted. The total amount of protein is still far less than would be needed using traditional methods.

Our results for both the rate of ATP-induced dissociation of...
The values of $V_0$ at 22 °C were calculated from the 12 °C data in Table I multiplied by 2 (in segment length, L, per second). The rates of dissociation of actomyosin as physiological salt conditions were calculated from the values of $K_{AD}$ in Table I multiplied by a correction factor of 3–5 for the decrease in ionic strength (0.5–0.2 M) and a concentration of 5 mM ATP as used in $V_0$ measurements. Numbers are quoted as the mean with a range of possible values. The rate of ADP release under physiological salt condition was calculated by dividing the experimental values of $K_{AD}$ in Table I by a correction factor of 3–7 for the decrease in ionic strength (0.5–0.2 M). This value was used in the equation $k_{AD} = K_{AD} \cdot k_{AD}$ with $k_{AD} = 10^7 \text{m}^{-1} \text{s}^{-1}$. Values are quoted as the mean with a range of possible values. $k_{min}$ was calculated from values of $V_0$ at 22 °C according to Equation 2 with $S_L = 1.36 \mu m$ and $d = 5–10 \mu m$.

In our experimental setup the binding of ADP to actomyosin can be described as a fast equilibrium with rate constants $k_{AD}$ and $K_{AD}$ in determining shortening velocity, such a correlation does not establish whether either one of the steps actually determines shortening velocity alone or the relative contribution of each of them. To evaluate more carefully the contribution of these events to shortening velocity, we must estimate $K_{AD}$ and $k_{AD}$ values under the more physiological conditions used to measure shortening velocities and quantitatively compare them. This will also allow the comparison of $k_{AD}$, which, we will suggest, can limit shortening velocity, with the value of $k_{min}$ (rate of the event limiting shortening velocity) estimated from the maximum shortening velocity of single muscle fibers (10).

Our values were measured in solution using whole myosin and were therefore made at a KCl concentration of 0.5 M to keep the myosin soluble. Both $K_{AD}$ and $K_{AD}$ values need to be corrected for the ionic strength in the fiber, which was 0.2 M in the $V_0$ measurements (4). In principle it would be possible to repeat the measurements with HMM or S1 and to work at physiological ionic strength. However, because of the very small amounts of myosin we have available, digestion of the protein would lead to unacceptable losses of protein. The corrections necessary can be estimated from a Debye-Hückel plot for $K_{AD}$ and $K_{AD}$ obtained using bulk preparations of skeletal myosin S1. Such measurements have been made for S1 isolated from rat soleus and edl muscle. The Debye-Hückel plot (not shown) for $K_{AD}$ shows a 3–7-fold decrease from 0.5 to 0.1 M KCl that corresponds to an ionic strength of 0.2 M. For the same change of ionic strength $K_{AD}$ is increased about 3–5-fold (29). The effect of these corrections is shown in Table II.

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**Table II**

| Parameters contributing to shortening velocity |
|-----------------------------------------------|
| $V_0$ | $K_{AD} \cdot [ATP]$ | $k_{AD}$ | $k_{min}$ |
|-------|----------------------|---------|---------|
| L s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | s$^{-1}$ |
| MHC-I | 2.1 | 3400 ± 860 | 600 ± 240 | 420 ± 140 |
| MHC-IIA | 4.7 | 4200 ± 1050 | 1150 ± 460 | 940 ± 310 |
| MHC-IIIX | 6.1 | 5200 ± 1300 | 1790 ± 715 | 1240 ± 420 |
| MHC-III | 7.4 | 5200 ± 1300 | 2220 ± 885 | 1490 ± 500 |

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2. N. Adamek and M. A. Geeves unpublished information.
The rate of ADP binding to \( k_{\text{AD}} \) actomyosin and dissociation from \( k_{\text{AD}} \) actomyosin are too fast to be measured using the method described here. At the limit we know the sum \( (\text{ADP})k_{\text{AD}} + k_{\text{AD}} \) is much greater than [ATP] \( k_{\text{AD}} \), which has measured values of up to 10 s\(^{-1}\) (see Fig. 2). Siemankowski and White (17) calculated the binding rate of ADP to actomyosin, \( k_{\text{AD}} \), from measured values of \( K_{\text{AD}} \) and \( k_{\text{AD}} \), to be 10\(^7\) m\(^{-1}\) s\(^{-1}\). This is close to the value expected for a diffusion-limited process. If we assume that ADP binding is a diffusion-limited process and is therefore similar for all myosins, then we can estimate the dissociation rate constant from \( k_{\text{AD}} = K_{\text{AD}}k_{\text{AD}} \). Thus, taking the corrected values of \( K_{\text{AD}} \), we can calculate the values of \( k_{\text{AD}} \) at physiological ionic strength to be about 600 s\(^{-1}\), 1150, 1790, and 2220 s\(^{-1}\) (with the range of possible values shown in Table II) for the isoforms MHC-I, MHC-IIA, MHC-IIIX, and MHC-IB, respectively.

Siemankowski et al. (10) propose that the minimum value of the rate constant \( (k_{\text{min}}) \) of the event limiting maximum shortening velocity \( (V_p) \) can be estimated from the half-sarcomere length \( (S_L) \) and the maximum allowed axial cross-bridge translation \( (d) \), Equation 2). The values of \( V_p \) from Table I were measured at 12°C. The \( Q_{10} \) for \( V_p \) in rat muscles is \( \sim 2 \) (30), which predicts that values will be doubled at 20°C, which is the temperature used in the kinetic measurements presented here (corrected \( V_p \) values given in Table II). The value of the half-sarcomere length \( (1.35 \mu m) \) is that of the fibers used in the \( V_p \) measurements by Bottinelli et al. (4), and the step size is assumed to be 5–10 nm. With these values, \( k_{\text{AD}} \) can be calculated, and the result is shown in Table II. Even within the limits of the measurements and corrections made here, the agreement between the values of \( k_{\text{min}} \) and \( k_{\text{AD}} \) is remarkably close for all four myosin isoforms, less than a factor of 2 in each case. The results are therefore compatible with the rate of ADP release limiting the shortening velocity for the four muscle fibers as predicted by Siemankowski et al. (10).

Under physiological conditions the concentration of ATP is of the order of 5 mM; thus, the rates of ATP binding and actin dissociation, \( [\text{ATP}]k_{\text{AD}} \), are approximated as shown in Table II. Taking the mid-range values of \( [\text{ATP}]k_{\text{AD}} \), these are \( \sim 4 \) fold faster than \( k_{\text{min}} \) for all MHC-II isoforms and 8-fold faster for MHC-I. Thus, it is clear that \( [\text{ATP}]k_{\text{AD}} \) is unlikely to contribute to \( k_{\text{min}} \) for the MHC-I isoform, but the situation is not clear cut for the faster isoforms. At the lower limits of the estimated range, the ATP binding step could be a significant contributor to \( k_{\text{AD}} \) and is of the same order as \( k_{\text{AD}} \). It is of interest to note that differences in \( k_{\text{AD}} \) (ATP) and \( k_{\text{AD}} \) are smallest for the fastest MHC-IIB (2.5-fold) and largest for the slow MHC-I (5.5-fold). Thus, any significant drop in the concentration of ATP (or an increase in the competing free (ADP)) could result in a slowing of A-M dissociation and lowering of \( V_p \).

Interestingly the maximum shortening velocity \( (V_p) \) of fast fibers has been shown to be more affected by a decrease in [ATP] than the \( V_p \) of slow fibers (31). It is possible that this is a contributor to fatigue-induced loss of muscle performance, which is more marked in fast muscles.

This result demonstrates that for four closely related muscle myosins, the shortening velocity is controlled by the rate at which ADP can escape from the cross-bridge after completion of the power stroke. A contribution from ATP dissociation of the cross-bridge remains possible for the fastest isoforms. It is of interest to note the ATP-induced dissociation varies by less than 2-fold between the four myosin types compared with a 3.5-fold variation in both \( k_{\text{min}} \) and \( k_{\text{AD}} \). The faster ATP-induced dissociation rate for the faster myosins has previously been reported for different muscle types of chicken muscle (9). It appears that this step must remain faster than the preceding ADP release step, but its precise value does not correlate as closely with the shortening velocity. It may not be surprising that both ATP binding and \( K_{\text{AD}} \) show a similar (but not identical) dependence on myosin isoform since both nucleotides are binding to the same pocket. Thus, changes in sequence which result in changes of the biochemical environment of the nucleotide binding pocket that lead to a faster ADP release may also produce faster binding of ATP.

Several comparisons between myosin isoforms have highlighted Loop1 (on the myosin surface at the entrance to the nucleotide pocket) as responsible for modulating ATP affinity to actomyosin and velocity of either the muscle fiber shortening or in \textit{in vitro} motility assays (11). These include scalar-striated and catch muscle myosin (32) and the phasic and tonic isoforms of smooth muscle myosin (12, 13) in addition to artificial myosin constructs (35). It is therefore of interest to consider the changes in sequence that produce these changes in the properties of the rat striated muscle myosin isoforms. Unfortunately the sequences of the MHC-II rat isoforms are not currently in the data base; only the MHC-I is known. However, given that the MHC-I sequences are very heavily conserved between the rat and human, a similar conservation may be expected in the MHC-II sequences. The human sequences are all known and have been compared by Weiss et al. (34). These show \( \sim 90\% \) identity between the three MHC-II sequences and \( 80\% \) identity comparing the MHC-I and II. Surprisingly, although there are changes in sequences in Loop 1 between MHC-I and -II, the sequence of Loop I is well conserved among the three MHC-II myosins, and it may be necessary to look for more subtle changes in structure to account for the 2-fold changes in \( V_p \) and \( K_{\text{AD}} \) reported here.

In conclusion we have confirmed the hypothesis proposed by Siemankowski et al. (10) for this closely related set of rat MHC isoforms in that ADP release can provide the limiting molecular event that limits shortening velocity. We have estimated that under normal conditions the rate of the ATP-induced dissociation of A-M remains up to 2–6-fold faster than the preceding step of ADP release from A-M-D. Further information on the sequence differences between the rat MHC isoforms will be required to understand how the ADP release rate is modulated by the myosin structure. Alternatively a similar study on the equivalent human isoforms for which sequence information is known will be of interest, and we are attempting such a study.

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