Transient Kinetic Analysis of the 130-kDa Myosin I (MYR-1 Gene Product) from Rat Liver

A MYOSIN I DESIGNED FOR MAINTENANCE OF TENSION?

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The 130-kDa myosin I (MI130), product of the myr-1 gene, is one member of the mammalian class I myosins, a group of small, calmodulin-binding mechanochemical molecules of the myosin superfamily that translocates actin filaments. Roles for MI130 are unknown. Our hypothesis is that, as with all myosins, MI130 is designed for a particular function and hence possesses specific biochemical attributes. To test this hypothesis we have characterized the enzymatic properties of MI130 using steady-state and stopped-flow kinetic analyses. Our results indicate that: (i) the Mg\(^{2+}\)-ATPase activity is activated in proportion to actin concentration in the absence of Ca\(^{2+}\); (ii) the ATP-induced dissociation of actin-MI130 is much slower for MI130 than has been observed for other myosins (–Ca\(^{2+}\), second order rate constant of ATP binding, 1.7 × 10\(^4\) M\(^{-1}\) s\(^{-1}\); maximal rate constant, 32 s\(^{-1}\)); (iii) ADP binds to actin-MI130 with an affinity of ~10 \(\mu\)M and competes with ATP-induced dissociation of actin-MI130; the rate constant of ATP release from actin-MI130 is 2 s\(^{-1}\); (iv) the rates of the ATP-induced dissociation of actin-MI and ADP release are 2–3 times greater in the presence of CaCl\(_2\), indicating a sensitivity of motor activity to Ca\(^{2+}\); and (v) the affinity of MI130 for actin (15 nM) is typical of that observed for other myosins. Together, these results indicate that although MI130 shares some characteristics with other myosins, it is well adapted for maintenance of cortical tension.

The myosin superfamily has no fewer than 15 members that share sequence homology in the amino-terminal, so-called “motor,” domain, which contains the ATP- and actin-binding sites (1–3). Although qualitatively myosins appear to share the same basic mechanism of coupling ATP hydrolysis to the movement of actin filaments, studies indicate that the ways in which they interact with actin and nucleotide are quantitatively different (4–6). These differences allow for myosins to function in a variety of situations including muscle contraction, vesicle transport, cytokinesis, and mediation of cell surface changes that accompany cell locomotion.

The class I myosins are small, single-headed, nonfilamentous ATPases (see Ref. 7). In lower eukaryotes, class I myosins appear in addition to being very slow kinetically, this myosin I may be designed to bind different loads and perhaps to perform different functions. Similarly, although homology in the motor domain among the 130-kDa polypeptide and two other mammalian isoforms, namely MM1β (20) or the 110-kDa myosin I (13) and MYR-4 (21) or the 105-kDa myosin I (14), is 70%, the amino acid differences in the motor domain might reflect distinct properties and hence disparate cellular roles for these myosins. As a result, detailed mechanistic analyses may assist in defining the cellular functions of these isoforms.

This report on MI130 is the first detailed kinetic analysis of a mammalian myosin I and represents the first step in a long-range plan designed ultimately to compare and contrast the kinetic properties of mammalian class I myosins. The study is made possible by adapting established transient kinetic methods to use the small quantities of myosin I that are available from rat liver. Our analysis shows that MI130 interacts with nucleotide and actin in much the same way as other myosins that have been studied. However, it is much slower than any other myosin II, the two Acanthamoeba class I myosins, and avian brush border myosin I (BBMI). Our results suggest that in addition to being very slow kinetically, this myosin I may be designed for efficient tension maintenance.

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The abbreviations used are: MI130, 130-kDa myosin I; BBMI, brush border myosin I; S1, subfragment 1; mant, 2′(3′)-O-(N-methylanthraniloyl)MOPS, 4-morpholinoepanesulfonic acid; A, actin; M, myosin; D, ADP.

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Myosin I Kinetics

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins and Reagents—**MI$_{130}$ was isolated from rat liver by gel filtration, anion, and cation exchange chromatography as described previously (13). Fractions containing purified MI$_{130}$ were pooled and, in some cases, concentrated in a Centricon 10 microconcentrator (Millipore Corp., Bedford, MA). After protein determination by colorimetry (Bio-Rad protein assay) using serum albumin as a standard, sucrose was added to 3 mg/ml, and the samples were frozen in liquid N$_2$ prior to storage at −80 °C. Rabbit skeletal myosin subfragment 1 (S1) was prepared by chymotryptic digestion as described by Weeds and Taylor (22). Actin was prepared according to Spudich and Watt (23) and, in some cases, labeled with pyrene at Cys-374 according to Criddele et al. (24). The Z(3')-O-(N-methylanilino) derivatives of ATP (mant-ADP) and ATP (mant-ATP) were prepared according to Hiratsuka (25).

**Enzyme Kinetics—**All kinetic experiments were performed at 19 °C in 20 mM MOPS, 100 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA, and 1 mM dithiothreitol at pH 7.0 with a Hi-Tech Scientific KV 389 nm cut-off. The stated concentrations refer to reactions involving actin-MI$_{130}$. ATP binds rapidly to actin-MI$_{130}$, anion, and cation exchange chromatography as characters refer to reactions involving MI$_{130}$, and bold terms of the model described by Bagshaw (232,000 for the myosin I heavy chain and its six associated calmodulins.

**ATP Hydrolysis—**The release of P$_i$ was measured for 2–5 μg of myosin I using a colorimetric assay described by Pollard (26). The Ca$_{2+}$-ATPase activity of myosin I was measured in 10 mM Tris, pH 7.0, 1 mM dithiothreitol, 5 mM CaCl$_2$, and KCl concentrations ranging from 30 to 500 mM. The K$^+$-EDTA-ATPase activity was measured in 10 mM Tris, pH 7.0, 1 mM dithiothreitol, 2 mM EDTA, and KCl ranging from 30 to 500 mM. The actin-activated Mg$_{2+}$-ATPase activity was determined in 10 mM Tris, pH 7.0, 30 mM KCl, 1 mM dithiothreitol, 1 mM MgCl$_2$, either 1 mM EGTA or 1 mM EDTA and 1.1 mM CaCl$_2$, and F-actin from 0–30 μM. All reactions were done at 37 °C. Standard curves were generated with known amounts of phosphate. Controls included samples containing no myosin I. For actin-activated ATPase measurements, the reported values were corrected for activity because of the presence of actin. Data are expressed as s$^{-1}$ assuming a molecular weight of 232,000 for the myosin I heavy chain and its six associated calmodulins.

**Data Interpretation—**As shown in Scheme 1, we interpret the kinetics of myosin I (M) interacting with nucleotide (T, ATP, D, ADP) in terms of the model described by Bagshaw et al. (27), where $k_{f,1}$ and $k_{r,1}$ are the forward and reverse rate constants, respectively, and $K_i$ ($k_{f,1}/k_{r,1}$) represents the equilibrium constant of the i th reaction. Normal characters are used to indicate reactions involving MI$_{130}$, and bold characters refer to reactions involving actin-MI$_{130}$. ATP binds rapidly to myosin in a two-step reaction before ATP is reversibly hydrolyzed on the protein. This results in a conformational change that limits phosphate release and the faster ADP release. The ATP-induced dissociation of actin-MI$_{130}$ and the inhibition of the reaction by ADP have been interpreted in terms of the models developed by Millar and Geves (28) and Siemankowski and White (29). As shown in Scheme 1B, ATP binds rapidly and reversibly to actin-MI$_{130}$ and is followed by a rate-limiting isomerization ($k_{i,4}$) of the complex, which leads to rapid dissociation of actin. ADP competes with ATP for the nucleotide binding site. The dissociation constants of actin for MI$_{130}$, actin for MI$_{130}$·ADP, and ADP for actin-MI$_{130}$ are $K_A$, $K_{AD}$, and $K_{AD}$, respectively.

**RESULTS**

**ATPase Measurements—**The Mg$_{2+}$-ATPase activity of MI$_{130}$ was very low and difficult to quantify with precision, but estimates suggest a $k_{cat}$ of < 0.01 s$^{-1}$ in both the presence and absence of Ca$^{2+}$. The Mg$_{2+}$-ATPase activity was significantly activated by actin only in the absence of Ca$^{2+}$. In 30 mM KCl the ATPase rate was approximately linearly related to the actin concentration over the range of 0–30 μM with a steady-state ATPase rate of 0.247 ± 0.003 s$^{-1}$ at 30 μM actin.

The ATPase activity was also determined in K$^+$-EDTA and Ca$^{2+}$, two nonphysiological conditions used historically to characterize conventional myosin II. At 30 mM KCl the $k_{cat}$ in EDTA of MI$_{130}$ was 1.43 ± 0.021 s$^{-1}$ and was inhibited at higher KCl concentrations. At 500 mM the activity was decreased to 25%. In contrast, the Ca$^{2+}$-ATPase activity was 0.46 ± 0.02 s$^{-1}$ and almost independent of KCl concentration up to 500 mM.

**Transient Kinetics—**The ATP-induced dissociation of MI$_{130}$-actin complexes can be conveniently followed by monitoring the fluorescence of a pyrene label covalently attached to Cys-373 of actin. Fig. 1A shows the resulting change in the fluorescence signal upon mixing 200 μM ATP with 25 mM MI$_{130}$ and 25 mM phalloidin-stabilized pyrene-actin. At this concentration of ATP the change in the fluorescence can be described by a single exponential with $k_{obs}$ = 1.6 s$^{-1}$ and an amplitude of +4%. At concentrations above 500 μM, the reaction is biphasic with both phases having similar amplitudes (Fig. 1B). The low concentrations of MI$_{130}$ used allowed the reaction to be followed over a wide range of ATP concentrations from 25 μM to 10 mM using only a few micrograms of protein. The dependence of the $k_{obs}$ for both phases of the reaction on ATP concentration is shown in Fig. 1C in both the presence and absence of calcium. The fast phase of both data sets shows a hyperbolic dependence on [ATP] with a maximal observed rate ($k_{2,1}$) of 74 and 32 s$^{-1}$ and 3.2 and 1.9 μM ATP required for half maximal saturation (1/2$K_1$) with and without Ca$^{2+}$, respectively. The values of 1/2$K_1$ are of the order measured for all other myosins studied, but $k_{2,1}$ is much slower than any other myosin characterized so far (Table I). The slow phase saturates at a $k_{obs}$ of 6 and 2 s$^{-1}$ with and without Ca$^{2+}$, respectively. The $k_{obs}$ corresponds to the rate constant of ADP dissociation from actin-MI$_{130}$ in each case (see below) and led us to believe that the protein was purified with ADP bound in the nucleotide pocket. The amplitude of the slow phase varied among preparations and was normally in the range of 10–60% of the total amplitude. Extensive treatment with apyrase only partially reduced the amplitude of the slow phase, raising the possibility that the slow phase may have two components (see “Discussion”).

The influence of ADP on the ATP-induced dissociation of actin-MI$_{130}$ is shown in Fig. 2. Addition of 2.5 μM ATP to apyrase-treated 25 mM pyrene-labeled actin-MI$_{130}$ results in a rapid change in fluorescence. The data for this preparation of protein fit a single exponential with $k_{obs} = 22.5$ s$^{-1}$. If, in place of apyrase, 25 μM ADP was added, the $k_{obs}$ was reduced to 5.8 s$^{-1}$. Increasing either ADP or ATP concentration did not change the $k_{obs}$ (data not shown), suggesting that it is the ADP release rate ($k_{ADP}$) that is being monitored. At lower ADP concentrations, a biphasic reaction was observed with $k_{obs}$ of 22 and 6 s$^{-1}$. Preliminary analysis of the amplitude dependence on ADP concentration suggests an affinity of <10 μM. Establishing the conditions under which the ADP concentration was less than 10 μM was difficult because of the presence of an unknown level of ADP contamination. Repeating the experi-
The amplitude of the ATP-induced dissociation reaction can be used to estimate the affinity of pyrene-actin for a myosin as shown by Kurzawa and Geeves (31). However, such a titration experiment requires a considerable amount of MI$^{130}$, which is limiting because it is present in cells in only small amounts. We therefore used a competition experiment with the well characterized rabbit skeletal muscle myosin S1 to provide an estimate of the affinity of MI$^{130}$ for actin. The addition of 50 µM ATP to 25 nM pyrene-actin and 50 nM MI$^{130}$ in the stopped-flow spectrophotometer led to a dissociation reaction with a $k_{obs}$ of 0.74 s$^{-1}$ (Fig. 3). The addition of 50 µM ATP to 25 nM pyrene-actin and 50 nM S1 gave a $k_{obs}$ of 0.8 s$^{-1}$. Both reactions had a similar amplitude of 3.8%, suggesting a comparable affinity if both myosins quench the pyrene fluorescence to a similar extent. When 25 nM actin was mixed with 50 nM MI$^{130}$ and 200 nM S1 before mixing with ATP, a biphasic reaction was observed with 68% of the reaction occurring at 80 s$^{-1}$ (the $k_{obs}$ for A-S1 dissociation) and 32% at 0.79 s$^{-1}$ (the $k_{obs}$ for A-MI$^{130}$ dissociation). The amplitudes of the dissociation reaction were dependent upon the S1 concentration as shown in Fig. 3B. It is important to note that in Fig. 3B it is the concentration of the proteins in the syringe that is plotted, because the system is at equilibrium before the ATP is added. This usage is the opposite of the normal convention used for all of the other figures. When the fast and slow reactions have the same amplitude (i.e. the two myosins bind the same amount of actin in the mixture), then $K_2/5K_{M1}^{130} = [S1]/[MI130] = ([S1]_0 - 0.5[A]_0[MMI130]_0 - 0.5[A]_0)$. This equation was valid at 100 nM S1 in the absence of ADP and at approximately 300 nM S1 in the presence of 30 µM ADP. Under the experimental conditions, the affinity of S1 for actin is 30 nM in the absence of ADP (31) and 600 nM in the presence of ADP, leading to an affinity of pyrene-actin for MI$^{130}$ of 13 nM (−ADP) and 60–110 nM (+ADP). The presence of Ca$^{2+}$ had little influence on the measured affinities (data not shown).

Mixing 2.5 µM mant-ATP with 250 nM MI$^{130}$ led to a 3.3% increase in fluorescence when the reaction was monitored by exciting the intrinsic protein fluorescence at 295 nm and measuring the energy transfer to the mant group (Fig. 4A). The observed reaction occurred at 0.28 s$^{-1}$, but it could only be followed over the concentration range of 1–2.5 µM mant-ATP. No reaction could be seen by monitoring the mant fluorescence directly. We could detect no reaction when monitoring mant-ADP binding over a similar concentration range. Addition of 5 µM ATP to 100 nM MI$^{130}$ resulted in a 1.2% increase in intrinsic protein fluorescence at 0.56 s$^{-1}$ (Fig. 4B). The reaction could be observed over the ATP concentration range of 1–15 µM (Fig. 4B, inset), and the data were consistent with a second order rate constant, $K_{2}/[A_0]/([S1]_0/(10^6 M)$, of 0.1 × 10$^6$ M$^{-1}$ s$^{-1}$. Thus, both ATP and mant-ATP bind with a similar rate constant and five times faster than the apparent rate of binding to actin-MI$^{130}$. No calcium dependence of the reaction was detected (data not shown).

### DISCUSSION

The ATPase activity and transient kinetics data presented here show that MI$^{130}$ interacts with both ATP and actin in a manner similar to that of other well characterized myosins, with the exception that many of the events are much slower. Although restricted in the quantity of available purified myosin I, the current studies were possible because of (i) the high sensitivity of pyrene-labeled actin to interactions between actin and myosin I complexes and (ii) the use of phalloidin to stabilize F-actin, thereby allowing many transient measurements using only microgram quantities of protein (31).

A major observation resulting from these studies is that the ATP-induced dissociation of actin-MI$^{130}$ ($K_{2}/[A_0] = 0.023 × 10^6 M^{-1} s^{-1}$) is slower than has been determined for other myosins.
As compared with BBM, the closest known relative to MI<sub>130</sub> (15), the rate of ATP-induced dissociation of the actin-MI<sub>130</sub> complex is 10% in the absence of Ca<sup>2+</sup> (32). This is largely attributable to a smaller value of <i>k</i><sub>a2</sub> as the I/<i>K</i><sub>a</sub> is comparable with that observed for BBM. Across the range of myosins listed in Table I, <i>k</i><sub>a2</sub> for BBM is similar to amoeboid myosin Ia (33) and Dictystelium myosin II (5) and much slower than the muscle myosin II <i>(5)</i> and much slower than the smooth muscle myosin II <i>(30)</i> and <i>D</i>cytostelium myosin I <i>(5)</i> and much slower than the muscle myosin II <i>(30)</i> and cardiac muscle myosins (29). Thus, MI<sub>130</sub> is by far the slowest myosin so far characterized. Similarly, the rate of ATP binding to MI<sub>130</sub> in the absence of actin is 10% that of BBM, which in this regard more closely resembles all of the other myosins listed in Table I. In contrast, both the dissociation rate constant for ADP for MI<sub>130</sub> in the absence of ADP (<i>k</i><sub>a2</sub>) and the affinity of ADP for MI<sub>130</sub> (<i>k</i><sub>a</sub>) are comparable for MI<sub>130</sub> and BBM, suggesting that these differences are an intrinsic property of the protein and not caused by the method of preparation.

What is responsible for the slow phase observed in the ATP dissociation of actin-MI<sub>130</sub> is unknown. Although the rate is similar to that of ADP dissociation, it could not be eliminated with extensive treatment with apyrase. Indeed, actin-MI<sub>130</sub> treated with 40 μM ADP to eliminate the fast phase, then treated with apyrase, resulted in restoration of the original fast phase, showing that the ADP had been removed; however, the slow phase remained. This slow component may represent a contaminating myosin in the preparation, damaged MI<sub>130</sub>, MI<sub>130</sub> without a full complement of calmodulin, or an alternatively spliced version of MI<sub>130</sub>. The examination of the kinetic properties of MI<sub>130</sub> expressed in baculovirus will soon allow us to rule out some of these possibilities.

The affinity of MI<sub>130</sub> for actin is similar to that observed for all myosins examined so far. Interestingly, the affinity of MI<sub>130</sub> for actin is reduced 5–7-fold in the presence of ADP. This behavior is similar to that displayed by smooth muscle myosin S1 (6) and differs markedly from skeletal muscle (30) and cardiac muscle myosins (29). Thus, coupling between the ADP and actin binding sites resembles that found with smooth muscle myosin II and Dictystelium cytoplasmic myosin II (<10-fold (5)) and differs from that of skeletal muscle and cardiac muscle myosin II (>20-fold). It has been proposed that this low

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**Fig. 2. Influence of ADP on the ATP-induced dissociation of actin-MI<sub>130</sub>.** Addition of 2.5 mM ATP to 25 mM pyrene-labeled actin-MI<sub>130</sub> in the absence of ADP (i.e., apyrase-treated) resulted in a rapid change in fluorescence. The data fit a single exponential with a <i>k</i><sub>a</sub> = 22.5 s<sup>-1</sup>. Addition of 25 μM ADP reduced the <i>k</i><sub>a</sub> to 5.8 s<sup>-1</sup>.
the rate of ATP hydrolysis of MI130 resembles qualitatively and although performed under slightly different buffer conditions, significantly faster than MI130 and more closely resembles that of ATP hydrolysis by the amoeboid myosin I family is significantly prohibited unless its cross-bridge loses its strain. Assuming a similar elasticity, this strain inhibition is more pronounced for MI130 than for smooth muscle myosin II because of the larger displacement but similar change in the minimum $\Delta G$.

In contrast to the inhibitory effect on both the actin activation of the steady state Mg$^{2+}$-ATPase activity and motility, the transient kinetic parameters measured show no major sensitivity to Ca$^{2+}$ (2–3-fold maximum) and, in fact, both $k_{12}$ and $k_{AD}$ are slightly accelerated by Ca$^{2+}$. MI130 appears similar to smooth muscle myosin II (Ref. 38; regulated by phosphorylation) or the scallop muscle myosins (Ref. 39; regulated by Ca$^{2+}$ binding to the light chains) in that the rate of $P_i$ release from M-D-P or A-M-D-P is primarily affected, although effects on ADP release are also reported. Other events such as ATP binding, actin binding, or ATP hydrolysis on the protein are relatively little affected.

Another feature that MI130 shares with smooth muscle myosin II and BBMI is that the molecules produce movement in two steps, as revealed by an optical tweezers transducer (40, 41). The second step is significantly greater for MI130 and BBMI (5.5 nm) than for smooth muscle myosin II (2–3 nm). It has been proposed that the first step in the power stroke is associated with $P_i$ release, whereas the second step represents ADP release. As expected, the time between the mechanical steps (300 ms, MI130; 120 ms, BBMI) is in good agreement with $1/k_{AD}$ (500 ms MI130, 125 ms BBMI) given the differences in experimental conditions. The ADP release step corresponds to the ADP-dependent structural changes observed in three-dimensional reconstructions of smooth muscle actomyosin II (42) and actin-BBMI (43, 44) generated by cryoelectron microscopy and helical image analysis and by spectroscopy for smooth muscle myosin S1 (45, 46). This argument predicts that a
similar or greater structural change should be observed for MI\textsuperscript{130}, studies to address this issue are in progress.

Cremo and Geeves (6) proposed that the high affinity of ADP for A-M, the structural change accompanying ADP release, and the weak coupling between actin and ADP affinities for smooth muscle myosin S1 could be indicative of a strain-sensing ADP release mechanism rather than the source of an additional power stroke in vivo (Fig. 5). These features of smooth muscle myosin II are common to MI\textsuperscript{130}. Because the free energy of ADP release from actin-MI\textsuperscript{130} is small (K\textsubscript{AD} < 10 μM) and may even be positive at cellular ADP concentrations, a large input of energy would be required to remove ADP from a myosin head bearing positive strain if the cross-bridge displacement is to be large (Fig. 5).

The amount of energy required to dissociate ADP can be estimated assuming the elastic energy of the MI\textsuperscript{130} cross-bridges is similar to that of skeletal muscle myosin II (0.5 milli-Newton m\textsuperscript{−1}). The energy (E) in the elastic element is related to the stiffness (k\textsuperscript{‘}) and the imposed stretch (x) by E = k\textsuperscript{‘}x\textsuperscript{2}/2. If the cross-bridge is already stretched by 5 nm (the size of the cross-bridge throw corresponding to the crossover of the A-M-D-P, and A-M-D parabolas), then an extra 3- or 5-nm stretch is required to release ADP for the smooth and MI\textsuperscript{130} cross-bridges, respectively. The ratio of the extra spring energy, k\textsuperscript{‘}Δx\textsuperscript{2}/2, to the thermal energy, RT, then defines the extent of the strain-induced change in K\textsubscript{AD} = k\textsuperscript{‘}rt/RT, where K\textsubscript{AD} is the equilibrium constant in the absence of strain, R is Boltzmann’s constant, and T is absolute temperature (47). This equation predicts that ADP would bind approximately 10-fold more tightly for an isometric smooth muscle cross-bridge bearing maximum tension and 100-fold more tightly for MI\textsuperscript{130}. If this change in equilibrium constant can be assigned to a strain-dependent inhibition of the rate of ADP release (the simplest assumption), then k\textsubscript{AD} is slowed from 20 to 2 s\textsuperscript{−1} for an isometric smooth muscle cross-bridge and from 2 to 0.02 s\textsuperscript{−1} for MI\textsuperscript{130}, giving an average lifetime for the isometric cross-bridge of 0.5 and 50 s, respectively.

If such a mechanism is contributing to the slow turnover lifetime for the isometric cross-bridge of 0.5 and 50 s, respectively remain trapped on MI130 cross-bridges bearing a significant load. This role would be also consistent with the slow ADP release mechanism rather than the source of an additional power stroke in vivo (Fig. 5). These features of smooth muscle myosin II are common to MI\textsuperscript{130}. Because the free energy of ADP release from actin-MI\textsuperscript{130} is small (K\textsubscript{AD} < 10 μM) and may even be positive at cellular ADP concentrations, a large input of energy would be required to remove ADP from a myosin head bearing positive strain if the cross-bridge displacement is to be large (Fig. 5).

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Lynne M. Coluccio and Michael A. Geeves

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