Native Myosin from Adult Rabbit Skeletal Muscle: Isoenzymes and States of Aggregation

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ABSTRACT: The globular heads of skeletal muscle myosin have been shown to exist as isoenzymes S1 (A1) and S1 (A2), and there are also isoforms of the heavy chains. Using capillary electrophoresis, we found two dominant isoenzymes of the whole native myosin molecule, in agreement with what has previously been found by various techniques for native and nondenatured myosin from adult rabbits. Findings about possible states of aggregation of myosin and its heads are contradictory. By analytical ultracentrifugation, we confirmed the existence of a tail−tail dimer. By laser light scattering, we found a head−head dimer in the presence of MgATP. Capillary electrophoresis coupled with analytical ultracentrifugation and laser light scattering led us to refine these results. We found tail−tail dimers in a conventional buffer. We found tail−tail and head−head dimers in the presence of 0.5 mM MgATP and pure head−head dimers in the presence of 6 mM MgATP. All the dimers were homodimers. Naming the dominant isoenzymes of myosin a and b, we observed tail−tail dimers with isoenzyme a (TaTa) and with isoenzyme b (TbTb) and also head−head dimers with isoenzyme a (HaHa) and with isoenzyme b (HbHb).

Studies of the possible states of aggregation of myosin and its heads (LC2-free S1[1] or Mg·S1, which contains LC2) report contradictory findings (*1*−*14*). Among the different data we must cite Winkelman et al. (*14*), who found that the crystallographic unit in S1 crystals is a S1 dimer. However, many specialists believe that neither the myosin tail−tail nor the S1 dimers exist. Margossian et al. (*15*) using purified cardiac Mg·S1 were unable to confirm the results obtained by Bachouchi et al. (*1*) with native rabbit skeletal Mg·S1, but they found that their Mg·S1 tended to self-associate into dimers under particular conditions. Grussaute et al. (*16*) recently confirmed the existence of the native LC2-free S1 dimer in reversible equilibrium with the monomer and showed it to be the refractory state of S1. They described a list of precautions required to keep both myosin and LC2-free S1 in their native states, i.e., states in which the dimerization sites are intact. For example, myosin should not be chromatographed, especially at room temperature, and should be prepared and studied in the same laboratory. Claire et al. (*17*) confirmed the existence of skeletal Mg·S1 dimers. We report a study of the different states of aggregation of the myosin molecule, and we show that the problems previously associated with dimerization of LC2-free S1 and Mg·S1 can be solved by using the whole myosin molecule.

Apparatus for high-performance capillary electrophoresis (HPCE) has been available on the market for about 6−7 years. However, the full potential of such equipment has not been fully tested. HPCE can separate isoenzymes that differ by only a small number or different arrangements of amino acids. Under nondenaturating conditions, the net charges on different isoenzymes are slightly different, and such isoforms can therefore be separated under high voltage (Beckman Company; personal communication). We present data obtained with adult rabbit skeletal native myosin by analytical ultracentrifugation and laser light scattering. The use of HPCE made it possible to obtain complementary informations when coupled with more traditional techniques. As far as we know, no general theory for the assignments of the peaks in HPCE has been suggested previously. In the Supporting Information, a theoretical approach to this problem is proposed. However, the simplified results are given in the main text.

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[1] Abbreviations: LC2-free S1, myosin subfragment 1 without the regulatory light chain LC2 (RLC); Mg·S1, myosin subfragment 1 containing RLC; ELC, essential light chain (A1 or A2); ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; BTP, Bis-Tris-propane (1,3-bis[tris(hydroxymethyl)methylamino]propane); DTT, DL-dithiothreitol; EDTA, Ethylenediaminetetracetic acid; Pi, inorganic phosphate; PPi, pyrophosphate; Tris, Trizma base (tris[hydroxymethyl]aminomethane); HPCE, high-performance capillary electrophoresis; rpm, revolutions per minute; K, monomer−dimer equilibrium constant; D_t, translation diffusion coefficient; t_r, time of retention in the HPCE capillary; $s_{20,w}$, sedimentation coefficient calculated at 20 °C in pure water.

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MATERIALS AND METHODS

Preparation of Native Myosin. Native myosin was prepared according to Grussaute et al. (*16*), who described the precautions required to obtain pure functional myosin. Only freshly dialyzed myosin was used: beyond 30−35 h after extensive dialysis (2 × 5 h) at 5 °C, myosin kept quiescent at 4 °C in the HPCE apparatus becomes denatured (as assessed by HPCE). The other properties (ATPase, binding to actin) are not affected. The myosin was extremely pure [$A_{280}/A_{260} = 2.5$; see also Grussaute et al. (*16*); no trace of actin was detected by SDS−PAGE (not shown); the index of polydispersity was zero; see Results]. Myosin concentration was measured by optical density, using the extinction coefficient of 0.53 mL/mg.

Electrophoresis. Myosin was extensively dialyzed for 2 × 5 h in the cold room (5.0 ± 1.0 °C) against the "electrophoresis" buffer: 600 mM NaCl, 15 mM Na_2HPO_4, 5 mM NaH_2PO_4, 1 mM EDTA, and 2 mM DTT, pH 8.7 (adjusted with NaOH). We used NaCl and not KCl because at low temperature (see below) small crystals of KCl stick to the inner wall of the capillary and impede the experiments. After dialysis, myosin was clarified by centrifugation at 40000 rpm for 1 h (Beckman 45Ti rotor).

There is a risk of total or partial dissociation of the essential light chains (ELCs) A1 and A2 at pH 8.7 (used in HPCE). To test the absence of dissociation, we assayed the MgATPase activity of our myosin preparations at different pH values (7.3, 8.1, 8.5, and 9.0), by measuring light scattered at 500 nm in a Hitachi F-2000 spectrofluorometer. Very high pH values lead to dissociation of the ELCs, and consequently all the ATPase activities are lost. Partial dissociation of the ELCs should lead to a curve presenting a maximum. Myosin was extensively dialyzed (see above) against a buffer at the appropriate pH similar to the electrophoresis buffer, except that it did not contain EDTA and NaPi but included 10 mM BTP and 6 mM $MgCl_2$. ATP was rapidly added to a final concentration of 6 mM. In the presence of 6 mM MgATP, head-to-head dimers are promoted (see Results), and the light scattered at 90° increased by about 30%. MgATP was split by myosin to give MgADP + Pi. The experiments were carried out at 15.0 ± 0.1 °C: at this temperature, MgADP is unable to induce head-to-head interactions (no variation in the scattered intensity upon addition of MgADP; not shown). Therefore, once all MgATP was hydrolyzed, the scattered light returned to its initial level. The MgATPase activity per head was calculated by using Chance's formula $k_{cat} = S_0/E_0t_{1/2}$, where S_0 is the substrate concentration (6000 μM), E_0 is the head concentration (21μM: 5 mg/mL of myosin, molecular mass 470 000 Da), and $t_{1/2}$ is the time corresponding to the inflection point. We found the following values (see Figure 1): 0.14 s^{-1} (pH 7.3); 0.47 s^{-1} (pH 8.1); 0.88 s^{-1} (pH 8.5); 1.58 s^{-1} (pH 9.0). These activities correspond mainly to those of dimers, the plateau following adjunction of MgATP being 75% horizontal (except near the completion of hydrolysis). Both the existence of a MgATPase activity and its correlation with pH indicate that the ELCs were not dissociated. In particular, the MgATPase activity increased regularly with pH (see above). Note that the steep increase in the MgATPase activity with increasing pH is a well-known result, which confirms the validity of our technique. The increase in scattered light intensity is new evidence for S1 dimerization, to be added to the other results described below.

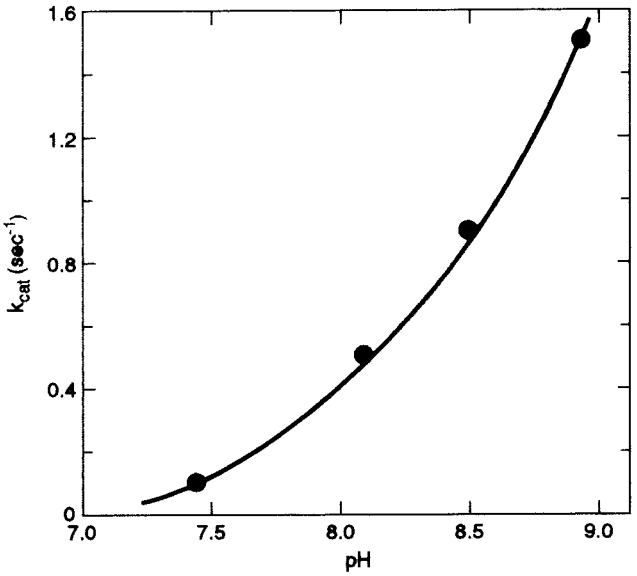

FIGURE 1: MgATPase activity per myosin head at various pH values and at 15 °C. This curve exhibits a steep but continuous increase in the activity as pH increases, indicating no dissociation of the ELCs.

The buffering capacity of our "electrophoresis" buffer at pH 8.7 is not excellent, but sufficient for reproducible results. As the ionic strength was high, which weakens head−head interactions, we used high pH, which strengthens such interactions in the case of LC2-free S1 (*1*, *2*, *10*, *11*). The lifetime of the myosin was around 40 h in the cold room, after the beginning of dialysis (see Results). However, the lifetime was less than 24 h if the myosin was frequently handled (even in the cold room). For capillary electrophoresis experiments, we used a P/ACE 2100 Beckman apparatus, which was placed in the cold room (especially equipped with a dehumidifier to maintain the relative hygrometry imperatively at 25 ± 2%). The working temperature was set at 4.0 ± 0.1 °C, and the wavelength was set at 280 nm (a wavelength of 214 nm, corresponding to absorption of the peptide bonds, is frequently used; however, in the case of myosin, the traces at 214 nm were extremely noisy). We used a low noise cartridge. The length of the capillary was 57 cm (detection length 50 cm), and its inner diameter was 75 μm. The voltage was 5.5 kV, and the current is 155−185 μA (hypersharp peaks can be obtained for high voltages, of 20−30 kV; however, in all cases, the maximum allowed current is 250 μA and because of the high ionic strength used, we could not increase the voltage. An alternative possibility would have been to use a capillary with a smaller inner diameter, say 20 μm, but this makes the experiments extremely difficult to perform). The duration of injection of myosin was 1 s. In preliminary experiments, we assessed BTP or Tris buffers, but the traces given by the photomultiplier were noisy. We therefore used NaPi. However, the traces were not satisfactory: the peaks corresponding to myosin were flat and difficult to interpret. This was probably due to the negative charges on myosin being screened by the Na^+ counterions and/or the net charges on myosin not being sufficient and/or the extinction coefficient of myosin being too weak (the most probable explanation; see below). ATP binds tightly to myosin (e. g., ref *18* and references

cited therein). Therefore, 0.5 mM ATP was added to the vials containing the dialysis buffer and to the vial containing myosin, such that the ATP concentration was uniform throughout all the HPCE apparatus. ATP bound to myosin gave a convenient extinction coefficient to myosin (see Results). We tried to use PPi in all the vials (instead of ATP), but the extinction coefficient of myosin was not high enough and no peaks were detected, confirming that the extinction coefficient for myosin was negligible. Pi is a competitive inhibitor of ATP at the enzymatic site. Thus, when MgATP is present (see Results), there is no problem of hydrolysis for many hours at low temperature (4 °C). Anyhow, at this temperature, MgATP and also MgADP and Mg^{2+} induce head−head dimerization (*10*, *16*). Finally, we chose pH 8.7 instead of pH 8.0, the traces being better at pH 8.7 than at pH 8.0: at pH 8.7, the peaks are clearly resolved and do not overlap the peaks corresponding to the blanks, which is not the case at pH 8.0.

In the Supporting Information, we develop a rigorous technique to assign the different peaks observed in HPCE. (i) Each molecule is characterized by a friction coefficient f and a measurable diffusion coefficient $D_t = kT/f$ [extrapolation of the straight line $D_t(c)$ at $c = 0$]. (ii) The mobility U_{app} of the molecule is the sum of the electrophoretic mobility U and the electroosmotic mobility U^*: $U_{app} = U + U^* = Z_{app}e/f$ (e is the charge of the electron; Z_{app} is the apparent net charge on the molecule; for more details see Supporting Information). The time of retention of the molecule in the capillary is $t_r = \chi/U_{app}$ (χ is a constant for a given apparatus, at a given temperature and a given voltage). We can write $t_r = \chi(kT/e)(D_tZ_{app})$. For oligomers containing N subunits, we have $Z^i_{app} \approx NZ^*_{app}$; i.e., $t_r^i = (D_t^i/D^*_t)(Z^i_{app}/Z^*_{app}) \approx (1/N)(D_t^*/D_t^i)$ (see Supporting Information). The asterisk symbol (*) corresponds to an a priori chosen peak to which all the other peaks (i) refer. Measuring the translation diffusion coefficients and giving to N the values 1, 2, 3, ..., all the peaks can be assigned if a peak is a priori chosen. For isoforms of a given species, Z_{app} are slightly different; and the different isoforms migrate with slightly different retention times for a given run.

Laser Light Scattering. Myosin was extensively dialyzed against a buffer which was the same as that used for electrophoresis, except that EDTA and NaPi were not included. However, 10 mM BTP was added and the pH was once more adjusted to 8.7 by adding HCl. For technical reasons, the scattering experiments could not be performed below 10.0 ± 0.1 °C, a temperature at which the head−head dimerization process is less efficient than at 4 °C (*10*, *16*). Therefore, we added 6 mM $MgCl_2$ to the dialysis buffer and 6 mM ATP (for the 1 mL sample of myosin). D_t was measured at a series values of c. ATP was added extemporarily for each value of c to avoid significant MgATP splitting. Each experiment lasted for ≈15−20 min, and we did not detect any denaturation of the myosin at the working temperature (10 °C). We measured the diffusion coefficients at 10 °C, in the same buffer as for electrophoresis. We converted the diffusion coefficients at 4 °C to compare HPCE and laser light scattering.

For the scattering experiments, we used a Spectra Physics laser (Argon; 514.5 nm) set at 250 mW. The scattered light was observed at 90°. The correlator was a Brookhaven 2030 AT apparatus. We chose a correlation time τ of 40 μs, and the corresponding values of Γ_1 and Γ_2 were recorded 20 times to deduce the translation diffusion coefficient D_t and the index of polydispersity (for more details see Suporting Information).

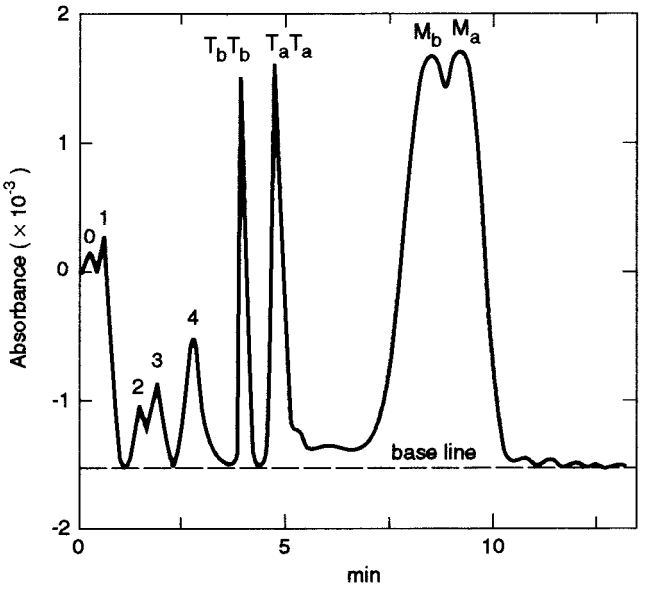

FIGURE 2: A typical trace obtained with 2.8 mg/mL myosin in the dialysis buffer + 0.5 mM ATP. The general shapes of the different peaks are highly reproducible. Peaks 0 and 1 are artifactual (see main text). Peaks 2−4 correspond exactly to ionic species observed in the blanks. The myosin peaks are clearly different from the "blank" peaks, at pH 8.7. M and TT are respectively the monomer (a, b) and the tail-to-tail dimer peaks (a, b). A reversal of these assignments leads to contradictory results, and therefore, the present assignments are the only possible ones. Experiments at pH 8.0 led to noisier traces and to myosin peaks overlapping the blank peaks. For this reason we did not use the traditional pH of 8.0.

Analytical Ultracentrifugation. We used a Beckman Type E ultracentrifuge. Myosin was dialyzed against a buffer identical to that used by Herbert and Carlson (*9*). Sedimentation coefficients of myosin were calculated from plots of ln distance ($r_{1/2}$) versus time, and the usual corrections were made for solvent density, viscosity, and temperature (we worked at 4 °C) to obtain $s_{20,w}$. The apparent specific volume of myosin was taken as 0.728 mL/g, irrespective of the state of aggregation of the protein.

RESULTS

Results in the Absence of Magnesium. Prior to experiments with myosin, the capillary was carefully equilibrated with the dialysis buffer (+ 0.5 mM ATP in all vials; see Materials and Methods) under voltage. Four to five runs of equilibration were carried out, each lasting for 40−60 min. Two to three blanks were recorded (not shown). We observed four peaks (absorbing at 280 nm and occurring in the range 0−3 min), probably corresponding to ATP, ADP, and perhaps impurities absorbing at 280 nm. At the beginning of each run (low retention times), we observed one or two peaks due to artifacts related to the voltage increase (≈0.17 min).

In Figure 2, we present a typical electrophoregram showing the peaks of myosin (loading concentration 2.8 mg/mL; $n = 8$). The peak corresponding to $t_r = 9.13 \pm 0.63$ min (± SD; $n = 8$) was assigned to a monomeric isoenzyme a (Ma). The peak corresponding to $t_r = 7.83 \pm 0.49$ min (± SD; n

= 8) was assigned to a monomeric isoenzyme b (Mb). There were also two other peaks at $t_r = 5.43 \pm 0.36$ min (± SD; $n = 8$) and at $t_r = 4.32 \pm 0.41$ min (± SD; $n = 8$), which we assigned to tail−tail homodimers: TaTa corresponds to $t_r = 5.43 \pm 0.36$ min and TbTb corresponds to $t_r = 4.32 \pm 0.41$ min. TaTa is a tail−tail homodimer composed of two Ma isoenzyme subunits. TbTb is a tail−tail Mb homodimer. Are these assignments suitable? A careful spectroscopic study of the translation diffusion coefficients of the monomer and the tail−tail dimer of myosin has been reported by Herbert and Carlson (*9*). We corrected their values for temperature and buffer conditions. Their data, under our conditions (4 °C; our buffer for electrophoresis, except that we used pH 8.7 and not pH 8.0 as in ref *9*) can be represented by

$$D_t^M \times 10^7\ (\mathrm{cm^2/s}) = 1.012\ (1-0.0056c) \qquad (1)$$

$$D_t^{TT} \times 10^7\ (\mathrm{cm^2/s}) = 0.683\ (1-0.0016c) \qquad (2)$$

We repeated the experiments of Herbert and Carlson (*9*) at pH 8.7 (see Materials and Methods) and found using their calculation method:

$$D_t^M \times 10^7\ (\mathrm{cm^2/s}) = 1.036\ (1-0.0040c) \qquad (3)$$

$$D_t^{TT} \times 10^7\ (\mathrm{cm^2/s}) = 0.773\ (1-0.0026c) \qquad (4)$$

We shall use these data for the interpretation of the HPCE peaks. The loading concentration of myosin was 2.8 mg/mL ($n = 8$; measured by optical density, but possibly slightly different in the capillary). For each M peak, the concentration was ≈1 mg/mL (calculated by the peak area in the electrophoregrams; $n = 8$) and for the TT peaks was ≈0.4 mg/mL ($n = 8$). From equations 3 and 4, we deduce D_t^M (1 mg/mL) = 1.032×10^{-7} cm²/s and D_t^{TT} (0.4 mg/mL) = 0.772×10^{-7} cm²/s. Thus, according to eq 14 in the Supporting Information and with $N = 2$, $t_r^{TT}/t_r^M \approx 0.5$ (1.032/0.772) = 0.668. For isoenzyme Ma, $t_r^{Ma} = 9.13 \pm 0.63$ min, and the calculated value of the retention time for TaTa is $(9.13 \pm 0.63) \times 0.668 = 6.10 \pm 0.42$ min. An analogous calculation leads to a retention time of $(7.83 \pm 0.59) \times 0.668 = 5.23 \pm 0.39$ min for TbTb. These values agree with the measured values. The difference of ≈0.67−0.91 min between the measured and the calculated values may be because either $A(c)$ (see eq 13 in the Supporting Information) is different for the monomers and the dimers or the values of D_t^M and D_t^{TT} are slightly erroneous. This could also be due to the dimers presenting minor rearrangements of the charges and leading, for example, to $Z^{TT} \approx 2.1\ Z^M$ instead of 2.0. Alternatively, the corrective factor $[\Phi + (L/V)(f/Ze)\Psi]$ may not be the same for M and TT. The slight differences between the observed and calculated values may be due to several or all of these factors.

As shown in ref. *10* for LC2-free S1, the monomer−dimer equilibrium depends on the hydrostatic pressure. Therefore, to confirm the existence of the tail−tail dimer, we studied the sedimentation coefficient of myosin in a buffer identical to that used by Herbert and Carlson (*9*) and at 4 °C. We performed sedimentation experiments at different speeds of rotation, i.e., at different hydrostatic pressures. For each speed of rotation, $s_{20,w}$ versus the myosin concentration c was analyzed (c varied from 2 to 5 mg/mL). Within the limits of experimental error, the $1/s_{20,w}$ plots (not shown) were straight lines. Extrapolation of $1/s_{20,w}$ versus c gave a value at $c = 0$ ($s^0_{20,w}$). Between 45 000 and 60 000 rpm, we obtained the usual value for the myosin monomer ($s^0_{20,w} = 6.8$ S). At all speeds of rotation between 15 000 and 45 000 rpm, a single peak was observed. This indicates the presence of a single species or a monomer−dimer mixture in rapid reversible equilibrium (in which case a single peak is observed and a single sedimentation coefficient is measured, having a value between that of the monomer and that of the dimer; for more details see ref *10*). It may appear that $1/s_{20,w}$ versus c cannot be a straight line for a monomer−dimer mixture. According to Morel and Garrigos (*10*), the apparent sedimentation coefficient for the mixture in rapid reversible equilibrium is given by $s_{app} = s_m + (s_d - s_m)(c_d/c)$, where s_m and s_d are the sedimentation coefficients for the monomer and the dimer, respectively, and c_d/c is the proportion of dimer. It is customary to write, for a single species, $s = s^0/(1 + kc)$. We observed that k is roughly independent of the hydrostatic pressure, i.e., $k_m \approx k_d$. We deduce $s^0_{app} = s_{app}(1 + kc)$, with $s^0_{app} = s^0_m + (s^0_d - s^0_m)(c_d/c)$. On the other hand, $c_d/c = 1 - 2/[1 + (1 + 4Kc)^{1/2}]$, where K is the equilibrium constant (*10*). A typical value for K is ≈1 mL/mg (see also refs *6* and *7*). If $c = 2$ mg/mL, then $c_d/c \approx 0.50$ and $s^0_{app} \approx 7.9$ S. If $c = 5$ mg/mL, then $c_d/c \approx 0.64$ and $s^0_{app} \approx 8.2$ S. Thus s^0_{app} is almost independent of c, and within the limits of experimental error, the curve $1/s_{20,w}$ versus c is a straight line (see above). For low rotation speeds, the curve ln $r_{1/2}$ versus t exhibited a slight downward curvature, indicating that the sedimentation coefficient s was not constant and decreased with the hydrostatic pressure. We treated the problem as described in ref *10*. The measured sedimentation coefficient was a function of the speed of rotation or more precisely of the hydrostatic pressure, which varies as the square of the speed of rotation. Figure 3 shows the "apparent" sedimentation coefficients versus (rpm)². The value extrapolated at atmospheric pressure (rpm = 0) was $s^0_{20,w} = 9.0$ S. We assign the value of 6.8 S to the myosin monomer and the value 9.0 S to the tail−tail dimer. The dependence of the sedimentation coefficients on the hydrostatic pressure is related to a displacement of the dimer toward the monomer when this pressure increases, which is due to a decrease in the equilibrium constant, i.e., to an increase in the apparent specific volume for the reaction dimer → monomer (for unknown reasons, the reverse phenomenon was observed for LC2-free S1 alone, ref *10*; possibly the variation in the specific volume of the tail is higher than that of the heads on dimerization: the whole molecule would have the same qualitative variations as the tails). Are the assignments of these two sedimentation coefficients correct? According to the Svedberg equation, $M = RTs/D_t(1 - \rho\upsilon)$ (M is the molecular mass; RT has the usual meaning; s is the sedimentation coefficient; D_t is the translation diffusion coefficient; ρ is the density of the buffer; υ is the apparent specific volume; all values measured at pH 8.0) and assuming that υ is approximately the same for the monomer and the dimer, we obtain $M_d/M_m = (9.0/6.8) \times (1.012/0.683) = 1.961$, which confirms the existence of an almost pure tail−tail dimer at 2−5 mg/mL, at atmospheric pressure, at 4 °C, and in a buffer identical to that used by Herbert and Carlson

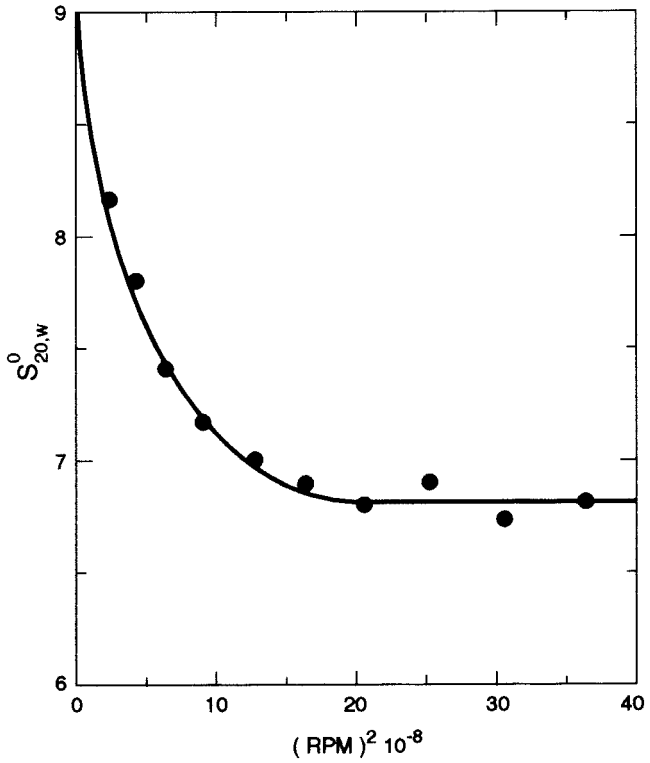

FIGURE 3: Sedimentation coefficients of myosin extrapolated to zero concentration as a function of $(rpm)^2$. The experimental points were fitted by a single-exponential $s^0_{20,w} = 6.8 + 2.2 \exp[-0.22\text{-}(rpm)^2\ 10^{-8}]$.

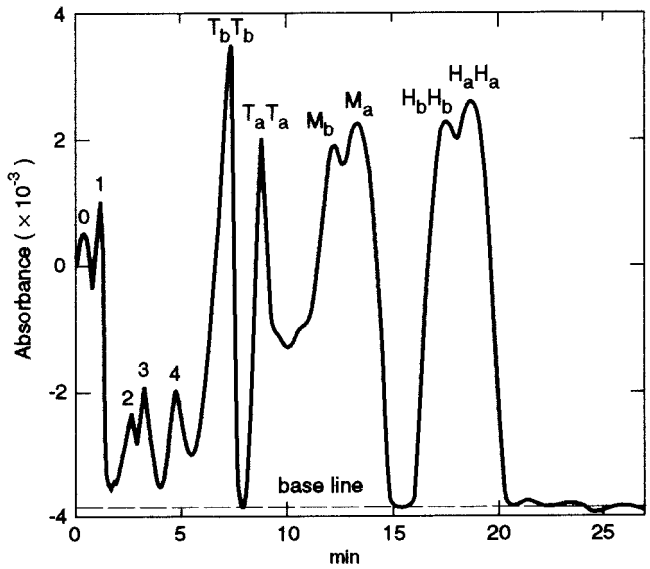

FIGURE 4: Typical trace obtained with 2.7 mg/mL myosin in the dialysis buffer + 0.5 mM ATP + 4 mM $MgCl_2$. The only possible assignments of M, TT, and HH are given. Other assignments lead to contradictory results. Note the higher retention times than in the absence of magnesium (see Supporting Information).

(*9*). More generally, this confirms the existence of the tail−tail dimer, with equilibrium constants varying according to the conditions [for example, Herbert and Carlson (*9*) observed a monomer−dimer mixture at atmospheric pressure under their experimental conditions]. This result and the results obtained by HPCE and laser light scattering support the existence of the tail−tail dimer. The most probable structure of this dimer is given in Figure 5a. Note that Cardinaud and Drifford (*4*) and Emes and Rowe (*5*) worked at 50000−60000 rpm, which explains why they only observed the myosin monomer. Obviously, a rigorous confirmation could be obtained by electron microscopy. However, as far as we know, no electron microscopy experiments have been able to demonstrate such structures. This is probably because such experiments require extremely low concentrations of myosin, which displaces the monomer−dimer equilibrium toward the pure monomer.

Results in the Presence of Magnesium. The myosin loading concentration was 2.7 mg/mL. Here again the capillary was equilibrated with the dialysis buffer for four or five runs under voltage in the presence of 4 mM $MgCl_2$ and 0.5 mM ATP (see Materials and Methods). Blanks were also recorded, as in the absence of magnesium. There were peaks in the range of 0−5 min. Peaks corresponding to voltage artifacts were observed as described above for low retention times (see Figure 4). Four peaks were observed corresponding to the different ATP or ADP ionic species, including MgATP. The Ma and Mb peaks appeared with retention times of 12.97 ± 1.01 min (± SD; $n = 8$) and 10.89 ± 0.96 min (± SD; $n = 8$), respectively. The TaTa and TbTb peaks had retention times of 9.02 ± 0.68 min (± SD; $n = 8$) and 7.26 ± 0.41 min (± SD; $n = 8$), respectively. The concentrations were ≈0.6 mg/mL for Ma and Mb ($n = 8$) and ≈0.3 mg/mL for TaTa and TbTb ($n = 8$) and $D_t^M = 1.032 \times 10^{-7}$ cm²/s and $D_t^{TT} = 0.770 \times 10^{-7}$ cm²/s. Therefore, according to eq 14 in the Supporting Information and with $N = 2$, $t_r^{TT}/t_r^M \approx 0.5$ (1.032/0.770) = 0.670. For isoenzyme Ma, $t_r^{Ma} = 12.97 \pm 1.01$ min, and the calculated value for TaTa is (12.97 ± 1.01) × 0.670 = 8.89 ± 0.68 min. A similar calculation leads to a retention time of (10.88 ± 0.96) × 0.670 = 7.29 ± 0.64 min for TbTb. Agreement between the observed and the calculated values is good, and the slight difference of ≈0.03−0.13 min is not significant.

The most interesting feature was the presence of two retarded peaks. We attribute these peaks to head−head myosin homodimers, which we call HaHa and HbHb. The respective retention times for HaHa and HbHb were 18.44 ± 1.32 min (± SD; $n = 8$) and 16.36 ± 1.19 min (± SD; $n = 8$). To confirm the presence of head−head dimers of myosin, we carried out laser light scattering experiments. The recorded values of D_t^{HH} (corrected for temperature conditions and buffer; 4 °C and our electrophoresis buffer) versus c (0.72−16.2 mg/mL) can be fitted by a straight line:

$$D_t^{HH} \times 10^7\ \text{cm}^2/\text{s} = 0.368\ (1-0.026c) \qquad (5)$$

These translation diffusion coefficients are very much lower than those of the monomer and the tail−tail dimer (see eqs 3 and 4). This can *only* be due to substantial asymmetric scattering particles, i.e., head−head dimer. Indeed, according to ref *20*, a large increase in the asymmetry of the molecule induces only a limited increase in the shape factor (e.g., the TT myosin dimer being assumed to fit reasonably well a prolate with axial ratio ≈30 has a shape factor $f/f_0 \approx 2.356$, whereas the HH dimer (same hydration) has an axial ratio of ≈80 and f/f_0 ≈3.658. The roughly calculated ratio for the diffusion coefficient is thus 2.356/3.658 ≈0.644 (measured from D_t, this ratio is 0.368/0.773 ≈0.476). The agreement is sufficient, due to the oversimplifications used here. The most probable structure of this dimer is shown in Figure 5b [the head−head dimer can indeed be formed, as

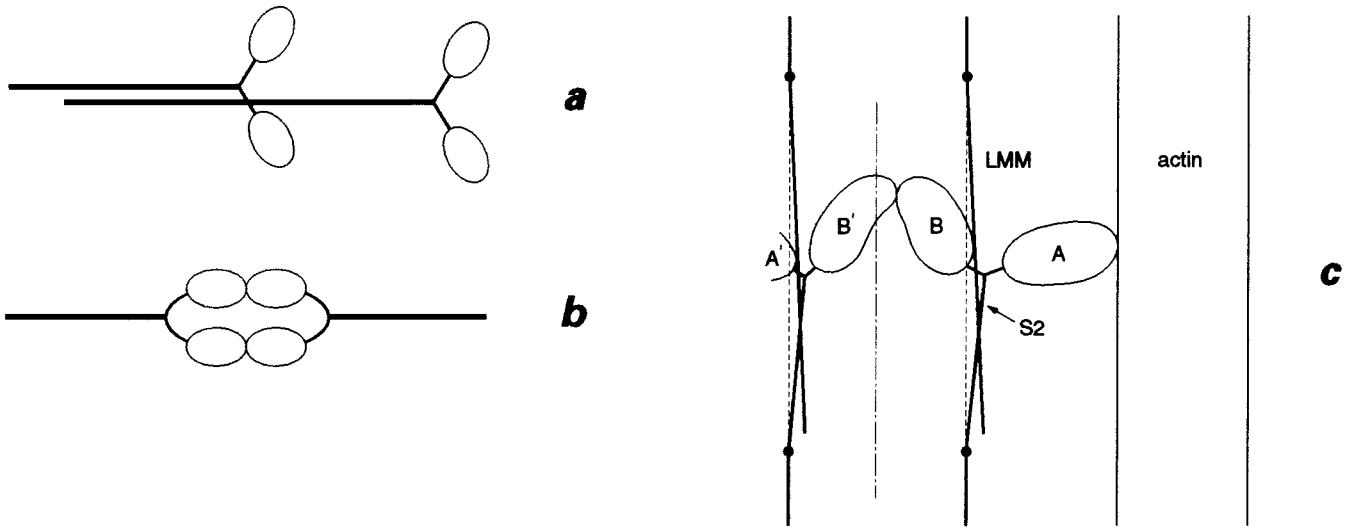

FIGURE 5: Most probable arrangements of the myosin dimers in vitro (panels a and b) and the thick filaments in vivo (panel c). (Panel a) In ref *3*, different arrangements of the myosin tail-to-tail dimers have been proposed. We selected the present scheme, inasmuch as it corresponds to the best structure for the formation of thick filaments. (Panel b) Due to the very low value of the diffusion coefficient for the head-to-head dimer (see text; presence of 6 mM MgATP) and assuming that the affinities of the heads for each other is similar, we propose this arrangement for the dimer in solution (only one conformation is possible due to the zero index of polydispersity; see text). In the HPCE experiments, the MgATP concentration is 0.5 mM plus 2.5 mM free magnesium (EDTA 1 mM, see Materials and Methods). At 4 °C, both MgATP and Mg^{2+} induce head−head interactions in vitro (*11, 16*). The good fit between HPCE and laser-light scattering indicates that this scheme is also applicable to myosin in the dimer buffer, in the capillary (see text). (Panel c) How can both the tail−tail and head−head dimers be combined to give the structure of the thick filaments? In vivo, at 37 °C, the MgATP concentration is ≈2−3 mM, and magnesium cannot induce head−head interactions (too high temperature, see ref *16*). Moreover, MgATP induces lower head−head interactions above 20 °C and no detectable interactions at 42 °C (unpublished). Thus, the head−head interactions are weaker than under the in vitro conditions, and only one head of two can attach to the head of the opposite myosin molecule as shown in the diagram (B, B′ heads). We do not know the behavior of the tail−tail dimer as the temperature increases, but we suggest that, at 20 °C and beyond, the tails can interact, leading to this plausible structure for the thick filaments. We suggest that the tail−tail and head−head interactions may depend on the conditions (temperature, pressure, composition of the medium, etc.). In this scheme, head A interacts with actin during the power stroke. This structure is valid for a two-stranded natural thick filament (structure in old rabbit muscles and in synthetic filaments; unpublished). The natural filaments of young rabbit muscles are three-stranded (unpublished), and the third myosin molecule has its two heads outside the core of the thick filament: the two heads can interact with actin. The new findings presented in the text support this model, suggested as long ago as 1982 (*11*). We assign different roles to the myosin heads, which could answer the old question: why two heads? It is yet to be shown whether this structure, which can vary slightly according to the conditions (tail−tail and head−head affinities; head−actin affinity, etc.), has implications for the molecular mechanisms of contraction. According to reference *11*, a swelling model can be developed. However, we believe a hydrid model could well account for the characteristics of a contracting muscle. From the present scheme for the thick filaments, we are currently working on the mechanical implications of the proposed structure of the thick filaments.

the isolated myosin heads can form dimers (*2*, *10*, *11*, *17*)]. We verified that the myosin was extremely pure: the index of polydispersity of our samples was typically close to zero [0.05 ± 0.05 (± SD; $n = 10$)], which confirms that the head−head dimer was pure, at least for $c > 0.72$ mg/mL. Due to the very high concentration of MgATP (6 mM), all the myosin molecules were in the form of head−head dimers, whereas in HPCE the concentration of MgATP was only 0.5 mM plus 2.5 mM magnesium, which allowed the simultaneous presence of tail−tail dimers. We tried to obtain electron micrographs of these head-to-head dimers, but due to the large dilution of myosin, we were unsuccessful, the dimers spontaneously splitting into monomers. At higher myosin concentrations, the population of myosin molecules was too dense, and no clear conclusion could be drawn. However, head-to-head interactions in the presence of MgATP have been directly observed between myosin filaments (electron microscopy; unpublished) and indirectly revealed in myosin (see above; enzymatic assays).

The concentrations of HaHa and HbHb were ≈0.6 mg/mL ($n = 8$). Thus, eq 5 leads to $D_t^{HH} = 0.362 \times 10^{-7}$ cm^2/s. According to eq 14 in the Supporting Information and with $N = 2$, $t_r^{HH}/t_r^{M} \approx 0.5$ (1.034/0.362) = 1.428. For isoenzyme Ma, $t_r^{Ma} = 12.97 \pm 1.01$ min, and the retention time for HaHa would be (12.97 ± 1.01) × 1.428 = 18.52 ± 1.43 min. Similarly, the retention time for HbHb would be (10.89 ± 0.96) × 1.428 = 15.55 ± 1.37 min. The agreement between the observed and calculated values is good. The difference of ≈0.08−0.81 min may be due to the factors discussed for the results in the absence of magnesium. We conclude that both electrophoresis and laser light scattering experiments support the existence of head−head dimers of myosin in the presence of MgATP. Electrophoresis shows that two populations of homodimers are present (tail−tail and head−head). We have not demonstrated that the head−head myosin dimer is in reversible equilibrium with the monomer: due to the presence of two types of dimers (tail−tail and head−head), the ultracentrifuge and laser light scattering experiments we performed in this field were extremely ambiguous. Nevertheless, the LC2-free S1 dimer is in rapid reversible equilibrium with the monomer in the presence of MgADP or Mg^{2+} (e.g., refs *10* and *16*), and there is no reason to expect that the myosin head−head dimer behaves differently.

In the Supporting Information, we present a new method for assigning the HPCE peaks and confirming the results obtained by this technique by other more traditional techniques (laser light scattering, ultracentrifugation). By com-

paring the different retention times of the peaks, we could deduce, for example, the "positions" of the different polymers. Having studied the myosin monomers and dimers at $c \approx 2.8$ mg/mL ($n = 8$), we can confirm and refine these assignments. With this loading concentration, we found approximately the following concentrations corresponding to the peak areas: Ma ≈ Mb ≈ 0.5 mg/L; TaTa ≈ TbTb ≈ 0.3 mg/mL; HaHa ≈ HbHb ≈ 0.6 mg/ml ($n = 8$). We deduce the presence of ≈21% TT dimers and ≈44% HH dimers. We performed other experiments with a loading concentration of 1.3 mg/mL and found the following values: ≈0.35 mg/mL (M); ≈0.05 mg/mL (T); ≈0.25 mg/mL (H) ($n = 8$). We deduce the presence of ≈8% TT dimers and ≈38% HH dimers. When c decreases, the percentages of dimers decrease, and in the light of the Supporting Information, we confirm that the different peaks are correctly assigned and that reversible monomer−dimer equilibria exist.

DISCUSSION

We confirm that there are two dominant isoenzymes of "adult" native myosin (forms a and b) and that they are present in similar proportions. Forms a and b correspond to adult fast and adult intermediate native myosin (e.g., ref *19*). We did not study neonatal myosin, which contains a third dominant isoenzyme (slow form; ref *19*). We did not intend to investigate less abundant isoenzymes obtained under various conditions. Homodimers of both tail-to-tail or head-to-head myosin molecules were detected. Both the tail-to-tail and head-to-head dimers are in rapid reversible equilibrium with the monomers (*1*, *2*, *3*, *6*−*8*, *10*, *11*). However, for unknown reasons, the dimer and monomer give clearly resolved HPCE peaks. This is a particularity of capillary electrophoresis. However, as far as we know, there are no experimental data that could elucidate this phenomenon. Notwithstanding, we have recently shown that in the presence of 2 mM $MgCl_2$ and at 4 °C, HPCE clearly separates S1(A1) and S1(A2) and also a heterodimer S1(A1)−S1(A2), which is in rapid reversible equilibrium with the monomers (unpublished). This particularity makes HPCE extremely useful as compared to other techniques. For example, in the case of hydrodynamic experiments (e.g., viscometry, laser light scattering or ultracentrifugation), there is no "separation" between the monomers and the dimers, and the observed experimental values represent "mean" values for the monomer−dimer mixtures. In the case of HPCE, all the dominant isoforms and aggregates can be observed. Thus, HPCE is a powerful tool but has to be coupled with other techniques. Here, we chose to measure the translation diffusion coefficients of the migrating molecules to identify the different peaks. The sedimentation coefficients could also be measured, with the drawback that the molecular weights of the molecules have to be known or measured (by means of sedimentation−diffusion equilibrium ultracentrifugation, for example) to determine the friction coefficients, given by $f = M(1 - \rho v)/N_A s$. Nevertheless, analytical ultracentrifugation per se is useful for some investigations. For example, we have confirmed the existence of tail−tail dimers by ultracentrifugations at various speeds of rotation.

Finally, we have used the present and older evidence to construct the most probable models for the tail−tail and head−head dimers and to give our opinions on their role in thick filament structure (see Figure 5).

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SUPPORTING INFORMATION AVAILABLE

We present a general theory for the assignments of the different peaks, based on the electrostatic and electroosmotic mobilities of the molecule and also its translation diffusion coefficient. We develop the equations presented in the main text and consider with particular interest the case of a momonomer−dimer equilibrium (9 pages). Ordering information is given on any current masthead page.

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