Dimerization of Native Myosin LC2(RLC)-Free Subfragment 1 from Adult Rabbit Skeletal Muscle

Jean-Emile Morel,*,‡,§ Karima Taouil,‡,§ Nathalie D'hahan,‡,§ Asuncion Aguilar,‡ Zalika Merah,‡,§ Jean-Pierre Dalbiez,|| Patrick Bayol,‡,§ Nathalie Guillo,‡,§ Louis Patard,§ Vincent Cabane,‡ Matteo Ferrari,‡ Gonzalo Figuera Picazo,‡ Hanh Dam Hieu,‡ and Mathilde Francin‡

Laboratoire de Biologie, Ecole Centrale de Paris, 92295 Châtenay-Malabry Cedex, France, and Service de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire and Département de Recherche sur l'Etat Condensé, les Atomes et les Molécules, CEA, CE-Saclay, 91191 Gif-sur-Yvette Cedex, France

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ABSTRACT: We reinvestigated whether the native myosin LC2-free-subfragment 1 (S1) dimer exists by using viscometry, capillary electrophoresis, and laser light scattering. We found that the intrinsic viscosity of the monomer is $[\eta]_m = 6.7$ cm^3/g and its translation diffusion coefficient is $D_{t,m}^{20,w}(c = 0) = 4.43 \times 10^{-7}$ cm^2/s. For the dimer, $[\eta]_d = 19.8$ cm^3/g and $D_{t,d}^{20,w}(c = 0) = 2.54 \times 10^{-7}$ cm^2/s. Using the Svedberg equation and introducing the values of the sedimentation coefficients (5.05 S for the monomer and 6.05 S for the dimer), we find the following molecular weights: $M_{r,m} = 108\,000$ Da and $M_{r,d} = 213\,000$ Da, which agree well with previous determinations. Capillary electrophoresis successfully separated S1(A1) and S1(A2), in a monomer buffer, and S1(A1) and S1(A2) and a heterodimer S1(A1)−S1(A2), in a dimer buffer. An interesting feature of the monomer−dimer equilibrium is the presence of temperature transitions, whose positions and widths depend upon the buffer conditions. At low temperatures, a pure dimer was observed, whereas at high temperatures only the monomer was present. The dimerization site on both myosin and S1 is extremely labile.

Morel and Garrigos (*1*, *2*) found that crude LC2-free S1 (called below S1) can form dimers in solution. Bachouchi et al. (*3*) directly visualized, by electron microscopy, myosin head dimers of LC2-free S1[1] and Mg.S1 (containing LC2). The existence of the dimeric state of S1 was not accepted for 12−13 years. However, Stewart et al. (*4*) described head-to-head interactions of heads belonging to different myosin molecules (*Limulus* and scorpion thick filaments), in agreement with Morel et al. (*5*), who found that native myosin can form head−head dimers. The work of Levine et al. (*6*) showing that it is possible to use bis_{22} ATP to crosslink axially sequential myosin molecules along each helical strand was not intended to demonstrate that myosin exists as dimers or in any other particular interactive form. However, cross-linking by bis_{22} ATP (a cross-linker specific for the enzymatic and dimeric sites on the heads) is highly in strong evidence of close head-to-head interactions of neighboring molecules. Despite these results, the existence of dimers has been challenged by Munson et al. (*7*) and Stafford III and Margossian (*8*). These authors probably overlooked the fact that the crystallographic unit in S1 crystals is a dimer (*9*), or considered that direct observation of the dimer by the techniques described by Bachouchi et al. (*3*) and Winkelmann et al. (*9*) may induce artifactual distortions, not occurring with hydrodynamic techniques. Furthermore, they could argue that the S1 routinely prepared in our laboratory was not sufficiently purified. Margossian et al. (*10*) were unable to confirm, with purified cardiac Mg.S1, the results obtained by Bachouchi et al. (*3*) with native Mg.S1 from rabbit skeletal myosin. However, Margossian et al. (*10*) found that purified cardiac Mg.S1 has a tendency to self-associate into dimers, under appropriate conditions. Grussaute et al. (*11*) have shown that it is better to work on native S1, which maintains the ability to form dimers, than on modified S1 (e.g., chromatographed S1; a full list of precautions required to keep S1 in its native form is presented in the Supporting Information). For all these reasons, we reinvestigated the problem of the existence of the S1 dimer, in native material, by viscometry, capillary electrophoresis, and laser light scattering. We confirm its existence and report some new factors influencing the monomer−dimer equilibrium (e.g., temperature). This work is complementary to studies presented in the 1980s by our group and more recently by Grussaute et al. (*11*) and Morel et al. (*5*). In

‡ Laboratoire de Biologie.

§ Département de Biologie Cellulaire et Moléculaire.

|| Département de Recherche sur l'Etat Condensé.

[1] Abbreviations: LC2-free S1, myosin subfragment 1, without the light-chain LC2 (regulatory light chain RLC); Mg.S1, myosin subfragment 1 with its RLC; S1(A1) and S1(A2), myosin subfragments 1 containing respectively the essential light chains (ELCs) A1 and A2 (A1 and A2 are also respectively named LC1 and LC3); BTP, Bis-Tris-Propane (1,3-bis(tris(hydroxymethyl)-methylamine) propane (Sigma); DTT, DL-Dithiothreitol (Sigma); ADP, adenosine 5′-diphosphate (Sigma); AMP-PNP, 5′-adenylylimido-diphosphate (Sigma); PMSF, phenyl-methyl-sulfonyl fluoride (Sigma); bis_{22} ATP, bifunctional crosslinker able to crosslink two S1 (the crosslinker was described in ref *7*); HPLC, high-performance capillary electrophoresis (Beckman); D_t, translation diffusion coefficient; $s_{20,w}$, sedimentation coefficient calculated at 20 °C in pure water; t_r, time of retention in the HPCE capillary; M_r, molecular weight; Z_{app}, apparent net charge on a protein; $[\eta]$, intrinsic viscosity; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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1996, Claire et al. (*12*) confirmed the existence of the skeletal Mg.S1 dimer. However, the properties they report for their Mg.S1 dimers are different from those observed for LC2-free S1 dimers. For example, LC2-free S1 cannot form a "natural" dimer in the absence of Mg nucleotides and at room temperature (our data), whereas Mg.S1 can (*12*). When S1 is prepared by digestion with either chymotrypsin or papain the heavy and light chains are more or less cleaved. These cleavages are different for the two species and are seen only on S1 or Mg.S1 denatured by SDS (for more details see ref *13*). Probably, the difference of behavior results from the different cleavages of RLC-free S1 and Mg.S1. Nonetheless, Bachouchi et al. (*3*) found that the major properties of Mg.S1 are qualitatively (not quantitatively) similar to those of LC2-free S1, in agreement with the fact that the myosin heads on whole myosin molecules can dimerize in the presence of MgATP (*5*). In light of the recent positive evidence for S1 dimers (*11*), Mg.S1 dimers (*12*), and myosin head−head dimers (*5*), we undertook further experimental studies on the S1 dimer.

MATERIALS AND METHODS

Preparation of Native Myosin and Native Chymotryptic S1. Preparations of native myosin and RLC-free S1 were derived from those of Margossian and Lowey (*13*). However, these techniques were improved to get native myosin and native RLC-free S1. Native myosin (around 30 mg/mL), less than 24 h old before storage in 50% glycerol at −20 °C (storage concentration of myosin around 15 mg/mL), was prepared by the technique described recently in ref *11*. To obtain native S1, we rapidly poured 9 volumes of distilled water, buffered at pH 7.0 by 50 mM imidazole, into 1 volume of glyceroled myosin. The diluted filaments were centrifiged at low speed (25 000 rpm) for around 30 min, and the pellets containing the myosin filaments were resuspended in the digestion medium (composition in ref *14*): 120 mM NaCl, 20 mM NaPi, 2 mM EDTA, pH 7.0; we added 2 mM DTT. The filaments (myosin around 10−12 mg/mL) were digested according to ref *11* to prepare minimally modified native myosin and RLC-free S1 (the absence of RLC is clearly indicated in Figure 1) (see also ref *5* and Supporting Information). We obtained 10 cc RLC-free S1 at around 3−4 mg/mL. Note that, for example, chromatography on myosin and S1 cannot be used. Therefore, other purification techniques should be used (ref *11* and Supporting Information). As described above, RLC-free S1 was digested from insoluble native myosin (10 cc at 10−12 mg/mL). To keep to a minimum impurities in S1 we digested insoluble myosin using a 1:1000 (w/w) ratio for chymotrypsin (Sigma)−myosin (room temperature 20−22 °C; digestion duration 40 min; stirring 300 rpm, in a 25 cc Becher). Digestion was stopped by adding 1% PMSF. S1 was partially purified up to 95% by ultracentrifugation (*11*).

Viscosity Measurements. Viscosity was measured in a Ubbelhode capillary using an automatic AVS 310 viscometer (Schott Geräte). The temperature was kept constant at 5.78 ± 0.01 °C, unless otherwise specified. We chose this low temperature to favor the stability of S1. All of the apparatus was placed in the cold room, the temperature of which was 5.0 ± 0.5 °C. The uncorrected and the true intrinsic viscosities were calculated as indicated in Yang and Wu (*15*) (see also ref *11*).

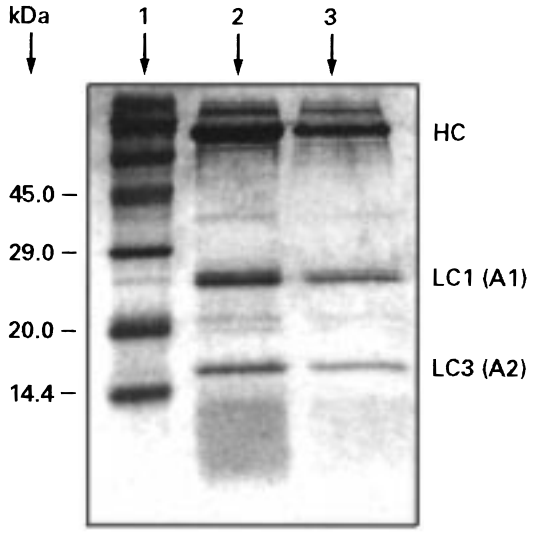

FIGURE 1: SDS−PAGE experiments on native LC2-free S1: separation gel, 15%; stacking gel 6%. Well 1: molecular weight markers. Well 2: S1 loading concentration 5 μg/mL. Well 3: 2.5 μg/mL S1. HC: heavy chain of S1. We see chymotrypsin at around 20 kDa. At 42−43 kDa we see trace amounts of actin. Our S1 is 95−97% pure (see also ref *11*).

In a first set of experiments we studied the S1 monomer in the following buffer (A): 120 mM NaCl, 50 mM BTP, 2 mM DTT, pH 7.3 at room temperature (7.5 at 5.8 °C). We determined the concentrations of S1 by OD (extinction coefficient at 280 nm: 0.75 mL/mg; ref *14*). Note that we used NaCl and not KCl: at our working temperatures, small amounts (crystals?) of KCl stick to the capillary, resulting in nonreproducible measurements of the flow durations. We also studied S1 in the following dimer buffer (B), with or without nucleotides: 38 mM NaCl, 4 mM $MgCl_2$, 50 mM BTP, 2 mM DTT, pH 7.3 at room temperature (7.5 at 5.8 °C). We used a lower ionic strength buffer to favor the dimer (*1*, *2*, *3*). Addition of 100 mM NaCl did not modify the properties of the dimer. However, at 138 mM NaCl the dimer no longer appeared at 0−2 °C (*11*). Therefore, to have coherent results we used lower ionic strength in the dimer buffers.

Laser Light Scattering. The methods used have been extensively described by Morel et al. (*5*). The index of polydispersity was around 0.1, and although very low, it is due to the 1% of heavy material in native S1 (*11*). D_t was measured at 10 °C, in buffers containing salts (mainly NaCl), and we made the usual corrections to obtain $D_t^{20,w}$ given by the following:

$$D_t^{20,w} = D_t \frac{293}{283} \frac{\eta}{\eta_{20,w}} \quad (1)$$

η is the viscosity (deduced from the Chemical Handbook) of the buffer at 10 °C, and $\eta_{20,w}$ is the viscosity of water at 20 °C. We measured $D_t^{20,w}$ for several concentrations and deduced $D_t^{20,w}(c = 0)$ by extrapolating $D_t^{20,w}(c)$ to $c = 0$ (see Results). Only $D_t^{20,w}(c = 0)$ is of interest since it corresponds to an infinite dilution (no particle interaction).

Capillary Electrophoresis. We used the HPCE apparatus described by Morel et al. (*5*), with the wavelength set at 214

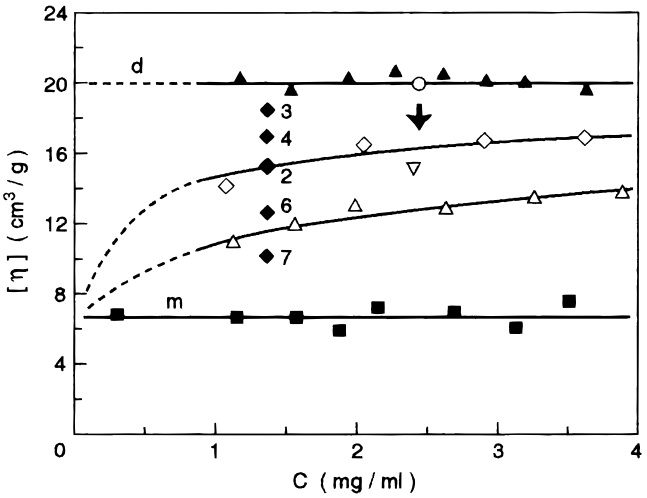

FIGURE 2: Intrinsic viscosity of S1 under various conditions at 5.78 °C. (■): intrinsic viscosity of S1 in the "monomer" buffer (A) (see Materials and Methods). The experimental points lie on a horizontal straight line corresponding to 6.7 cm^3/g and to $K' = 0$. (△): intrinsic viscosity of S1 in the "dimer" buffer (B), without nucleotides (see Materials and Methods). The experimental points fit a curve as follows. According to Tanford (*27*) and Burke and Harrington (*28*) and since $K' = 0$, $[\eta]_{app} = [\eta]_m + ([\eta]_d - [\eta]_m)(c_d/c)$, where $[\eta]_m = 6.7$ cm^3/g and $[\eta]_d = 19.8$ cm^3/g (see main text), c_d/c is the proportion of dimer, given by $c_d/c = 1 - 2/[1 + (1 + 4Kc)^{1/2}]$ (ref *1*) and the value of K corresponding to the best fit is $K = 0.7$ mL/mg. Note that, in the presence of magnesium alone (2 mM), a pure monomer was observed above 6−8 °C and a pure dimer at 0−2 °C (*11*). Here we used an intermediate temperature. (◇): intrinsic viscosity of S1 in buffer (B) + 2 mM ADP where the best fit gives $K = 3.7$ mL/mg. (▲): viscosity in buffer (B) + 4 mM AMP-PNP. (○): viscosity in buffer (B) + 2 mM ATP; only 3 measurements were made at the beginning of the experiments (after 3 measurements, the viscosity began to decrease, owing to the splitting of ATP into ADP + Pi). (▽): same experiment as (○), but at the end of the run, corresponding to buffer (B) + 2 mM ADP + 2 mM Pi. The arrow indicates the decrease in viscosity, due to the disappearance of ATP. (◆): intrinsic viscosity in buffer (B) + 2 mM ADP versus the age of the S1 solution (age is indicated by numbers corresponding to the numbers of days after digestion). For these experiments we added 1 mM NaN_3 to the S1 solution and kept the solution in the viscometer for 7 days. It appears that S1 solutions more than around 50 h old are unsatisfactory (best age: 10−15 h after the beginning of digestion).

nm, the voltage at 20 kV, and the injection duration at 8 s. The current was, in all cases, 115−120 μA. We used two buffers for S1: "monomer" buffer, 50 mM NaCl, 50 mM BTP, pH 7.6 (at room temperature), 2 mM DTT; and "dimer" buffer, 38 mM NaCl, 4 mM $MgCl_2$, 50 mM BTP, pH 7.6 (at room temperature), 2 mM DTT. S1 was dialyzed for only 2 × 3 h against these buffers in the cold room and was used within 15−16 h after digestion. Capillary electrophoresis was performed at 4.0 ± 0.1 °C.

RESULTS

Viscometry. The intrinsic viscosities of S1 solutions were measured at a fixed temperature of 5.78 °C (Figure 2). First we studied the intrinsic viscosity of S1 in the monomer buffer (A). We obtained $[\eta]_m = 6.7$ cm^3/g and $K' = 0$ (Huggins coefficient), in good agreement with previous determinations (*15*, *16*). Second, we studied the intrinsic viscosity in the dimer buffer (B), in the presence or absence of nucleotide. In the absence of nucleotide, we confirmed the findings of Morel and Garrigos (*1*) and Grussaute et al. (*11*) that Mg^{2+} alone is able to induce dimerization (see Figure 2). We

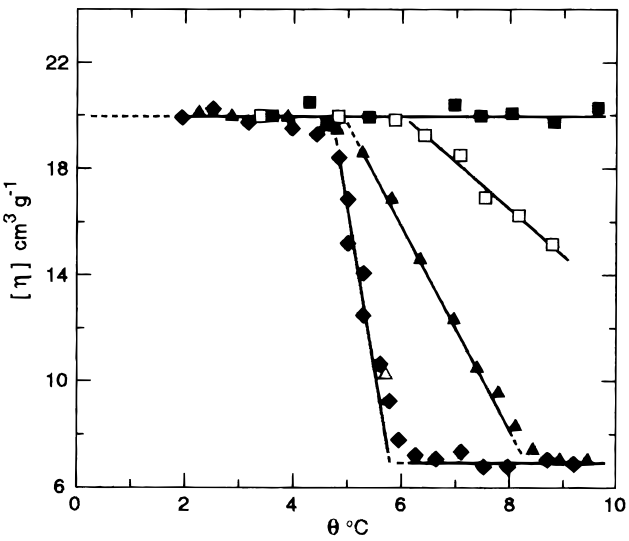

FIGURE 3: Intrinsic viscosity of S1 versus the temperature θ. The conditions were the following: 38 mM NaCl, 4 mM $MgCl_2$, 2 mM DTT, 50 mM BTP, pH 7.7 (at 5 °C). The open triangle corresponds to the absence of nucleotide: (◆) AMP-PNP 0.5 mM, (▲) AMP-PNP 2 mM, (□) AMP-PNP 6 mM, (■) ATP 6 mM. Note that the maximum value of the intrinsic viscosity (19.8 cm^3/g) is independent of the temperature and the composition of the medium and corresponds to a pure dimer (see text). Note also that the temperature transitions depend on the concentration of nucleotide. All the experiments were carried out at an S1 concentration of 1.5 ± 0.2 mg/mL.

repeated the experiments in the presence of 2 mM ADP, to allow comparison with results reported by Morel and Garrigos (*1*), who found that S1 was in the form of a pure dimer under these conditions. Here, we found that the presence of 2 mM ADP in buffer (B) increased the intrinsic viscosity, but we did not find a pure dimer. This particularly important point is discussed below. We also studied the intrinsic viscosity in buffer (B) + 4 mM AMP-PNP. At all S1 concentrations, and within the limits of the experimental error, we obtained the same value for the intrinsic viscosity, corresponding most probably to the pure dimer, $[\eta]_d = 19.8$ cm^3/g (with $K' = 0$). We confirmed (see below) that under these conditions S1 is a pure dimer. We performed other series of experiments. (i) We studied the intrinsic viscosity of S1 in buffer (B) + 2 mM ATP. The intrinsic viscosity was again 19.8 cm^3/g. During the course of the experiments, ATP was split into ADP + Pi and we could follow the progress in the hydrolysis as a decrease in the intrinsic viscosity. The final value of the intrinsic viscosity was only slightly lower than the values obtained in the presence of 2 mM ADP. The difference was probably due to the presence of 2 mM Pi, which very likely competes with ADP at the dimerization site, thereby partially inhibiting dimerization. (ii) Bachouchi et al. (*17*) have shown that the MgATPase activity of S1 varies with the age of the protein. There is first an increase in the MgATPase activity, corresponding to an irreversible oligomerization, and then a steep decrease. We studied this problem in buffer (B) + 2 mM ADP, by viscometry. We observed the same phenomenon (Figure 2): first an increase in the intrinsic viscosity and then a steep decrease. Note that the variations in the intrinsic viscosity as a function of the age of S1 are both qualitatively and quantitatively comparable to those in k_{obs} versus the age of S1. The two kinds of behavior may both correspond to a

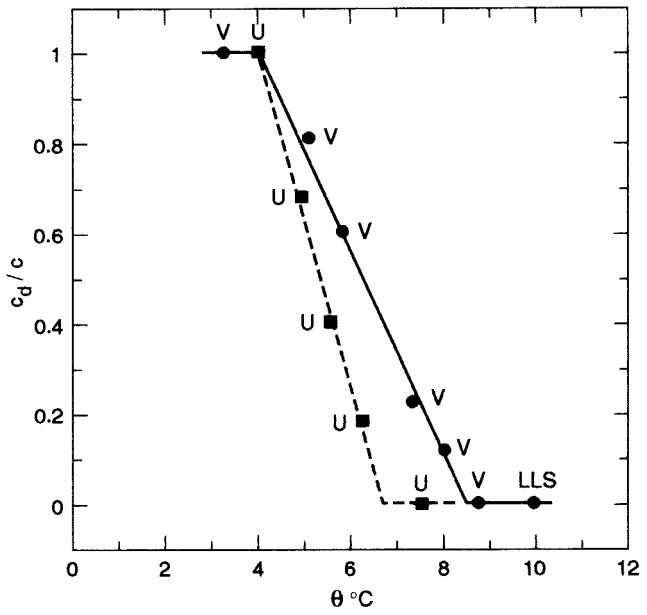

FIGURE 4: Proportion of dimer c_d/c versus the temperature θ, in buffer (B) + 2 mM ADP: V = viscometry, U = equilibrium ultracentifugation, LLS = laser light scattering (see Figure 4; same symbol (●) for the three series of experiments V and LLS). The S1 concentration was 1.5 ± 0.2 mg/mL, except for U (0.38 mg/mL; symbol ■). Note the coherence of the results obtained by different techniques. Note also that the present results are consistent with the observation by Morel and Garrigos (*1*) of monomer−dimer mixtures or a pure dimer, at 20 °C, in the same buffer and in the ultracentrifuge: the hydrostatic pressure in the ultracentrifuge is sufficient to displace the monomer−dimer equilibrium toward the dimer (*1*).

rapid formation of irreversible oligomers, followed by a rapid loss of the dimerization site in too old S1 solutions (say 30 h).

For technical reasons, we could not perform laser light scattering experiments below 10 °C. Therefore, before carrying out these experiments, we measured the intrinsic viscosity of S1 at various temperatures. There appeared to be temperature transitions in the monomer−dimer equilibrium (Figure 3). For instance, in buffer (B) + 0 or 0.5 mM AMP-PNP, there was a large decrease in the intrinsic viscosity when the temperature increases from about 5 to 6 °C. As found by Yang and Wu (*15*), the intrinsic viscosity of the monomer was independent of the temperature and the same was true for the dimer (see Figure 3). Therefore, the sharp decrease in the intrinsic viscosity was presumably due to the dissociation of the dimer into monomers. This phenomenon is most likely related to conformational changes in the subunits, which lead to an inhibition of the dimerization site. On decreasing the temperature, the dimer reappeared. Similar results have also been reported by Grussaute et al. (*11*). We then measured the proportion of dimer, at various temperatures, in buffer (B) + 2 mM ADP, which is the dimer buffer used by Morel and Garrigos (*1*) and Munson et al. (*7*). Again, small temperature changes had large effects (see Figure 4). Thus the exact temperature at which the experiments are carried out has major consequences on the results as also shown in the presence of AMP-PNP (see above).

At higher AMP-PNP concentrations, the temperature transition occurred at higher temperatures and was less steep (Figures 2 and 3). To have a pure dimer at 10 °C, the MgAMP-PNP concentration would have to be above 6 mM. However, for economy we replaced AMP-PNP by ATP (which is less expensive). Also, at a given concentration and temperature, MgATP is more efficient than MgAMP-PNP in inducing dimerization (see Figure 3; MgAMP-PNP can be compared to MgADP). In buffer (B), we added 6 mM ATP. MgATP is in large excess, and its concentration should remain noticeably high during the course of both the viscosity and the laser light scattering experiments. The MgATPase activity of S1 in the dimeric form and at 20 °C is 0.047 s^{-1} (*16*) and can be estimated to be around 0.018 s^{-1} at 10 °C ($Q_{10} \approx 2.6$, unpublished results). For a S1 concentration of ≈1.5 mg/mL (≈14 μM) and a MgATP concentration of 6 mM, we get $t_{1/2} \approx 6$ h (maximum time for total splitting of MgATP). The maximum duration of the viscosity and laser light scattering experiments being around 45 min, the loss of ATP is only about 1 mM. The concentration of ATP hydrolyzed being around 1 mM, the concentration of MgATP remains near 4−5 mM throughout the experiments. Under these conditions, we obtained constant values for the intrinsic viscosity (19.8 cm^3/g) up to 10 °C (Figure 3), indicating that the intrinsic viscosity of the dimer (see below) is independent of the temperature, as in the case for the monomer (*15*).

Laser Light Scattering. The question arises as to whether an intrinsic viscosity of 19.8 cm^3/g corresponds to a pure RLC-free S1 dimer. We used the laser light scattering technique described under Materials and Methods and in Morel et al. (*5*) to study $D_t^{20,w}$ for both the S1 monomer (buffer A) and the S1 dimer (buffer B + 6 mM ATP). The results giving $D_t^{20,w}(c)$ are shown in Figure 5. For the S1 monomer, we have $D_t^{20,w}(c = 0) = 4.43 \times 10^{-7}$ cm^2/s and, for the S1 dimer, $D_t^{20,w}(c = 0) = 2.54 \times 10^{-7}$ cm^2/s. These values correspond to a pure monomer and to a pure dimer, respectively. Indeed, the Svedberg equation can be written:

$$M_r = \frac{RTs_{20,w}(c = 0)}{D_t^{20,w}(c = 0)(1 - \rho\bar{V})} \qquad (2)$$

Morel and Garrigos (1) have shown that, for the monomer, $s_{20,w}(c = 0) = 5.05$ S and that, for the dimer, $s_{20,w}(c = 0) = 6.05$ S. On the other hand, Garrigos et al. (*18*) have shown that $\bar{V}_m = 0.743$ mL/g (20 °C), that is, $\rho\bar{V}_m = 0.742$. Introducing the above value of $D_t^{20,w}(c = 0)$ for the monomer, we get $M_{r,m} = 108\,000$ Da, in good agreement with previous determinations (107 000 Da; refs *1*, *18*). Morel and Garrigos (*1*) found that, upon dimerization, there is a noticeable decrease in $\bar{V}$ and that $\bar{V}_d = \bar{V}_m - 0.015 = 0.728$ mL/g, that is, $\rho\bar{V}_d = 0.727$. Introducing $D_t^{20,w}(c = 0)$ for the dimer, we get $M_{r,d} = 213\,000$ Da, which corresponds to a pure dimer and which is in agreement with $2 \times 108\,000 = 216\,000$ Da and with 215 000 Da (*1*). These experiments and calculations clearly demonstrate that the S1 dimer exists and that, in buffer (B) + 6 mM ATP, S1 is in the form of a pure dimer. In this buffer, the intrinsic viscosity is 19.8 cm^3/g (see Figures 2 and 3), and we deduce that $[\eta]_d = 19.8$ cm^3/g.

Capillary Electrophoresis. The results obtained by capillary electrophoresis in the monomer and dimer buffers are shown in Figures 6 and 7. In Figure 6 (monomer buffer) two clearly resolved peaks are seen, corresponding to

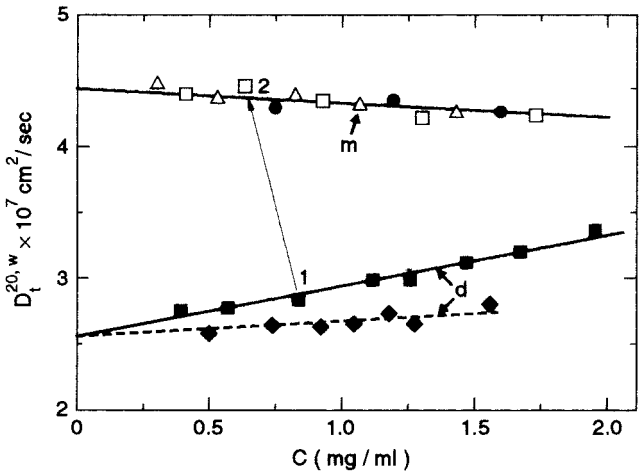

FIGURE 5: Translation diffusion coefficients of the S1 monomer (m) and dimer (d). The S1 monomer was studied in buffer (A) and the dimer in buffer (B) + 6 mM ATP. For the monomer $D_{t,m}^{20,w} = 4.43 \times^{-7}(1 - 0.020c)$ cm^2/s (ionic strength around 40 mM). The slight negative slope indicates that the monomers (m) are in weak attraction. For the dimer $D_{t,d}^{20,w} = 2.54 \times 10^{-7}(1 + 0.143c)$ cm^2/s. The marked positive slope indicates that, under the conditions used (ionic strength around 50 mM), the dimers (d) are in strong repulsion. As pointed out by Pusey (*29*) an increase in the ionic strength should reduce the slope (increase in the screening effects), but extrapolation to $c = 0$ should be independent of the buffer conditions. This fact was verified by experiments (◆) in buffer (B) + 6 mM ATP + 30 mM NaCl (ionic strength around 80 mM), where $D_{t,d}^{20,w} = 2.54 \times 10^{-7}(1 + 0.048c)$ cm^2/s. The points (□) were obtained in buffer (B), whereas the points (●) were obtained in buffer (B) + 2 mM ADP and correspond to a pure monomer ($K = 0$) at 10 °C. At 5.78 °C, under the same conditions, K was 3.7 mL/mg (Figure 2). This confirms the steep decrease of K with temperature (Figure 2). Points (△) correspond to S1 previously concentrated by ammonium sulfate and purified by HPLC (Bio-Sil TSK 350 column; gel filtration) studied in the dimer buffer (B) + 6 mM ATP. We see that the dimerization site is lost (pure monomer: we confirm that chromatography leads to a modified S1; see main text and Supporting Information). Point "2" corresponds to the addition of 5 mM ATP to buffer (B) + 6 mM ATP (point "1") and indicates the disruptive power of free ATP on the dimer (2).

retention times $t_r^{A2} = 32.61 \pm 1.21$ min (±SD; $n = 5$) and $t_r^{A1} = 34.93 \pm 1.17$ min (±SD; $n = 5$). Moreover, two other peaks, extremely close to the A2 and the A1 peaks, respectively, are also seen and they correspond to $t_r^{A2'} = 31.78 \pm 0.91$ min (±SD; $n = 5$) and to $t_r^{A1'} = 33.88 \pm 0.99$ min (±SD; $n = 5$). The different ratios are $t_r^{A1'}/t_r^{A1} = 0.9699 \pm 0.0052$, $t_r^{A2}/t_r^{A1} = 0.9336 \pm 0.0117$, and $t_r^{A1}/t_r^{A2} = 1.0711 \pm 0.0117$. As we shall see under Discussion, the peaks A1 and A2 correspond to S1(A1) and S1(A2) respectively and the subpeaks A1′ and A2′ to presumably two isoenzymes of S1(A1) and S1(A2), S1(A1′) and S1(A2′). As shown by Morel et al. (*5*), two dominant isoenzymes a and b of the whole native myosin molecule can be detected by capillary electrophoresis and correspond to rapid and intermediary myosin isoenzymes. Most likely A1 and A1′ on the one hand and A1 and A2′ on the other correspond to the two dominant myosin isoenzymes. There is no consensus about the proportion of A1 and A2 in myosin. Note that it is widely suggested from SDS−PAGE experiments that the amount of A1 is around twice that of A2 (e.g., Figure 1). In our HPCE study, A1 (and A1′) appears to be more abundant than A2 (and A2′) (see peak areas in Figures 1 and 7).

In the dimer buffer three clearly resolved peaks were obtained (Figure 7): peak A1, with $t_r^{A1} = 45.15 \pm 1.21$ min (±SD; $n = 5$); peak A2, with $t_r^{A2} = 47.08 \pm 1.36$ min (±SD; $n = 5$); and peak D with $t_r^{D} = 43.23 \pm 0.98$ min (±SD; $n = 5$). Note the reversal of peaks A1 and A2, as compared to those in the absence of magnesium (Figure 6; the presence of magnesium modifies the electro-osmotic flow; see ref *5*). The different ratios are $t_r^{A1}/t_r^{A2} = 0.9590 \pm 0.0203$ and, for example, $t_r^{D}/t_r^{A2} = 0.9182 \pm 0.0312$. A1′ and A2′ were not clearly resolved, although, in the main figure, peaks A1 and A2 are significantly asymmetrical (this fact is more obvious in the inset). Peak D is a single peak and very likely corresponds to a heterodimer S1(A1)−S1(A2) and/or S1(A1′)−S1(A2′). In the case of a mixture of heterodimers D and D′, the resolution of the technique would be too low for resolving D and D′ peaks. We cannot dismiss the possibility of a single heterodimer [S1(A1) + S1(A1′)]−[S1(A2) + (S1(A2′)]; the molecular weight of the dimer being twice that of the monomer, this is the most probable composition of the dimer. As described in the legend to Figure 7, the S1 loading concentration for the main trace was 2.3 mg/mL. To check that peak D corresponds to a dimer (at least an oligomer), in equilibrium with the monomers, it was necessary to verify that the area of peak D decreased on increasing S1 concentration. This was done by carrying out experiments at a S1 loading concentration of 0.6 mg/mL. A typical trace is shown in the inset in Figure 7: the D peak area is considerably lower than those of A1 and A2 (in the main figure, the three areas are comparable). Therefore D corresponds to a heterodimer (hetero-oligomer?) in reversible equilibrium with the monomers (mass action law). However, as pointed out by Morel et al. (*5*), we do not know the reason for the clear separation between the dimer and the monomers.

DISCUSSION

Interpretation of the Capillary Electrophoresis Experiments. The aim of this discussion is to assess whether the different assignments of the peaks we suggest under Results are reasonable. Let us first study Figure 6. The S1 monomer being a mixture of S1(A1) and S1(A2) in a ratio chosen at 2:1 (traditional SDS−PAGE and our results), we can write: $D_t^{app} = (2/3)D_t^{A1} + (1/3)D_t^{A2}$. Assuming that the degrees of hydration and shapes of S1(A1) and S1(A2) are comparable, we can write $D_t^{A1}/D_t^{A2} \approx (M_r^{A2}/M_r^{A1})^{1/3}$. SDS−PAGE experiments show that the molecular weights of S1(A1) and S1(A2) are 112 000 and 106 000 Da, respectively (*13*) from which we deduce $D_t^{A1}/D_t^{A2} = 0.982$. As for the apparent charges on S1(A1) and S1(A2), we shall assume, as a first approximation, that the net charges are proportional to M_r. Thus, we can write $Z_{app}^{A1}/Z_{app}^{A2} \approx M_r^{A1}/M_r^{A2} = 1.057$ (this estimation is very rough) we can deduce that $t_r^{A1}/t_r^{A2} \approx (Z_{app}^{A2}/Z_{app}^{A1})(D_t^{A2}/D_t^{A1}) \approx 0.963$, as compared to 1.0711 found experimentally. Therefore, we confirm that peaks A1 and A2 should appear almost simultaneously, but we cannot predict the exact order of appearance of A1 and A2. It is possible that $Z_{app}^{A1} \approx Z_{app}^{A2}$. In this case, we find $t_r^{A1}/t_r^{A2} \approx 1.037$, which is a good prediction (experimental value of 1.0711; see above).

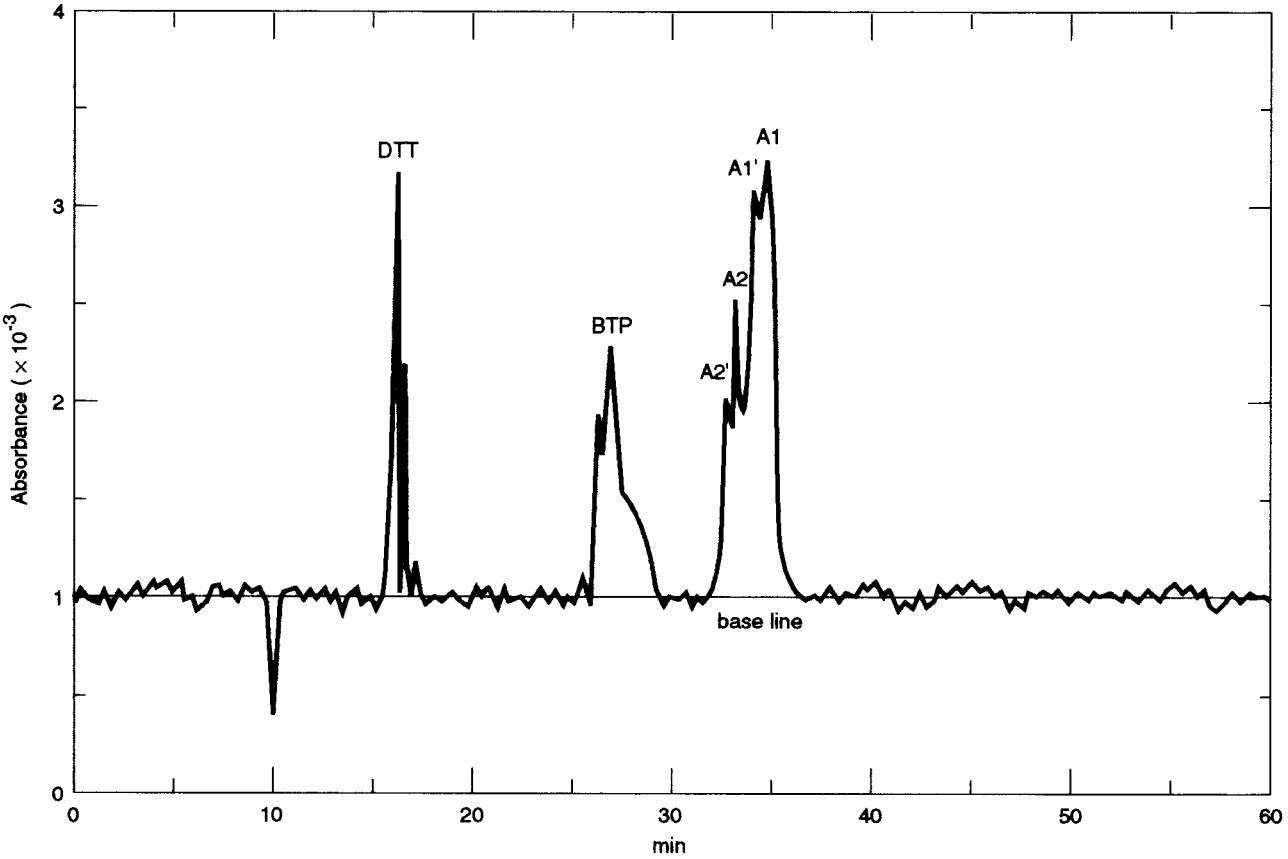

FIGURE 6: Capillary electrophoresis of S1 (loading concentration 2.5 mg/mL) in a monomer buffer (see main text for composition). Before the experiments we carefully equilibrated the capillary under voltage with a freshly prepared monomer buffer (for more details see ref *5*). Both the blank and the protein buffer are rather noisy, in sharp contrast with what was observed at 280 nm (*5*). Fortunately, it was possible to smooth the trace by substracting the blank (the protein-loaded buffers exhibit approximately the same noise level as the blank). The negative peak at a retention time around 10 min corresponds to α-chymotrypsin treated by PMSF. Note that we found the same time of retention when chymotrypsin was present in the blank. However, this "blank" peak was positive, for unknown reasons. Although the blank is substrated, we see a double peak corresponding to DL-DTT and two peaks corresponding to BTP (which possesses two p*K*'s). This indicates that the composition of the buffer is slightly different outside and inside the dialysis membrane tubing (a few percent; osmotic effects; Donnan equilibrium).

Let us now turn to Figure 7. We found that $D_{t,m}^{20,w}(c = 0) = 4.43 \times 10^{-7}$ cm^2/s and $D_{t,d}^{20,w} = 2.54 \times 10^{-7}$ cm^2/s. From the above relations between D_t^{app}, D_t^{A1}, and D_t^{A2}, we deduce $D_{t,A1}^{20,w}(c = 0) = 4.40 \times 10^{-7}$ cm^2/s and $D_{t,A2}^{20,w}(c = 0) = 4.48 \times 10^{-7}$ cm^2/s. On the other hand, for the heterodimer S1(A1)−S1(A2), we can assume that $Z_{app}^{D} \approx Z_{app}^{A1} + Z_{app}^{A2}$. We deduce that $t_r^D/t_r^{A2} \approx [Z_{app}^{A2}/(Z_{app}^{A1} + Z_{app}^{A2})](D_{t,A2}^{20,w}/D_{t,D}^{20,w})$. We have assumed above that $Z_{app}^{A1}/Z_{app}^{A2} \approx 1.057$. Ignoring the dependence of $D_{t,D}^{20,w}$ and $D_{t,A1}^{20,w}$ on the concentration, we deduce $t_r^D/t_r^{A2} \approx 0.890$, as compared to 0.918 found experimentally and peak D should appear before peaks A1 and A2. We have seen above that peak D corresponds to an oligomer. We see here it is a dimer, the parameters characterizing the dimer (M_r, D_t, etc.) leading to a good prediction of the time of retention for a dimer.

Arrangement of the Subunits in the Dimer. Using a technique of freeze-fracture in solution, followed by electron microscopy observations, Bachouchi et al. (*3*) found that the S1 dimer is end-to-end and that its maximum chord is 25 nm, that is, twice the maximum chord of the S1 monomer (*3*, *18*, *19*). However, Bachouchi et al. (*3*) pointed out that concluding that the dimer has an end-to-end nature "does not rule out the possibility of the presence of some amounts of side-by-side dimers or intermediary arrangements". It is possible, indeed, that the freeze-fracture technique used by these authors leads to distortions. Such distortions might result in modifications in the angle between the two subunits. The end-to-end and side-by-side arrangements would correspond to the maximum distortions. In spite of this possible difficulty, the results of these authors are valuable, since they show that the observed maximum chord of the end-to-end dimer is twice that of the monomer and that the two subunits are comparable to individual monomers. In particular, the maximum chord of the subunits is 12 nm and their axial ratio $p = 2.3$ (*3*, *18*, *19*). Morel et al. (*20*) and Morel (*21*) have shown that the rigid globular part of S1, ignoring the thinner S1/S2 joint (lever arm) is 12 nm long. Only this globular part is "visible" by hydrodynamic techniques. From neutron scattering data, the whole S1/S2 joint may be visible (*21*), but this conclusion is unclear owing to a misinterpretation of the Guinier region (*21*). Crystallized S1 contains the RLC and the S1/S2 joint becomes visible (RLC is located on this joint). Therefore, all of the apparently scattered data are compatible.

Although the freeze-fracture technique leads to valuable results, its drawback is that it does not allow the measurement of the exact angle between the subunits. The intrinsic viscosity of the dimer is 19.8 cm^3/g and its translation diffusion coefficient 2.54×10^{-7} cm^2/s. According to the

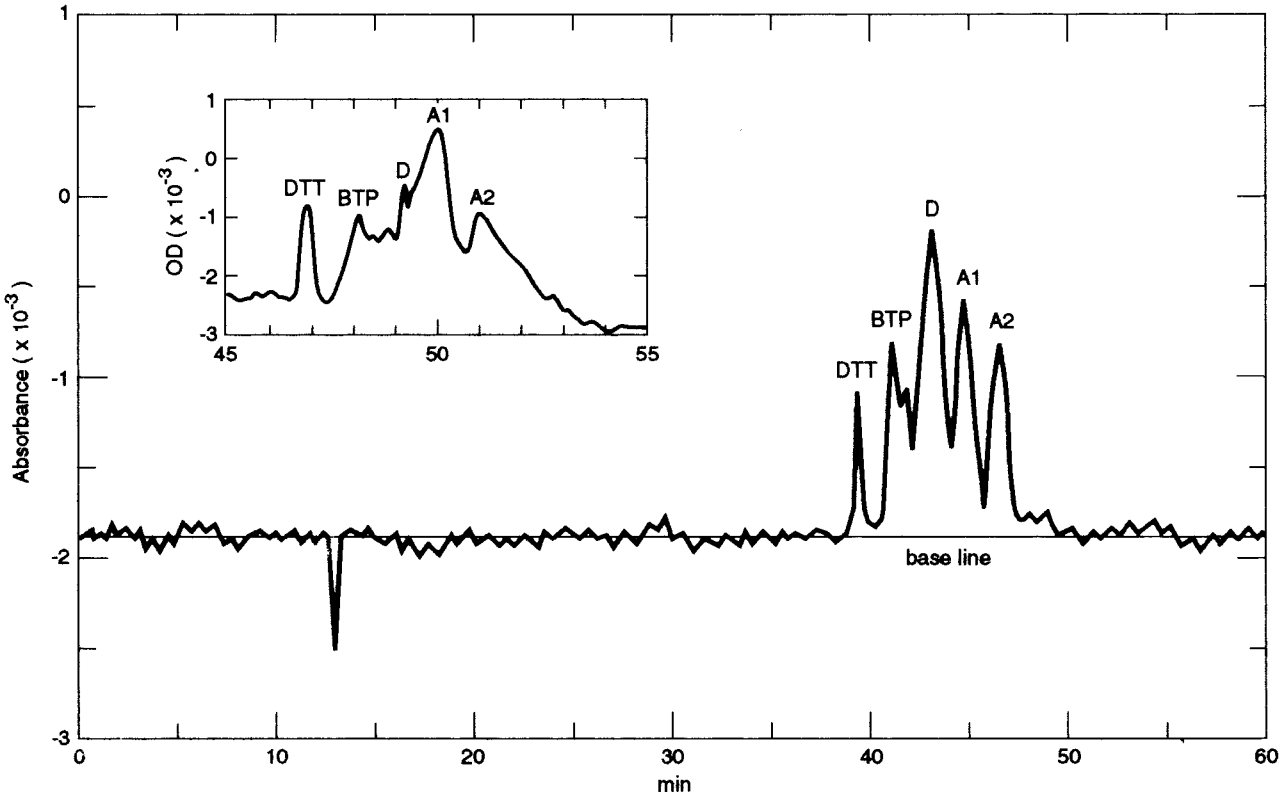

FIGURE 7: Capillary electrophoresis of S1 (loading concentration 2.3 mg/mL) in a dimer buffer (for composition see the main text). The experiments are analogous to those described in the legend to Figure 6. Inset: magnification of the trace obtained with S1 at a loading concentration of 0.6 mg/mL between 45 and 55 min. Note the considerable reduction in the peak area for D as compared with the main trace (see main text).

theoretical approach of Lopez Martinez & Garcia de la Torre (*22*) we can write

$$D_t^* = D_t 6\pi\eta_o b/k_B T \quad \text{and} \quad [\eta]^* = [\eta]M_r/N_A b^3 \qquad (3)$$

where D_t is the translation diffusion coefficient of the oligomer; b is the semi-minor axis of the hydrated subunit; η_o is the solvent viscosity; k_B, T, and N_A have the usual meanings; and M_r is the molecular weight of the oligomer. Eliminating b between these two equations gives the following:

$$M_r = 5.92 \times 10^{-15} \frac{[\eta]^* D_t^{*3}}{[\eta](D_t^{20,w})^3} \qquad (4)$$

$$[\eta] = 5.92 \times 10^{-15} \frac{[\eta]^* D_t^{*3}}{M_r(D_t^{20,w})^3} \qquad (5)$$

The interest of these equations is that they are independent of b, that is, the state and degree of hydration of the protein. Lopez Martinez and Garcia de la Torre (*22*) have calculated the reduced intrinsic viscosity $[\eta]^*$ and the reduced diffusion coefficient D_t^* as functions of the axial ratio p of the equivalent prolate ellipsoid. Garrigos et al. (*18*) have shown that the best model for the LC2-free S1 monomer in solution is a prolate with $p = 2.3$, corresponding to the rigid globule of 12 nm long. Using the tables presented by Lopez Martinez and Garcia de la Torre (*22*) and linear interpolations between $p = 2.0$ and $p = 3.0$, equation (4) leads to $M_m = 108\,000$ Da (with $[\eta]_m = 6.7$ cm^3/g and $D_{t,m}^{20,w} = 4.43 \times 10^{-7}$ cm^2/s; see Results). We confirm that the prolate is a sufficient model for the LC2-free S1 rigid part of the monomer (for a full discussion, see ref *21*). Lopez Martinez and Garcia de la Torre (*22*) have studied the cases of the end-to-end-dimer and the side-by-side dimer and the case where the subunits form an angle of 90°. By using their results and introducing $M_r = 215\,000$ Da, $[\eta]_d = 19.8$ cm^3/g, and $D_{t,d}^{20,w} = 2.54 \times 10^{-7}$ cm^2/s, we obtain the values of the angle α between the two subunits for $p = 2.3$ (the values of the reduced viscosities and diffusion coefficients for 2.0 and 3.0 were previously calculated):

$$\alpha = 51.14([\eta]_d - 17.9)^{0.962} \qquad (6)$$

$[\eta]_d = 19.8$ cm^3/g and equation (6) leads to $\alpha = 95°$. The length of the myosin head (observed on a whole myosin molecule by electron microscopy) is around 19 nm, including the S1/S2 joint of 7−8 nm (*23*). Using the same reasoning as above, the value of the angle between the subunits is 100°. Therefore, the value of the angle is almost independent of the length of the head. We will not discuss the various data concerning S1 conformations. This was available in refs *20* and *21*. In any case the angle between the subunits is around 90−100°.

By viscometry, capillary electrophoresis, and laser light scattering, we confirm that the LC2-free S1 dimer exists. Morel et al. (*5*) have also shown that myosin can undergo head-to-head dimerization (LC2 present). Therefore, dimerization is a *genuine* property of the myosin heads. We studied various parameters and found that temperature has a very large effect; the dimer does not form at high

temperatures. The exact position of the temperature transition depends upon the nature of the buffer. Using ^{31}P-NMR, Shriver and Sykes (*24*) found that at 0 °C S1.MgADP gives rise to two clearly resolved peaks. At 25 °C, only one peak is observed. Since we have shown that S1 is in the dimeric form at 0 °C and in the monomeric form at 25 °C, we think the observation by Shriver and Sykes (*24*) could be related to dimerization. Other experimental work has corroborated and extended their results (see ref *25* and references therein). By means of proteolysis, Redowicz et al. (*26*) have shown that conformational transitions in the myosin heads are induced by temperatures between 0 and 25 °C. They digested S1 in a buffer of composition comparable to that of our dimer buffers, and we deduce that at 0 °C S1 is in the dimeric form, whereas it is in the monomeric form at 25 °C, leading possibly to different digestion products at the two temperatures. S1 dimerization and myosin head−head dimerization are important phenomena, probably involved in the structure of synthetic and native filament (*2*). We also found isoforms of S1(A1) and S1(A2) (two dominant isoforms as was the case for whole myosin). Our purpose is not to study these isoforms in detail, but it is highly probable that the two isoforms of myosin are found in the rod and in the heavy chains of S1. One may assume that, very probably, the two alkali light chains A1(LC1) and A2-(LC3) also present two isoforms each. Finally RLC is not quantitatively involved in the dimerization process.

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SUPPORTING INFORMATION AVAILABLE

A full list of precautions to be taken routinely when preparing functional LC2-free S1 (and also Mg.S1?) (13 pages). In the past years, these precautions were not systematically applied and the consequences included widely diverging experimental results between the authors. Ordering information is given on any current masthead page.

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