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Structure of the actin–myosin complex in the presence of ATP

(electron microscopy/negative staining/crosslinked actomyosin/cross-bridge cycle/arrowhead structure)

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ABSTRACT **The structure of the complex between actin and myosin subfragment 1 (S1), designated the acto-S1 complex, in the presence of ATP was examined by electron microscopy. This was accomplished by using negative staining to study a complex of S1 covalently crosslinked to actin by the zero-length crosslinker, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide. Two levels of S1 binding were studied, with a molar ratio of crosslinked S1 to total actin of either 20% or 50%. The lower percentage was used to observe individual S1 molecules attached to actin, while the higher percentage was used to look at the overall pattern of S1 decoration of the actin filament. In the absence of ATP, the appearances of both the 20% and 50% crosslinked filaments closely resembled the rigor appearances obtained with noncrosslinked proteins. The arrowheads observed had the conventional structure, and individual S1 molecules were elongated and curved and appeared to make an angle of 45° with the thin filament. Addition of ATP to the crosslinked acto-S1 complex caused a radical change in the structure of the cross-bridges. At both 20 and 170 mM ionic strengths, individual S1 molecules appeared to be attached at variable angles which, in contrast to rigor, did not center on 45°. In addition, the S1 molecules often appeared shorter and fatter than in rigor. The 50% crosslinked acto-S1 preparation no longer showed the arrowhead pattern of S1 decoration but instead appeared to be disordered with little obvious polarity. Control experiments with ADP suggest that these effects were not due simply to a weakening of the binding of S1 to actin in the presence of nucleotide but most likely were ATP-specific. The crosslinked acto-S1 complex, which hydrolyzes ATP at about the same rate as the maximal actin-activated ATPase of S1 (V_{max}), is composed of a mixture of states A·M·ATP and A·M·ADP·P_i (in which A = actin and M = myosin), with more than 50% of the crosslinked S-1 occurring in state A·M·ATP. Therefore, it appears that both states A·M·ATP and A·M·ADP·P_i have a very different conformation from the classic arrowhead conformation of the A·M state.**

Contraction of muscle results from the sliding of the thick (myosin-containing) filaments past the thin (actin-containing) filaments. The sliding force is generated by cyclic interaction between the myosin heads (the cross-bridges) and actin subunits of the thin filament. With each cycle of interaction between a myosin head and actin, one molecule of ATP is hydrolyzed, providing the energy for sliding. Although it has long been thought that the structural changes in the cycle involve an attachment of the cross-bridge to actin, a change in the angle of attachment (providing the driving force) followed by detachment, and then reattachment to actin at a different point (1–3), clear evidence for such a scheme remains scant. In general, cross-bridges in the absence of ATP have been observed to bind to actin at a uniform angle of approximately 45° (4, 5), with a direction of tilt such that the actin-binding end of the cross-bridge points towards the center of the sarcomere (4). Recent studies do suggest that when geometric constraints occur (e.g., in the filament lattice) rigor cross-bridges may be distorted from this basic form (6, 7). In any event, the rigor state is thought to be related to the structure of the cross-bridge at the end of the power stroke, when the cross-bridge has lost the products of ATP hydrolysis.

Evidence for the structure of the attached cross-bridge at other stages of the cycle has been difficult to obtain. One of the most direct approaches would be to use electron microscopy to study actin filaments that have been decorated with subfragment 1 of myosin (S1) and then negatively stained in the presence of ATP. This has not proved successful, however, owing to the low affinity of myosin heads for actin in the presence of nucleotide. The high concentrations of actin or myosin heads necessary to maintain head binding in the presence of ATP are incompatible with the low concentrations necessary for electron microscopy. Recently, however, Mornet *et al* (8) developed a method for covalently crosslinking S1 to actin using the zero-length crosslinker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. This prevents dissociation of S1 from actin even in the presence of ATP, which should allow the structure of the complex between actin and S1, designated the acto-S1 complex, to be studied by electron microscopy in the presence of ATP.

The crosslinked acto-S1 complex hydrolyzes ATP at a rate comparable to the maximal actin-activated ATPase of S1 (V_{max}), implying that the crosslinked S1 has not been denatured and is behaving like S1 that is at infinite actin concentration (8). By selected cleavage of the crosslinked acto-S1 complex, Sutoh has shown that it is the NH_2-terminal segment of actin (residues 1–12) that is crosslinked to either the 20-kDa or 50-kDa domain of S1 (9–11). Although the original crosslinking studies of Mornet *et al.* suggested that one S1 was crosslinked to two actin monomers (8), it now appears that the stoichiometry of S1 to actin in the crosslinked complex is 1:1 (11–13).

We report here on the structure of the crosslinked acto-S1 complex observed by negative staining in the absence of nucleotide and on the large scale change in structure that occurs when ATP is present.

METHODS

Actin and chymotryptic S1 were prepared from rabbit muscle by the methods of Spudich and Watt (14) and Weeds and Taylor (15), respectively.

Crosslinking of actin to S1 was carried out as described by Stein *et al.* (16), essentially by the procedure of Mornet *et al.* (8). This resulted in a ratio of crosslinking of about one S1 per five F-actin monomers ("20% crosslinked"), determined by using trace amounts of iodo[^{14}C]acetamide-modified S1 in the S1 population (16). To increase the ratio of crosslinking, the

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Abbreviation: S1, subfragment 1 of myosin.

initial acto-S1 crosslinked preparation was passed through five cycles of actin depolymerization, followed by centrifugation. This was shown not to significantly affect the ATPase activity of the crosslinked S1, but it did preferentially remove noncrosslinked actin (13). The final crosslinked preparation had an S1/F-actin monomer ratio of about 1:2 [40–50% crosslinked, henceforth called "50% crosslinked" (13)].

Crosslinked acto-S1 was negatively stained with 1% uranyl acetate by a modification of the method described by Craig *et al.* (6). A drop of crosslinked acto-S1 (≈0.1 mg of actin per ml) in rigor solution (1 mM $MgCl_2$/1 mM NaN_3/5 mM NaP_i, pH 7.0) was placed on a 400-mesh electron microscope grid coated with a holey carbon film. After 30 s the grid was rinsed with the appropriate solution—rigor solution, high-salt ATP solution (0.15 M KCl/2 mM $MgCl_2$/1 mM ATP/1 mM NaN_3/5 mM NaP_i, pH 7.0), low-salt ATP solution (same as high-salt ATP solution but without KCl), or high-salt ADP (0.5 M KCl/5 mM $MgCl_2$/3 mM ADP/1 mM NaN_3/5 mM NaP_i, pH 7.0)—and then stained. Alternatively, the filaments were suspended directly in the appropriate solution (as just defined), a drop was applied to the grid for 30 s, the bulk of the filament droplet was drawn off with filter paper, and the grid was stained without rinsing. All procedures were carried out at room temperature (20°C) with solutions at this temperature. Staining and drying of the grids were carried out at 85–95% relative humidity to aid spreading of the stain. Filaments were observed in films of stain suspended over holes in the support film since this gave the greatest clarity. This advantage must be weighed against the fact that most of the films of stain were broken and shrank in the electron beam, causing distortion of the filaments (see Fig. 1 legend). Micrographs were recorded at a nominal magnification of ×50,000 on a JEOL 100-CX electron microscope operated at 80 kV with a 60-μm objective aperture and anticontamination cold finger.

RESULTS

In this study we crosslinked S1 to actin to observe the structure of the acto-S1 complex in the presence of ATP. Before we can accept the appearance observed with ATP, however, we must first establish that, in the absence of ATP, the crosslinked acto-S1 complex has the normal rigor structure observed with noncrosslinked proteins. A diagnostic feature of the rigor complex is the arrowhead pattern obtained when the actin filament is fully decorated with S1. The crosslinked acto-S1 preparation, in which the ratio of S1 to total actin was 1:2 (i.e., 40–50% crosslinked), was examined to determine whether it showed normal arrowheads. At this level of crosslinking we observed many filaments with some full, or almost full, arrowheads (all actin subunits in one crossover occupied by an S1), together with partial arrow-

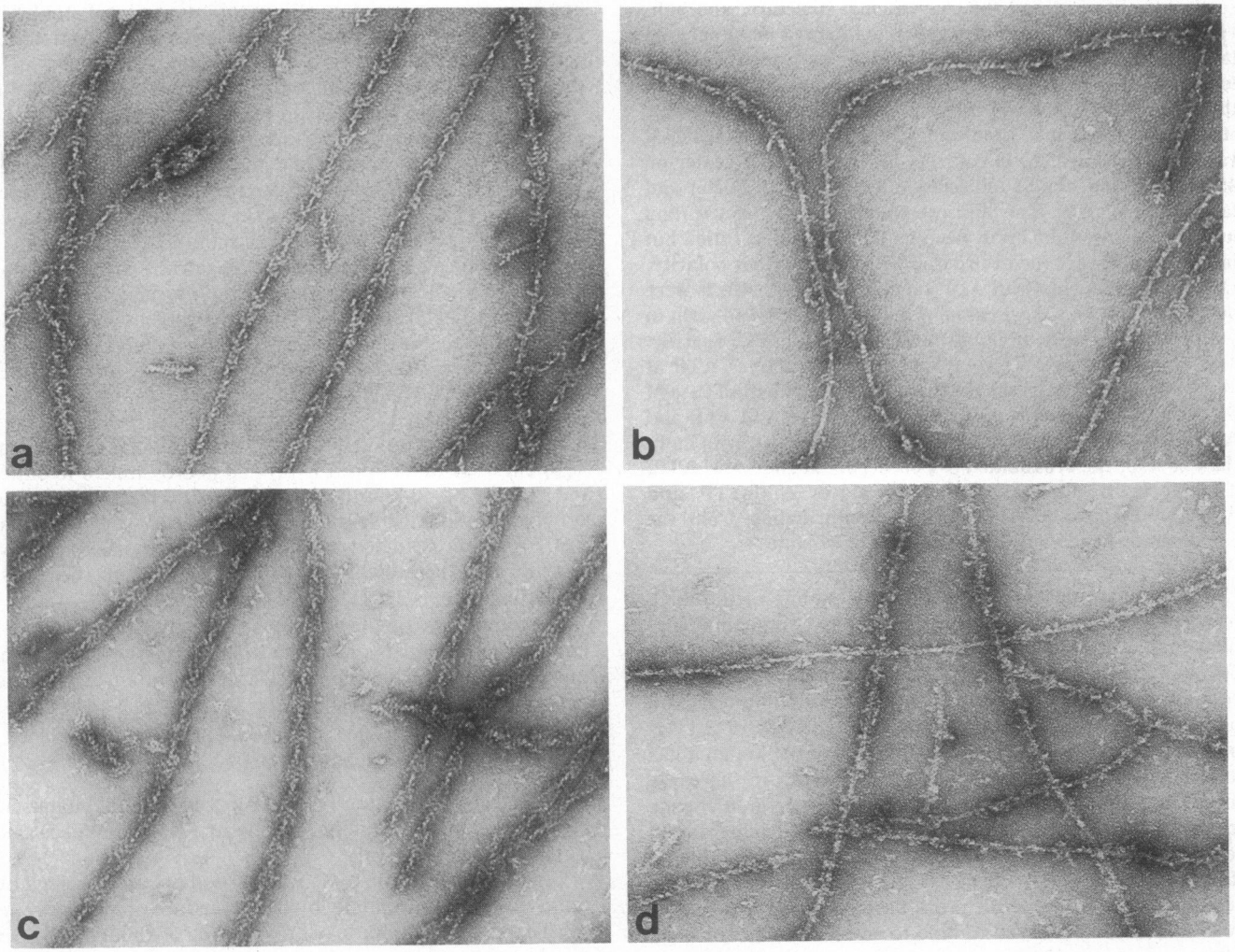

FIG. 1. Crosslinked acto-S1 negatively stained with 1% uranyl acetate. (*a*) Fifty percent crosslinked, in rigor solution (for compositions of solutions, refer to *Methods*). (*b*) Twenty percent crosslinked, in rigor solution. (*c*) Fifty percent crosslinked, treated with exogenous S1 in rigor solution (6). (*d*) Fifty percent crosslinked, treated with exogenous S1 and then with ATP. Note that although the filaments are distorted due to shrinkage of the film of stain (see *Methods*), arrowheads and angled individual heads are still clearly recognizable in the absence of ATP (*a* and *b*), and this polarity clearly disappears in the presence of ATP (see Fig. 2). (×190,000.)

heads and isolated attached S1 molecules (Fig. 1*a*). The full arrowheads have an appearance similar to the "blunt" appearance obtained when actin filaments are decorated with chymotryptic S1 (no regulatory light chain present) without crosslinking (6). In addition, the background of this and the other preparations that follow showed variable amounts of very short actin filaments with little decoration (Fig. 1*a*) and occasionally large rafts of long parallel actin filaments, separated by 10–20 nm, also with little decoration (not shown).

The appearance of individual S1 molecules crosslinked to actin also was examined to determine whether their structure resembled that obtained with noncrosslinked proteins. This was done by using a lower percentage (20%) of crosslinking, where superposition of one S1 molecule on another was greatly reduced. Many filaments in the 20% crosslinked acto-S1 in the absence of ATP showed, on average, two-to-three myosin heads bound per actin crossover repeat (Fig. 1*b*). Individual S1 molecules were attached to actin at a constant angle of approximately 45° to the filament axis and appeared to be slightly curved. This appearance is very similar to that observed with actin and chymotryptic S1 without crosslinking (6).

The similarity in the rigor appearance obtained with crosslinked acto-S1 to that obtained with noncrosslinked proteins was further shown by adding exogenous S1 to either 20% or 50% crosslinked acto-S1 to produce completely decorated actin filaments. This gave a full array of arrowheads of conventional appearance, with the crosslinked S1 being indistinguishable from the exogenous S1 (Fig. 1*c*). The addition of MgATP to these arrowheads reduced the decoration to about the level observed prior to addition of the S1 (Fig. 1*d*).

The preceding experiments indicate that, in the absence of nucleotide, crosslinking does not alter the structure of the actin or the S1 at the level of detail observable by our techniques. With this established, we examined the structure of the crosslinked acto-S1 in the presence of ATP. The addition of 1 mM MgATP to the 50% crosslinked preparation caused a clear change in the structure of the crosslinked acto-S1 (Fig. 2). Furthermore, this new structure was similar at low (20 mM) and high (170 mM) ionic strengths (compare Fig. 2 *a* and *b*). The polarity of the filaments shown by the arrowheads in rigor was generally not apparent in ATP, but instead the decoration pattern appeared to be disordered. Often it appeared that myosin heads were collapsed onto each other or onto the actin filament or that they were attached to actin at variable angles which, unlike rigor, did not center on 45°.

The appearance of individual crosslinked S1 molecules, observed on 20% crosslinked filaments, was likewise changed by the presence of ATP, at both 20 and 170 mM ionic strengths (Fig. 2 *c* and *d*). The polarity of attachment of individual heads to actin generally disappeared, with the angles of attachment of the S1 molecules being variable. Individual heads often appeared to be shorter and fatter

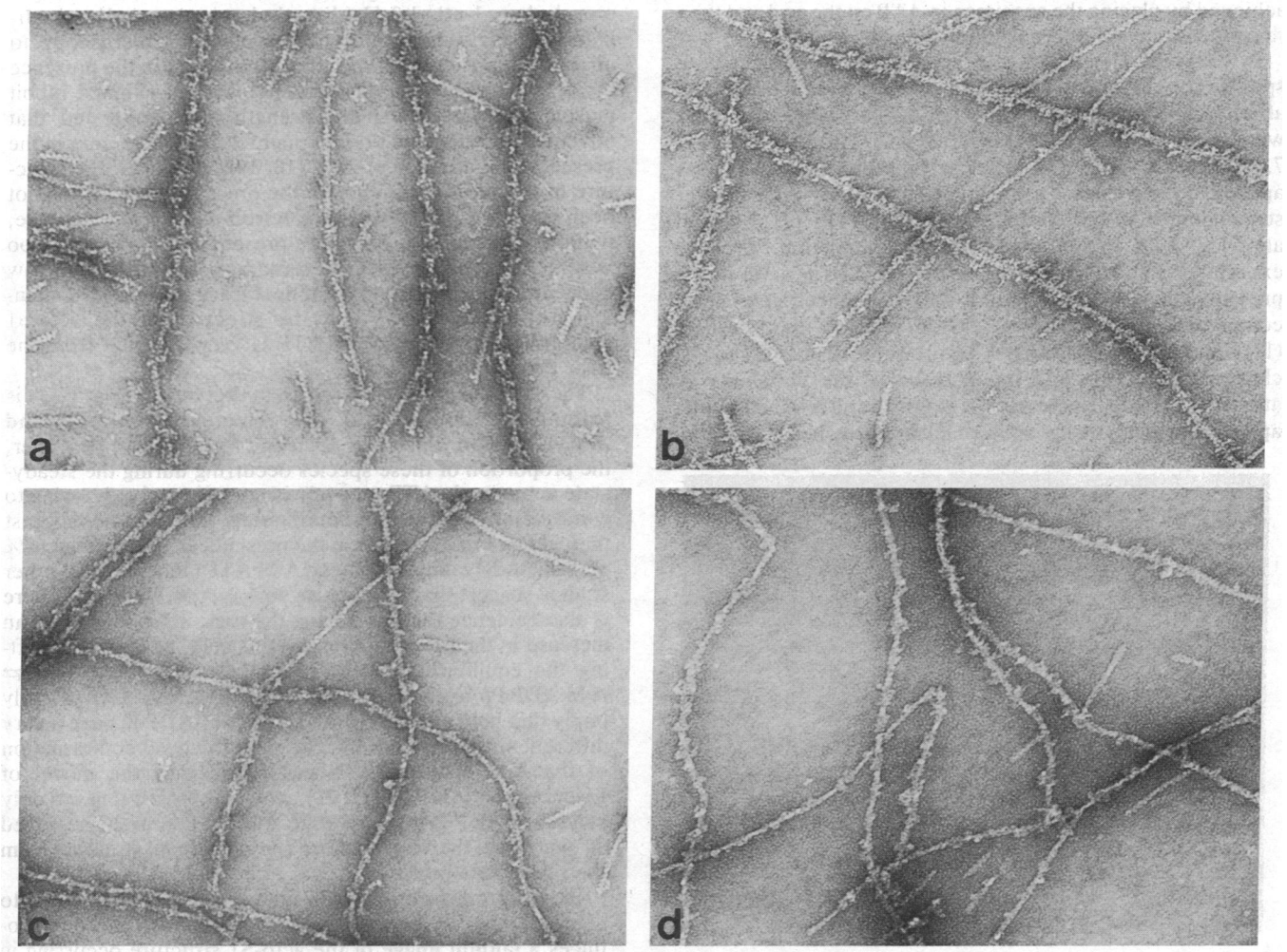

FIG. 2. Crosslinked acto-S1 in the presence of MgATP, negatively stained with uranyl acetate. (*a* and *b*) Fifty percent crosslinked, in 20 mM ionic strength (low-salt ATP solution) (*a*) and 170 mM ionic strength (high-salt ATP solution) (*b*). (*c* and *d*) Twenty percent crosslinked, in 20 mM (*c*) and 170 mM (*d*) ionic strengths. (×190,000.)

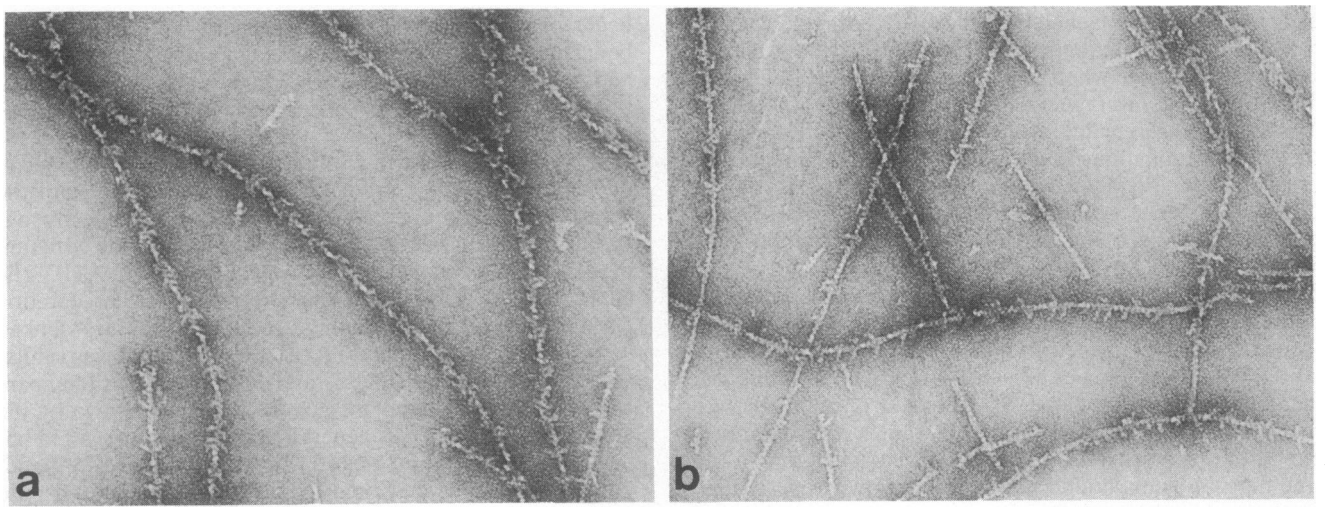

FIG. 3. Crosslinked acto-S1 negatively stained after treating with ATP (low-salt ATP solution) and allowing the hydrolysis to go to completion. (*a*) Fifty percent crosslinked. (*b*) Twenty percent crosslinked. (×190,000.)

than they did in rigor. As with the 50% crosslinked preparation, the level of decoration remained approximately the same in the presence and absence of ATP.

The ATP-induced changes in both the 20% and 50% crosslinked acto-S1 preparations were generally reversed by allowing ATP hydrolysis to go to completion before applying specimens to the grid (Fig. 3 *a* and *b*). Reversal could also be achieved by placing the specimen in ATP on the grid and then rinsing with an ATP-free solution prior to staining.

In order to check that the changes we observed were specific to ATP and did not simply reflect a reduction in the affinity of S1 for actin, both 20% and 50% crosslinked acto-S1 were stained in the presence of 0.5 M KCl/3 mM MgADP, pH 7.0 (high-salt ADP; Fig. 4). The crosslinked acto-S1 was applied to the grid after suspending it in high-salt ADP or after suspending it in 0.5 M KCl/3 mM MgATP, pH 7.0, and allowing ATP hydrolysis to go to completion. *In vitro* experiments showed that the affinity of S1 for actin in the presence of ADP and 0.5 M KCl is reduced to a level comparable to that obtained with ATP at low ionic strength (17). Under these conditions (Fig. 4), some filaments showed clumping of heads and an increase in the variability of attachment angle. However, the majority still retained a polar appearance and many showed clear arrowheads indistinguishable from those observed at low ionic strength either in the absence of nucleotide (compare Fig. 4 with Fig. 1*a*) or after all of the ATP has been hydrolyzed (Fig. 3*a*).

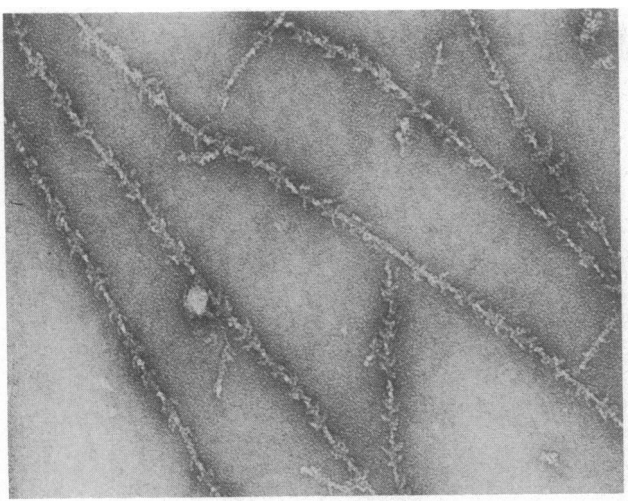

FIG. 4. Fifty percent crosslinked acto-S1 in the presence of 0.5 M KCl/3 mM MgADP/5 mM NaP_i, pH 7.0, negatively stained with uranyl acetate. (×190,000.)

DISCUSSION

By covalently attaching S1 to actin with the zero-length crosslinker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, we have been able to use electron microscopy to observe the structure of the acto-S1 complex in the presence of ATP. Recent x-ray diffraction studies of skinned rabbit muscle fibers at low ionic strength have suggested that attached myosin cross-bridges might differ in structure in the presence and absence of ATP (18, 19). However, the structure of the acto-S1 complex in the presence of ATP has not been observed previously by electron microscopy because, without crosslinking, S1 in the presence of ATP binds too weakly to actin to form a ternary complex at the low concentrations of S1 and actin necessary for negative staining. This study shows that the structure of the acto-S1 complex in the presence of ATP is very different from the rigor complex.

The crosslinked acto-S1, which hydrolyzes ATP at V_{max}, is primarily composed of the species, A·M·ATP and A·M·ADP·P_i, in which A is actin and M is myosin. However, the proportion of these species occurring during the steady-state actomyosin ATPase cycle is not yet resolved, owing to controversy over the rate-limiting step. Some studies suggest that, at low ionic strength in the presence of ATP, about 60% of the acto-S1 complex may be A·M·ATP (16, 20), while other studies suggest that as much as 90% is A·M·ATP (21). There is also evidence that increasing the ionic strength causes an increase in the proportion of A·M·ADP·P_i, possibly by shifting the equilibrium of the hydrolysis step (A·M·ATP $\rightleftarrows$ A·M·ADP·P_i) to the right (16). Therefore, our data strongly imply that both states A·M·ATP and A·M·ADP·P_i have a very different structure from the classic arrowhead conformation of the A·M state. This is consistent with the model of Eisenberg and Greene (22, 23), which proposed that not only state A·M·ADP·P_i but also state A·M·ATP is in the so-called 90° state and, therefore, differs markedly in its structure from the rigor cross-bridge.

In interpreting our electron micrographs, it is essential to consider whether negative staining with uranyl acetate produces a faithful image of the acto-S1 structure occurring in solution, and whether crosslinking has modified the structure significantly. X-ray diffraction patterns of glycerinated rigor muscle incubated with S1 (so that the thin filaments become

fully decorated) have intensity distributions similar to those of computed diffraction patterns of negatively stained conventional arrowheads (24), suggesting that the arrowheads normally observed by negative staining have a structure similar to that occurring in solution. A number of other electron microscope techniques (4, 25, 26) also provide images that are consistent with the structure deduced for the negatively stained arrowhead, further supporting the fidelity of the images. Other muscle components, such as thick filaments, also have structures in uranyl acetate that closely resemble the structures deduced from x-ray diffraction of intact muscle (27). Both the 20% and 50% crosslinked acto-S1 specimens have negatively stained appearances in the absence of ATP that are very similar to those of noncrosslinked acto-S1 (compare Fig. 1 *a* and *b* with ref. 6). Thus, it appears that uranyl acetate staining produces an image faithful to the rigor structure in solution and that crosslinking does not materially alter this structure.

Are the images of the negatively stained crosslinked acto-S1 in the presence of ATP also faithful to the structure in solution? First, there can be little doubt that before staining, all of the crosslinked S1 is bound to actin in the presence of ATP; it is not simply held near the actin by the tethering action of the crosslinker. The finding that the ATPase activity of the crosslinked acto-S1 is about equal to V_{max} shows that each crosslinked S1 spends essentially all of its time actually bound to actin just as noncrosslinked S1 would do at very high actin concentrations (20, 28). However, it still remains possible that the negative staining itself disrupts the relatively weak bonds that occur between S1 and actin in the presence of ATP, so that in our electron micrographs the S1 is simply tethered to the actin filament. We have tried to test this possibility by carrying out a control experiment in which the interaction of S1 with actin is reduced by means other than ATP. In the presence of 0.5 M salt and 3 mM MgADP, where the affinity of S1 for actin is similar to that in the presence of 1 mM MgATP at low ionic strength (17), the crosslinked acto-S1 still generally shows a fairly normal rigor appearance (Fig. 4). Although salt undoubtedly weakens the interaction of S1 with actin in a different way from nucleotide, this result does suggest, to a first approximation, that a weak interaction in conjunction with an effect of the negative staining is not the cause of the change in structure of acto-S1 induced by ATP. This view is supported by preliminary experiments done in collaboration with John Heuser using the rapid-freeze/freeze-etch technique (29). Crosslinked acto-S1 prepared by this method had an appearance in the presence of ATP similar to that which we observed by negative staining. Thus, we suggest that the disordered appearance of crosslinked acto-S1 that we observe may reflect the true structure of S1 that is bound (not tethered) to actin in the presence of ATP.

The acto-S1 structure we observe in the presence of ATP is clearly different from that in its absence. The heads appear to be more flexibly attached to actin, giving rise to a range of angles not centered on 45°, compared with the constant 45° rigor angle. In addition, the heads often appear shorter and fatter than in rigor, which may indicate an active shortening or a less-rigid structure in the S1 itself. Thus, there may be a whole range of structures of the crosslinked complex in the presence of ATP. It is of interest that no evidence has been published showing any change in the head structure of rotary shadowed myosin molecules in the presence and absence of ATP. This would suggest that the differences we observe occur only when the heads are attached to actin. It will be important to use negative staining (with its higher resolution) to study isolated molecules in order to check this point. It will also be important to use crosslinked acto-S1 to study other biochemically defined states produced, for example, by the use of ATP analogs, and, if sufficiently ordered "arrowheads" are obtained, to use three-dimensional reconstruction techniques to determine the structure of the acto-S1 complex. Crosslinked acto-S1 appears to have significant potential for answering structural questions that have, until now, proved impossible to study.

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1. Pringle, J. W. S. (1967) *Prog. Biophys. Mol. Biol.* **17,** 1–60.
2. Eisenberg, E., Zobel, C. R. & Moos, C. (1968) *Biochemistry* **7,** 3186–3194.
3. Huxley, H. E. (1969) *Science* **164,** 1356–1366.
4. Reedy, M. K., Holmes, K. C. & Tregear, R. T. (1965) *Nature (London)* **207,** 1276–1280.
5. Thomas, D. D. & Cooke, R. (1980) *Biophys. J.* **32,** 891–906.
6. Craig, R., Szent-Gyorgyi, A. G., Beese, L., Flicker, P., Vibert, P. & Cohen, C. (1980) *J. Mol. Biol.* **140,** 35–55.
7. Taylor, K. A., Reedy, M. C., Cordova, L. & Reedy, M. K. (1984) *Nature (London)* **310,** 285–291.
8. Mornet, D., Bertrand, R., Pantel, P., Audemard, E. & Kassab, R. (1981) *Nature (London)* **292,** 301–306.
9. Sutoh, K. (1982) *Biochemistry* **21,** 3654–3661.
10. Sutoh, K. (1982) *Biochemistry* **21,** 4800–4804.
11. Sutoh, K. (1983) *Biochemistry* **22,** 1579–1585.
12. Heaphy, S. & Tregear, R. (1984) *Biochemistry* **23,** 2211–2214.
13. Greene, L. E. (1984) *J. Biol. Chem.* **259,** 7363–7366.
14. Spudich, J. A. & Watt, S. (1971) *J. Biol. Chem.* **246,** 4866–4871.
15. Weeds, A. G. & Taylor, R. S. (1975) *Nature (London)* **257,** 54–56.
16. Stein, L. A., Greene, L. E., Chock, P. B. & Eisenberg, E. (1985) *Biochemistry* **24,** 1357–1363.
17. Greene, L. E. (1982) *FEBS Lett.* **139,** 233–236.
18. Brenner, B., Yu, L. C. & Podolsky, R. J. (1984) *Biophys. J.* **46,** 299–306.
19. Matsuda, T. & Podolsky, R. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2364–2368.
20. Stein, L. A., Chock, P. B. & Eisenberg, E. (1984) *Biochemistry* **23,** 1555–1563.
21. Rosenfeld, S. S. & Taylor, E. W. (1984) *J. Biol. Chem.* **259,** 11908–11919.
22. Eisenberg, E. & Greene, L. E. (1980) *Annu. Rev. Physiol.* **42,** 293–309.
23. Eisenberg, E. & Hill, T. L. (1985) *Science* **227,** 999–1006.
24. Amos, L. A., Huxley, H. E., Holmes, K. C., Goody, R. S. & Taylor, K. A. (1982) *Nature (London)* **299,** 467–469.
25. Heuser, J. E. & Cooke, R. (1983) *J. Mol. Biol.* **169,** 97–122.
26. Begg, D. A., Rodewald, R. & Rebhun, L. I. (1978) *J. Cell Biol.* **79,** 846–852.
27. Vibert, P. & Craig, R. (1983) *J. Mol. Biol.* **165,** 303–320.
28. Stein, L. A., Schwarz, R. P., Chock, P. B. & Eisenberg, E. (1979) *Biochemistry* **18,** 3895–3909.
29. Heuser, J. E. (1983) *J. Mol. Biol.* **169,** 155–195.