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Visualization of freeze-dried and shadowed myosin molecules immobilized on electron microscopic films

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The mica replication technique first described by Hall [5] has produced myosin molecules which were heterogeneous in appearance in terms of shadowing, decoration, contrast and background. Therefore, an alternative technique for the visualization of myosin molecules was developed: Myosin molecules are sprayed directly onto glow discharged or silicium-monoxide coated carbon filmed grids, omitting glycerol. After washing several times with distilled water, rapid freezing, and freeze-drying, the immobilized myosin molecules are visualized by shadow-casting at low temperature and at varying angles. After backing with carbon the "in situ" shadowed molecules are observed in the electron microscope. This technique has several advantages over the standard method in that it yields more reproducible results. It is potentially useful for investigating interactions of myosin binding proteins with myosin and for visualizing unshadowed myosin in the STEM.

Introduction

The visualization of filamentous proteins of high M_r, like myosin ($M_r \sim 470000$, consisting of a 140 nm long α-helical coiled-coil rod and two pear-shaped heads of some 20 nm in length and 7-10 nm in width [9]), in the electron microscope has been hampered by the fact that many of these molecules are soluble only at relatively high salt concentrations, thus making adsorption to support films and subsequent negative staining or freeze-drying impossible.

Nevertheless, using a mica replication technique first described by Hall [5], the morphology of such filamentous protein molecules has been the object of many electron microscopic studies. After slight modification, this technique has been used as the only successful method for the visualization of myosin molecules by several investigators [1, 2, 3, 6, 8, 11].

With this method a myosin solution containing 30 to 70% glycerol is sprayed onto freshly cleaved mica (either at 20 °C or precooled to − 196 °C) and subsequently dried at $p \approx 10^{-5}$ mbar and room temperature. After rotary shadowing with platinum/carbon at a very low elevation angle (5-10°) and carbon backing, the replica is floated onto distilled water and mounted on EM grids.

Depending on non-controllable conditions, this standard method has produced myosin molecules which were heterogeneous in appearance in terms of shadowing, decoration, contrast and background. Hence, a precise estimation of the exact molecular dimensions of myosin, especially the shape of the heads and the width of the rod as attempted by Elliott and Offer [2] was difficult.

Therefore, a solution of myosin molecules was sprayed and immobilized directly onto electron microscopic support films without glycerol [14]. These molecules were frozen, freeze-dried, and shadowed under controlled conditions which results in a more reproducible appearance of the shadowed molecules and the background.

Materials and methods

Myosin was isolated from chicken skeletal muscle by high ionic strength extraction and ammonium sulfate precipitation [13] followed by chromatography on DEAE-Sephadex A-50 [7] and stored at 4 °C after dialysis against 40% ammonium sulfate, 5 mM P_i, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 to 0.5 mM β-mercapto-ethanol, 1 to 2.5 mM ATP [13]. The myosin, stored at a concentration of 5 mg/ml, was dialyzed overnight against 40 mM NaCl, 3 mM NaN_3, 1 mM $MgCl_2$, 0.5 mM β-mercapto-ethanol, 5 mM P_i, *p*H 6.8, centrifuged, and resuspended in 0.7 M ammonium acetate, *p*H 7.0. Immediately before spraying the myosin was diluted into 0.3 M ammonium acetate, *p*H 7.0, at 0 °C. For air-drying experiments myosin dissolved in 0.7 M ammonium acetate was diluted into 0.3 M ammonium acetate containing either 50% glycerol or 1 µg/ml bacitracin in order to reduce surface tension [4]. In other experiments glycerol was omitted or H_2O was replaced by D_2O.

For freeze-drying experiments a solution of myosin in 0.3 M ammonium acetate without glycerol, D_2O, or bacitracin, was used.

In air-drying experiments, droplets were sprayed onto freshly cleaved mica or onto glow discharged carbon coated grids by means of a spray gun device (Desaga, Heidelberg). After drying at $p \leqq 5 \cdot 10^{-7}$ mbar and room temperature for about 1 h the specimens were rotary shadowed (about 100 turns/min: Balzers commutator unit BCM 101) at 6° elevation angle with 0.4 nm Ta/W.

In freeze-drying experiments, droplets were sprayed onto glow discharged carbon coated grids or onto carbon coated grids

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backed with a 3 to 5 nm thick silicon-monoxide film. After washing the grids several times with droplets of bidistilled water and blotting off excess water the grids were frozen in supercooled nitrogen at −210 °C. The frozen grids were then inserted into a magnetic table [16] immersed in liquid nitrogen, transferred via the counterflow loading device onto the precooled stage (Balzers BAF 300) at −80 °C and subsequently freeze-dried for 1 h at −35 °C and $p \leqq 5 \cdot 10^{-7}$ mbar. The grids were then rotary shadowed (about 100 turns/min: Balzers BCM 101 with cooling device) at low elevation angle (5-15°) or unidirectionally shadowed at 15 to 45° with different amounts of Ta/W, depending on the shadowing angle. In all experiments the grids were backed with 5 nm carbon, the specimens then slowly thawed to room temperature and withdrawn from the BAF 300 device.

The grids were examined in a Jeol JEM 100C electron microscope equipped with an anticontamination device at 100 kV and at 50000×. Pictures were taken on Agfa-Gevaert Scientia films. Magnifications were calibrated with catalase crystals. The negatives were used directly for enlarged positives (shadows are white).

Results

Using the replication technique on mica, myosin molecules were visualized by rotary shadowing with 0.4 nm Ta/W at an angle of 6° after drying at room temperature in vacuo from droplets containing myosin in different solvents (Figs. 1a-c). Molecules air-dried from droplets containing 0.3 M ammonium acetate and 50% glycerol (Fig. 1a), 0.3 M ammonium acetate in D_2O, without glycerol (Fig. $1b_1$), 0.3 M ammonium acetate in H_2O, without glycerol (Fig. $1b_2$), 0.3 M ammonium acetate in H_2O containing 1 µg/ml bacitracin (Fig. 1c) were clearly recognizable. Molecules prepared according to the standard method, which includes glycerol (Fig. 1a), are comparable to those published by other investigators and very similar to myosin prepared in D_2O (Fig. $1b_1$) or even H_2O (Fig. $1b_2$), omitting glycerol. Myosin molecules were also clearly visualized if bacitracin was used at a concentration of 1 µg/ml (Fig. 1c). After this treatment a distinct repetitive "banding pattern" of heavy metal deposits seen along the rod seemed especially pronounced [14, 15]. Higher concentrations of bacitracin resulted in a drastic increase of the dimensions of the myosin heads and in a clumping of molecules. As is obvious from these experiments myosin can be visualized with the mica replication technique in the absence of glycerol (Fig. 1b, c).

In the next series of experiments a more direct method was used (Figs. 1d, e). The myosin solution with glycerol (Fig. 1d) and without glycerol (Fig. 1e) was sprayed directly onto glow discharged carbon coated grids, air-dried at room temperature and at $p \leqslant 5 \cdot 10^{-7}$ mbar, then rotary shadowed at 6° elevation angle. Molecules prepared according to this method were also clearly recognizable and composed of a rod portion and two pear-shaped heads. If glycerol was omitted, myosin molecules and background of the specimens (Fig. 1e) seemed to exhibit smaller grain size and smoother granularity as compared to samples derived from glycerol containing solutions (Fig. 1d). Most importantly myosin molecules can more clearly be seen when prepared directly on the EM grid omitting glycerol. Encouraged by the results obtained with the more direct technique omitting glycerol, a solution of myosin in 0.3 M ammonium acetate was sprayed onto glow discharged carbon coated grids, or onto carbon coated grids backed with a 3 to 5 nm thick silicon-monoxide film. The grids were then washed several times with distilled water, frozen, freeze-dried, and shadowed (Figs. 1f-h, Fig. 2). At 6° elevation angle rotary shadowed molecules are clearly visible (Fig. 1f) and of a finer granularity than air-dried rotary shadowed molecules.

Interestingly, freeze-dried molecules prepared directly on silicium-monoxide coated carbon filmed grids are now recognizable when shadowed unidirectionally at elevation angles of 15° (Fig. 1g; overview in Fig. 2) and even of 45° (Fig. 1h). This has never been achieved with the standard method. The heads are visible as two pear-shaped structures, and the rod appears as a narrow ribbon lined by a white shadow which has been cast by the rod. The length of the shadow, which is cast by the molecules in 45° unidirectionally shadowed specimens, directly gives the height of the corresponding molecule parts (and also the width assuming rotational symmetry of the rod and heads [2]).

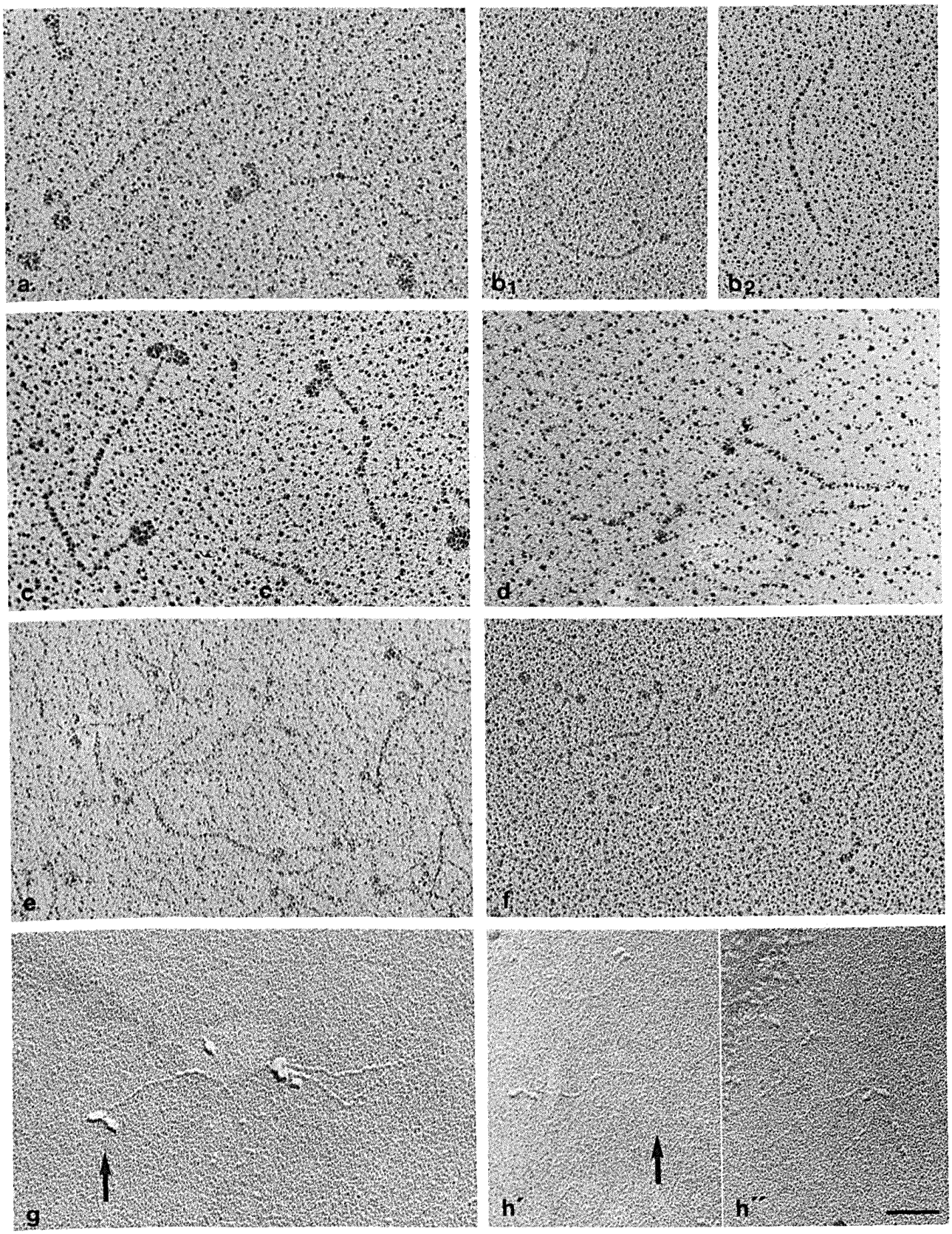
The following mean values were measured: rod width 2.4 ± 0.5 nm, rod length 132 ± 11 nm, maximal head width 6.2 ± 0.6 nm, minimal head width 3.4 ± 0.7 nm, head length 22.6 ± 3.2 nm.

When myosin molecules were rotary "shadowed" at low elevation angles (6-10°) a relatively large proportion of the contrast was due to decoration and subsequent self-shadowing effects by the growing Ta/W grains. As a consequence the repetitive "band pattern" was amplified and more pronounced in rotary shadowed samples (Fig. 1a-f). On the other hand, if myosin was unidirectionally shadowed, a clear shadowing effect, in the sense of portrayal of relief, was observed and self-shadowing was minimal (Fig. 1g, h, Fig. 2).

Discussion

In the past, the mica replication technique used with glycerol has produced myosin molecules which were quite heterogeneous in appearance. The alternative method reported here of spraying the myosin molecules directly onto EM support films in combination with freeze-drying pro-

Fig. 1. 10 µg/ml myosin in 0.3 M ammonium acetate prepared by different methods. — **a to c.** Myosin sprayed onto mica, rotary shadowed with 0.4 nm Ta/W at 6° elevation angle after drying at room temperature and $p \leqslant 5 \cdot 10^{-7}$ mbar from droplets containing 50% glycerol (**a**), in D_2O ($\mathbf{b_1}$), in H_2O ($\mathbf{b_2}$), in H_2O containing 1 µg/ml bacitracin (**c′, c″**). — **d, e.** Myosin sprayed onto glow discharged carbon coated grids, rotary shadowed with 0.4 nm Ta/W at 6° elevation angle after drying at room temperature and $p \leqslant 5 \cdot 10^{-7}$ mbar from droplets containing 50% glycerol (**d**) and without glycerol (**e**). — **f to h.** Myosin sprayed onto carbon coated grids backed with a 3 to 5 nm thick silicon-monoxide film, freeze-dried at −35 °C and $p \leqslant 5 \cdot 10^{-7}$ mbar, and rotary shadowed at 6° elevation angle (**f**) or unidirectionally shadowed at 15° (**g**) and 45° (**h′, h″**) with Ta/W. *Arrow* shows shadowing direction. — Bar 50 nm.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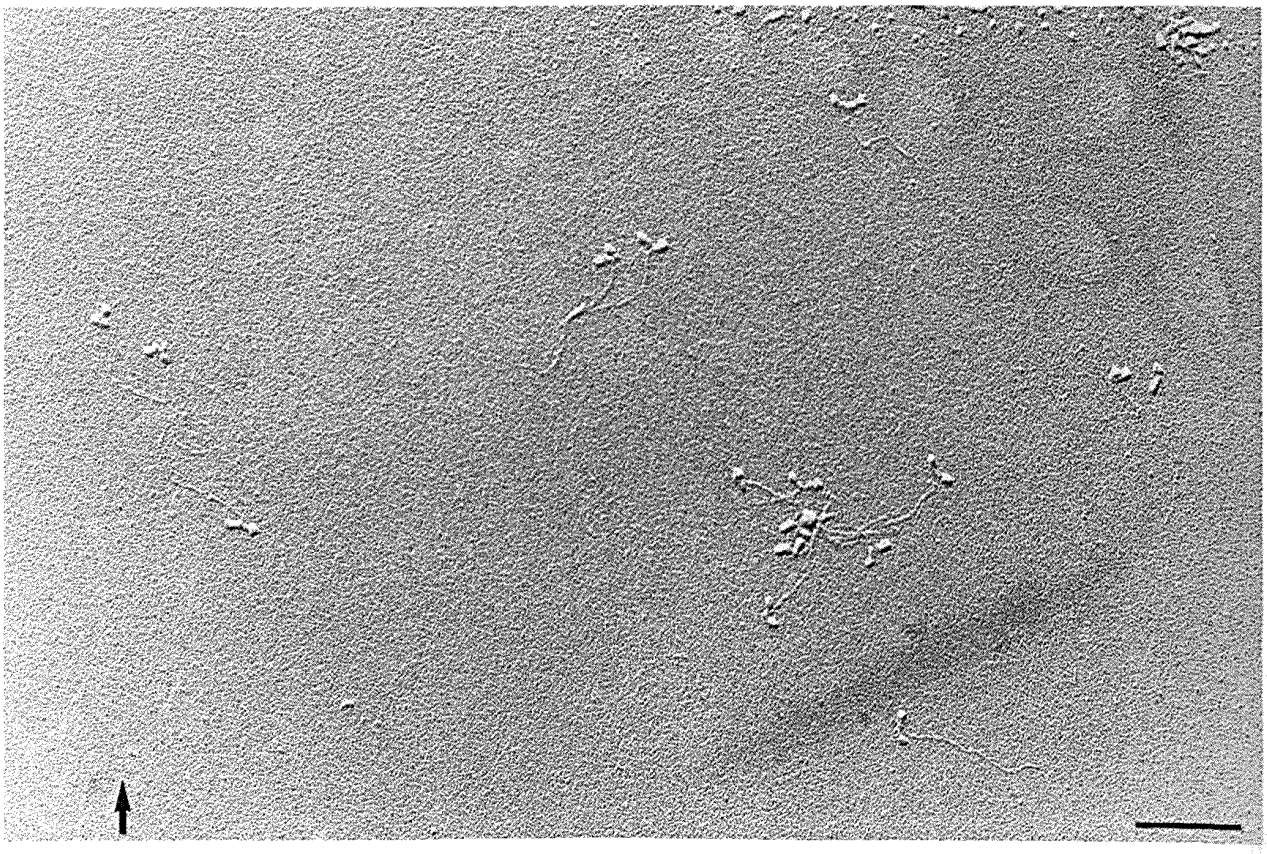

Fig. 2. 10 μg/ml myosin in 0.3 M ammonium acetate were sprayed onto carbon coated grids backed with a 3 to 5 nm thick silicon-monoxide film, freeze-dried at −35 °C and $p \leqslant 5 \cdot 10^{-7}$ mbar and unidirectionally shadowed at 15° with Ta/W. *Arrow* shows shadowing direction. — Bar 0.1 μm.

vides several improvements even though a few thicks filaments are occasionally formed. Myosin is directly immobilized on specially pretreated EM support films and can directly be observed in the EM after shadowing, because no further handling of metal replicas is required. The omission of glycerol significantly improves the reproducibility of the appearance of shadowed myosin. Although the influence of glycerol on myosin molecules in the drying process is not thoroughly investigated it is believed that glycerol makes drying more gentle because it modifies the surface tension during drying and because it slows the drying process [12]. Nevertheless, in view of investigating "in vitro" interactions of myosin with other proteins of the contractile apparatus, omitting glycerol is potentially useful. The role of D_2O is not adequately understood; however, there is empirical evidence that it may stabilize the structure of proteins and in this respect be superior to H_2O. Bacitracin, an antibiotic which reduces surface tension [4], seems to bind directly to myosin enhancing both contrast and decoration of the periodical "band pattern" along the rod [14, 15]. The effect of water surface tension is minimized by freeze-drying thus decreasing the possibility of structural damage [10, 16]. Shadowing is performed at low temperature causing less lateral mobility of the condensing heavy metal atoms resulting in improved resolution due to the formation of a larger number of smaller sized grains. The high elevation angle that could be used after freeze-drying for unidirectional shadowing portrays the relief better than does the low shadowing angle needed in the standard method because the self-shadowing effect of the growing heavy metal grain, which enlarges the diameter of the rod to about twice its size, is minimized. From the length of the shadow cast by the rod and the heads in 45° unidirectionally shadowed specimens, the diameters can be estimated with less deviation since the shadowing angle can be fixed exactly in the freeze-etch unit. The width of the rod measured in this way corresponds well to the theoretical value of an α-helical coiled-coil structure (~2 nm).

In contrast to air-drying, freeze-drying has a stabilizing effect on the three-dimensional structure of myosin, as has been shown for other specimens [10, 16]. Consequently, all additional stabilizing substances (glycerol, D_2O, or bacitracin) can be avoided.

Pictures of air-dried and freeze-dried rotary shadowed molecules indicate that myosin is not only shadowed but also decorated by Ta/W atoms which, exhibiting a certain lateral mobility, are able to migrate to and settle at sites with the highest binding energy along the myosin molecules thus forming a certain periodicity in the decoration pattern along the myosin rod. This will be described and

discussed elsewhere [15]. All methodological improvements reported in this paper are now being applied to study the interactions of myosin molecules with myosin binding proteins under a variety of conditions directly on the EM grids. This method is also being applied to the direct visualization of unshadowed myosin molecules in the STEM.

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