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Shadowing of elongated helical molecules (myosin, tropomyosin, collagen, and DNA) yields regular molecule-dependent heavy metal grain patterns

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Dedicated to Professor Dr. Kurt Mühlethaler, Zürich, on the occasion of his 65th birthday

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Myosin and other α-helical molecules (tropomyosin, collagen) can now directly be adsorbed on EM support films, washed, air-dried, or frozen and freeze-dried [56, 57]. Using this method, the molecules were rotary or unidirectionally shadowed with different heavy metals (Pt/C, Ta/W, Ag) or with C alone [31]. After shadowing at low elevation angles with Ta/W or Ag, myosin, tropomyosin, collagen, and DNA showed strikingly regular patterns of either single or coalesced heavy metal grains (bands) along their entire lengths. Even after shadowing with C alone, repetitive, granular accumulations or bands of C were found along the molecules. The different heavy metals and C displayed distinctive banding patterns on the molecules examined, all of which are characterized by different surface charge periodicities and pitch values. The patterns were quantified on the basis of the distances between grains or bands. Two most frequently measured distances between bands were found after shadowing with heavy metals. After shadowing with Ag the prevalent distances between grains were about twice as large as those after Ta/W shadowing. By evaporating a thin layer of carbon on the molecules before shadowing with heavy metals or by evaporating C alone (with no heavy metal) at 6°, one of these two most prevalent distances between bands was attenuated or disappeared. It was demonstrated that the remaining most frequently measured distances between grains seemed to be related to relief periodicities, to the pitch of the double-coiled (myosin, tropomyosin) and triple-coiled α-helices (collagen) and fractions thereof. The attenuated distances between grains agreed very well with distances of periodic surface charges on the molecules examined. The investigation of the grain or band patterns showed that their characteristic appearance was molecule-dependent and caused both by periodic chemical (repeats of positive and negative surface charges) and periodic structural features (coiling of the helical strands). The examination confirmed the existence of periodic positive and negative surface charges along the myosin rod and suggested a value of about 17.0 nm for the hitherto undetermined pitch of the double-coiled myosin rod.

Introduction

Myosin and other filamentous proteins of high M_r can now be directly attached to and immobilized on pretreated EM support films without glycerol [56, 57] thereby avoiding the indirect replication technique first described by Hall [16] that was used until now as a standard method for the visualization of large molecules at the electron microscopic level [5, 10, 11, 12, 13, 23, 41, 50, 52]. By the new method, molecules, like myosin, which are soluble only at relatively high salt concentrations are adsorbed in high salt buffer to support films, washed with low salt buffer and water, without significant loss of molecules, frozen, freeze-dried and then either contrasted directly by heavy metal shadowing or processed without staining for the STEM [58]. After rotary shadowing at a low elevation angle with Ta/W or Pt/C, myosin molecules prepared in this way showed a periodic banding pattern of heavy metal grains along the entire length of the coiled-coil α-helical rod portion. The heavy metal grains had coalesced to form parallel bands that were roughly perpendicular to the rod axis [56, 57]. The characteristic appearance of heavy metal bands led us to investigate whether physico-chemical periodicities along the myosin molecule were related to the observed banding pattern. The regularly spaced heavy metal bands could be due to: 1) a periodic relief structure of the coiled-coil α-helices with grooves and loops alternating along the rod [44]; 2) the 3,4-heptapeptide repeat with clusters of positive and negative surface charges alternating every 28 amino acids [25, 26, 27]; or 3) a combination of both, the periodic charge distribution and the periodic relief structure of the coiled-coil α-helices.

In an attempt to resolve the question as to which of the above cited factors are responsible for the banding pattern of regularly spaced heavy metal grains on myosin, three test molecules with known surface charge periodicities and pitch values were examined for periodic patterns after shadowing with different heavy metals (e.g., Ta/W or Ag) or with C alone [31]. Both of the metals have shown to decorate sites on biological specimens with certain chemical properties [32, 49], whereas C alone probably does not decorate and, therefore, may mask one of these periodic properties.

Myosin, composed of two heavy chains and two pairs of different light chains [2, 20, 24, 60], is an asymmetrical molecule consisting of an α-helical coiled-coil rod 140 nm long [22, 23, 46] and two pear-shaped heads of some 20 nm in

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length and 3.5 to 6.5 nm in width [11, 42, 57, 58]. The amino acid sequence of the rod region shows a 3,4 heptapeptide repeat of 28 amino acids (~4.1 nm) with an alternating positively and negatively charged surface [25, 26, 27]. Each positive and negative cluster of amino acids is separated by 14 amino acids (~2.0 nm). The pitch of the double-coiled molecule is estimated to be between 14.0 and 18.0 nm [4, 9, 30, 44]. The molecules used as test specimens were the following:

1. Tropomyosin, an α-helical coiled-coil molecule [4, 34, 47, 48] showing a pseudo-repeat of 19 amino acids (~2.8 nm) with alternating charge distribution [17, 28, 29, 35, 47], the pitch of the double-stranded molecule being 13.7 nm [29, 36].
2. Collagen, an α-helical triple-coiled molecule [6, 38, 39, 40] showing a negative charge periodicity of 12.0 nm [1, 3, 14, 18, 19, 38, 51, 53, 54], the pitch of the triple-coiled molecule estimated to be between 21.0 and 30.0 nm [1, 38, 44].
3. DNA (rDNA), a double-stranded molecule, showing a pitch of 3.4 nm [7, 59].

After shadowing, the distances between grains on myosin and on the test specimens, e.g., tropomyosin, collagen and DNA, were measured and compared. The results suggest that shadowing with heavy metals reveals information on both the surface charge distribution and the periodicity of relief structure, while shadowing with carbon alone at a low elevation angle or covering the molecules with a thin layer of carbon prior to shadowing with heavy metals revealed information mostly on the structural aspects, e.g., the pitch values.

Materials and methods

Isolation and preparation of proteins and DNA

Myosin, kindly provided by M. Bähler, was isolated from chicken breast muscle by high ionic strength extraction, low salt precipitation and ammonium sulfate fractionation [55] followed by chromatography on DEAE-Sephadex A-50 [33, 45]. Myosin was pooled, low salt precipitated and dialyzed 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 [55] in which it was stored at 4 °C at a concentration of 5 mg/ml. Before use, myosin was dialyzed over night 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 to 10 μg/ml in 0.3 M ammonium acetate, *p*H 7.0 at 0 °C, and used without further dilution for freeze-drying experiments. For air-drying experiments myosin was diluted to 10 μg/ml in 0.3 M ammonium acetate containing 50% glycerol.

Tropomyosin was isolated from an acetone powder according to the method of Eisenberg and Kielley [8]. Immediately before spraying, tropomyosin was diluted to 10 μg/ml in 0.3 M ammonium acetate, *p*H 7.0 at 0 °C, and used without further treatment for freeze-drying experiments. For air-drying experiments the protein was diluted to 10 μg/ml in 0.3 M ammonium acetate containing 50% glycerol.

Calf skin collagen (No. C-3511, Sigma) was first mechanically dissociated into fibres and then dissolved at a concentration of 10 mg/ml in 0.15 M NaCl, *p*H 6.4 at 0 °C. The collagen was dialyzed overnight against 0.15 M NaCl, *p*H 6.2, and stored at 4 °C. Aliquots of the sample were diluted to 1 mg/ml in 0.7 M ammonium acetate and stored also at 4 °C. Before spraying, the samples were diluted to 10 μg/ml in 0.4 M ammonium acetate without glycerol for freeze-drying experiments or with 50% glycerol for air-drying experiments.

DNA (rDNA), isolated from Dictyostelium discoideum and prepared on freshly cleaved mica for electron microscopy as described by Koller et al. [21], Portmann and Koller [37] and Sogo et al. [43], was kindly provided by Dr. J. M. Sogo (Institute for Cell Biology, ETH-Hönggerberg, Zürich).

Air-drying experiments

Droplets of the protein solution containing glycerol or without glycerol were sprayed onto glow discharged carbon coated grids by a spray gun device (Desaga spray gun, Desaga, Heidelberg). After drying at $p \leqslant 5 \cdot 10^{-7}$ mbar and room temperature for about one hour the specimens were rotary shadowed (about 100 turns/min: Balzers commutator unit BCM 101) at a 6 °C elevation angle with 0.2 to 0.4 nm Ta/W, 0.3 to 0.4 nm Ag, or 0.2 to 0.3 nm C, and unidirectionally shadowed with 0.1 to 0.2 nm Ta/W, or 0.2 nm Ag. In all experiments the grids were backed with 3.0 to 10.0 nm C. In additional experiments, the specimens were first covered with a 1.0 nm thick carbon layer and then rotary or unidirectionally shadowed with either Ta/W or Ag at a 6 ° elevation angle.

Freeze-drying experiments

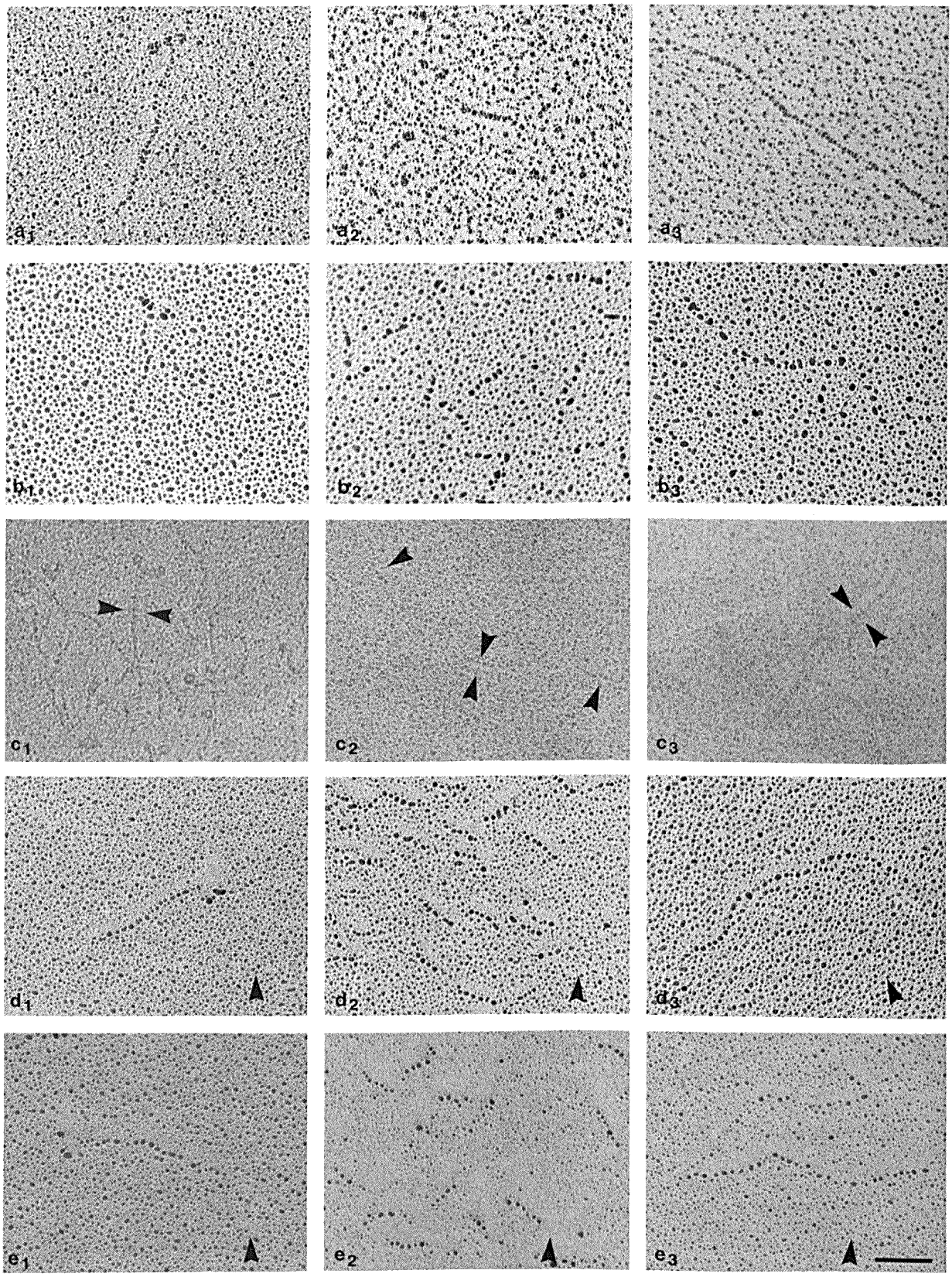
Droplets of the protein solution were sprayed directly onto glow discharged carbon coated grids or onto carbon coated grids backed with a 3 to 5 nm thick silicium-monoxide film. After washing several times with droplets of distilled water and blotting off excess liquid, the grids were frozen in supercooled liquid nitrogen at −210 °C, inserted under liquid nitrogen into a magnetic table [61] transferred via counterflow loading device onto the precooled stage (Balzers BAF 300) at −80 °C and subsequently freeze-dried for one hour at −35 °C and $p \leqslant 5 \cdot 10^{-7}$ mbar. The grids were then rotary shadowed (about 60–80 turns/min: Balzers commutator unit with cooling device) or unidirectionally shadowed at 6° with the same amounts of heavy metal as described for air-drying experiments. In all freeze-drying experiments the grids were backed with 5 to 10 nm C. The specimens were then slowly heated to room temperature.

Electron microscopy

The grids were examined in a Jeol JEM 100C electron microscope equipped with an anticontamination device at an acceleration voltage of 100 kV. Pictures were taken at 50000× on AGFA-Gevaert Scientia film. The magnification was calibrated using catalase crystals as a reference. The negatives were used directly to produce enlarged positive prints (shadows are white).

Measurements

The EM negatives with the shadowed molecules were projected onto a white wall by means of a slide projector and enlarged to a final magnification of 2.5×10^6. The distances between grains or bands (coalesced grains) as in the case of heavy metals, or the distances between granular accumulations or stripes/bands as in the case of C, were measured from the centre of each grain/band or stripe on straight molecules (myosin rod, tropomyosin) or on straight parts of molecules (DNA, collagen). Using a computer, means, variances, standard deviations and 95% confidence intervals of all distances between bands were calculated, the distances measured between bands grouped into classes to the nearest multiple of 0.5 nm and the frequencies of these classes plotted against the distance classes for each protein or DNA molecule and each experiment.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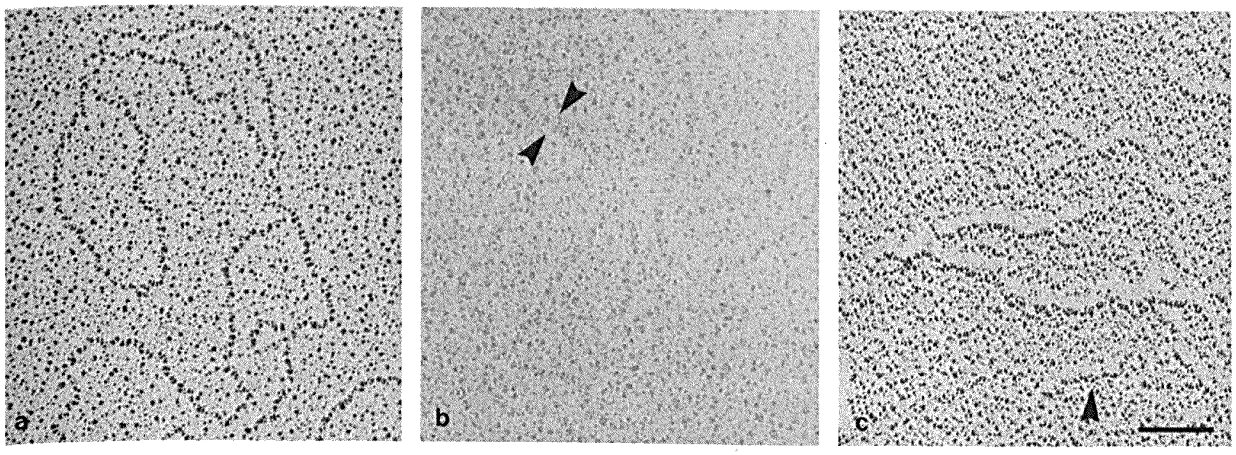

Fig. 2. DNA (rDNA) molecules spread on mica and air-dried. The molecules were rotary shadowed with Ta/W (**a**), C alone (**b**), or unidirectionally shadowed with Ta/W (**c**). — Elevation angle: 6°. — *Arrow* at the bottom: shadowing direction. — Bar 50 nm.

Distances between bands or grains along the myosin molecules were also measured by scanning EM negatives with a Joyce-Loebl densitometer. The calculated means, variances, standard deviations, 95% confidence intervals and number of bands obtained by this method were compared with the values obtained by direct measurements of enlarged, projected negatives at 2.5×10^6 magnification. Parallel to the examination of distances between grains or bands along the molecules, background grains were also taken into consideration. Measurements on grains appearing in the background included the mean grain distance, the mean distance of each nearest grain by the method of nearest neighbour analysis, the mean grain distance along a random line of about 150 nm length (length of myosin molecule), and the mean size of background grains. For this purpose all dark spots of approximately round shape appearing in the background of heavy metal shadowed specimens and all granular accumulations in the background of specimens shadowed only by carbon were considered to be heavy metal grains and carbon "granules", respectively. In addition, the mean grain distances of short strings of heavy metal deposits which occasionally appeared also in the background were measured as well. The background values were compared to the values obtained with the molecules.

Results

Banding patterns

All molecules which were rotary shadowed at a low elevation angle with Ta/W, Pt/C, Ag, or C alone showed a periodic pattern of either single grains or grains that had coalesced to bands roughly perpendicular to the axis of the molecules (Figs. 1a–c, 2a, b, 3a–c). This banding pattern was also visible on myosin molecules that were sprayed in the presence of 1 to 10 µg/ml Bacitracin (Fig. 3c) or were shadowed with Ta/W first in the unidirectional and subsequently in the rotary mode (Fig. 3d) [56, 57]. Even when shadowed solely unidirectionally with Ta/W, Pt/C or Ag, the heavy metal grains seemed to be regularly arranged along the filamentous portions of the molecules (Figs. 1d, e, 2c). Bacitracin was used to reduce surface tension during the drying process [15].

Fig. 1. Solutions of myosin (a_1–e_1), tropomyosin (a_2–e_2) and collagen (a_3–e_3) sprayed onto glow discharged carbon coated grids followed by air-drying or by freezing and freeze-drying. The molecules were rotary shadowed with Ta/W (**a**), Ag (**b**), C alone (**c**), or unidirectionally shadowed with Ag (**d**), or covered with a 1.0 nm thin layer of C evaporated at 90° prior to unidirectional shadowing with Ag (**e**). — Elevation angle: 6°. — *Arrow* at the bottom: shadowing direction. — Bar 50 nm.

Distances between grains in the background

The values of distances between grains and sizes of grains in the background are summarized in Table I.

The distances between grains in the background obtained from samples shadowed with Ta/W or Ag at a 6° elevation angle were both similar, Ta/W grains being somewhat smaller in diameter than Ag grains. The distances between grains and size of grains obtained from samples shadowed with Ta/W at a 90° angle were smaller than those obtained with Ag at the same angle.

The mean distances measured between Ta/W grains in the background were about twice as large as those on the molecules when shadowed at a 6° elevation angle, and somewhat smaller than those on the molecules when shadowed at a 90° angle. The size of Ta/W grains in the background of samples shadowed at a 6° or 90° angle corresponded to that on the molecules.

The mean distances measured between Ag grains in the background when shadowed both at a 6° or 90° angle were similar to those on the molecules, whereas the size of Ag grains in the background was somewhat smaller than the size of grains measured on the molecules. However, the mean distances between nearest grains in the background were about half the mean distance between grains on the molecules in the case of Ag shadowed samples, and somewhat smaller in the case of Ta/W shadowed samples. When shadowed at both 6° and 90° angles, there was a difference in the mean distance between grains in the background and on the molecules on Ta/W shadowed samples, or, a difference in the size between grains in the background and on the molecules on Ag shadowed samples.

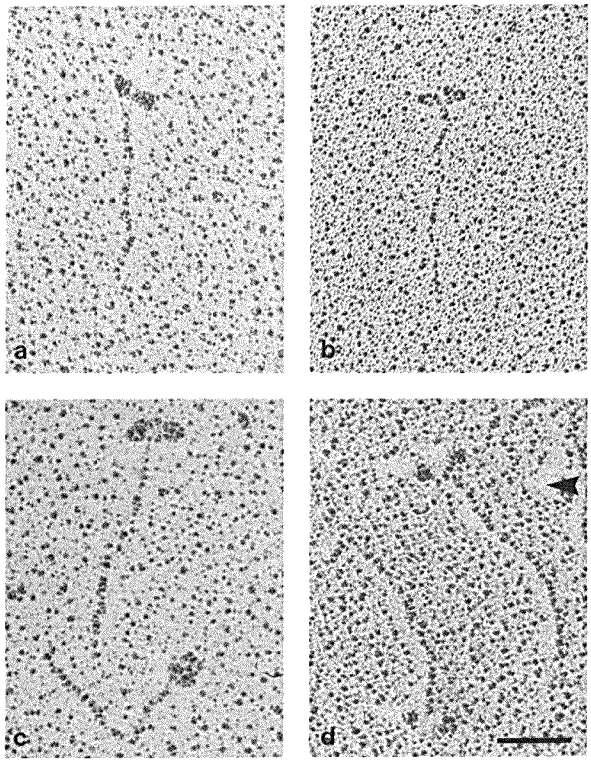

Fig. 3. Solution of myosin sprayed onto glow discharged carbon coated grids, washed, frozen, freeze-dried and rotary shadowed with Ta/W (**a**), Pt/C (**b**), or sprayed onto mica in the presence of 1 μg/ml Bacitracin, air-dried and rotary shadowed with Ta/W (**c**), or sprayed with 50% glycerol on mica, air-dried and shadowed with Ta/W first in the unidirectional and subsequently in the rotary mode (**d**). — Elevation angle: 6°. — *Arrow:* shadowing direction. — Bar 50 nm.

Although the molecules were always shadowed at a 6° elevation angle, the angle made to the source of evaporation by the surface of the molecules was not 6°, but rather 90° or somewhat smaller. Therefore, the values of mean distances between grains and sizes of grains in the background of samples shadowed at a 90° angle were also compared to those of distances between grains and sizes on the molecules.

Although background regions showing strings of grains with periodicities that may look similar to those on the molecules, the mean grain distance for this short strings of grains in the background was about twice to three times as large when shadowed with Ta/W or C, or about half as large as those measured on the molecules when shadowed with Ag.

Distances between grains/bands on different molecules. Distribution of distances between the bands after shadowing with Ta/W, Ag or with C alone

Since no significant differences in the mean distance between bands on both air-dried and freeze-dried myosin molecules were observed (see Tab. II), their values were combined for all subsequent evaluations. Measurements on molecules projected and enlarged to a final magnification of 2.5×10^6 yielded the same distances between bands as measurements on densitometered molecules. In addition, the values of the mean distances between corresponding bands along the rods of several myosin molecules did not reveal any pronounced irregularity (unpublished data).

The mean of all distances between bands were calculated separately for each experiment and each protein or DNA molecule (Tab. II). As seen, these values did not differ very much after shadowing with the same heavy metal or carbon among the molecules tested, while there was a significant difference between background and molecules (Tabs. I, II). Therefore, not only the means were compared but also the distances measured between the bands were grouped into classes to the nearest multiple of 0.5 nm and histograms were constructed with the frequencies of these classes plotted against the distances between the bands (Fig. 4).

The distributions of distances between heavy metal grains or bands on all molecules after rotary shadowing with Ta/W (Fig. 4a) and Ag (Fig. 4b) were bimodal, revealing two most frequent distances, while the most frequent distances between bands of Ag shadowed molecules were about twice as large as those of Ta/W shadowed molecules (Figs. 4a, b, Tab. II). Correspondingly, the mean distance between Ag bands on all molecules was twice that between Ta/W bands.

Covering the molecules first with a thin layer of carbon (~ 1.0 nm) evaporated from an angle of 90°, and then shadowing with Ta/W at a low angle, changed the mean distances between Ta/W bands on the molecules examined only slightly (Tab. II). However, one of the two most

Tab. I. Grain distances and grain sizes in the background.

	Samples shadowed with Ta/W at 6° (nm)	Samples shadowed with Ag at 6° (nm)	Samples shadowed with Ta/W at 90° (nm)	Samples shadowed with Ag at 90° (nm)
Mean grain distance per unit of background area	9.0–12.0	7.0–10.0	2.0–4.0	5.0–8.0
Mean grain distance along a random line of 150 nm length	6.0–11.0	6.0–10.0	3.0–5.0	6.0–9.0
Mean distance of nearest neighbours	3.0–4.0	3.0–3.5	2.0–2.5	3.0–5.0
Mean grain size	2.0	2.5–4.0	2.0	3.0–4.0
Mean grain distance for short strings of grains	6.0–9.0	4.0–4.5	—	—

frequently measured distances between bands yielded reduced frequency or even disappearance (Fig. 4c).

The same was found in experiments in which the molecules were rotary shadowed at a 6° elevation angle with C alone omitting heavy metal, according to Müller et al. [31]. Interestingly, although of lower contrast compared to Ta/W, a banding pattern was found on all molecules examined after such a treatment (Figs. 1c, 2b, 4d). The value of the mean distance between bands or stripes was in the same range as seen after heavy metal shadowing (Tab. II). By comparing the distribution of distances between the bands after rotary shadowing with Ta/W (Fig. 4a) and shadowing with C alone (Fig. 4d), it was obvious that one of the two most frequently measured distances seen after shadowing with Ta/W corresponded to the most frequent distances observed after shadowing with C alone (Fig. 4c, 4.0–4.5 nm on myosin, 3.5 nm on tropomyosin, 4.5 nm on collagen, 3.5 nm on DNA), whereby the others (3.0–4.0 nm on myosin, 2.5–3.0 nm and 4.5 nm on tropomyosin, and 6.0–7.0 nm on collagen) showed no apparent correlation. The latter distances were also the same ones that were attenuated or disappeared when a thin layer of carbon was evaporated onto the molecules prior to shadowing with Ta/W.

After unidirectional shadowing with Ag (Fig. 4e) as with rotary shadowing (Fig. 4b), not one but two classes of distances were most frequently measured.

When a carbon layer of 1 nm thickness was evaporated onto the molecules from an angle of 90° before unidirectional shadowing with Ag at 6° (Fig. 4f), one of the two most frequent distances between the Ag bands which was still visible in Figure 4e was attenuated or disappeared.

The 3.5 nm distance obtained with DNA after rotary shadowing with carbon alone (Fig. $4d_4$) corresponds well to the pitch value of 3.4 nm for DNA [62], and the 3.5 nm (Fig. $4d_2$) and the 7.0 nm distances (Fig. $4f_2$) obtained with tropomyosin correspond to ¼ and ½, respectively, of the 13.7 nm pitch value for tropomyosin [29, 36]. The 4.5 nm (Fig. $4d_3$) and 9.0 nm distances (Fig. $4f_3$) obtained with collagen may agree with ⅙ and ⅓, respectively, of the pitch value for collagen that lies between 21.0 and 30.0 nm [1, 38, 44]. From these findings it was concluded that the most frequent distances between bands after shadowing with C alone (Fig. 4d), or shadowing first with C and subsequently with heavy metals (Figs. 4c, f) were due to periodic relief structures, e.g. to ¼ and ½ pitch, respectively, of α-helical double-coiled molecules (myosin, tropomyosin) and to ⅙ and ⅓ pitch, respectively, of α-helical triple-coiled molecules (collagen). Accordingly, pitch values of ~17.0 nm (between 4×4.5 nm and 4×4.1 nm, 4.1 nm: mean band distance of molecules rotary shadowed with C alone) for myosin, and of ~27.0 nm (6×4.5 nm, 3×9.0 nm) for collagen were estimated by this method.

The other distances between bands measured after heavy metal shadowing (Figs. 4a, b) that were attenuated or no longer visible after pre-treatment with C were in good agreement with reported charge periodicities: 3.0 nm (Ta/W) and 7.0 nm (Ag), repeating unit ≅4.0 nm, correspond to the ~4.0 nm charge repeat on myosin [25, 26, 27]; 2.5 to 3.0 nm, 4.5 nm (Ta/W) and 6.0 nm, 9.0 nm (Ag), re-

Tab. II. Band/grain distances on the molecules.

		Myosin (nm)	Tropomyosin (nm)	Collagen (nm)	DNA (nm)
Rotary shadowed with Ta/W	Mean band distance	4.1±1.2 (±0.13) AD[a] 4.2±1.5 (±0.19) FD[b]	3.5±1.2 (±0.11)	5.0±1.6 (±0.16)	3.6±0.9 (±0.08)
	Most frequent distances	3.0 4.0–4.5	2.5 3.5 4.5	4.5 6.0–7.0	3.5
	Number of bands	35–36	11–12	—	—
Rotary shadowed with Ag	Mean band distance	7.8±1.9 (±0.13)	6.9±1.3 (±0.14)	8.8±1.9 (±0.21)	
	Most frequent distances	7.0 9.0	6.0 7.0	9.0 12.0	—
	Number of bands	17–19	5–6	—	
C layer before rotary shadowing with Ta/W	Mean band distance	4.4±1.3 (±0.13)	4.1±1.3 (±0.12)	4.8±1.2 (±0.12)	3.7±0.9 (±0.12)
	Most frequent distances	4.0–4.5	2.5 3.5 4.5	4.5 6.5–7.0	3.0–3.5 4.5
Rotary shadowed with C	Mean band distance	4.1±0.9 (±0.10)	3.6±1.0 (±0.07)	5.2±1.4 (±0.20)	3.5±0.8 (±0.09)
	Most frequent bands	4.0–4.5	3.5	4.5 6.0	3.5
	Number of bands	33–36	11–12	—	—
Unidirectionally shadowed with Ag	Mean band distance	7.8±1.8 (±0.16)	6.9±1.2 (±0.12)	8.6±1.7 (±0.22)	
	Most frequent distances	7.0 9.0	7.0	7.0–8.0 9.0	—
C layer before unidirectional shadowing with Ag	Mean band distance	8.3±1.4 (±0.14)	7.5±1.4 (±0.26)	8.9±1.5 (±0.22)	
	Most frequent distances	7.0 9.0	7.0	9.0	—

[a] AD Air-dried. — [b] FD Freeze-dried. — Values between brackets: 95% confidence intervals.

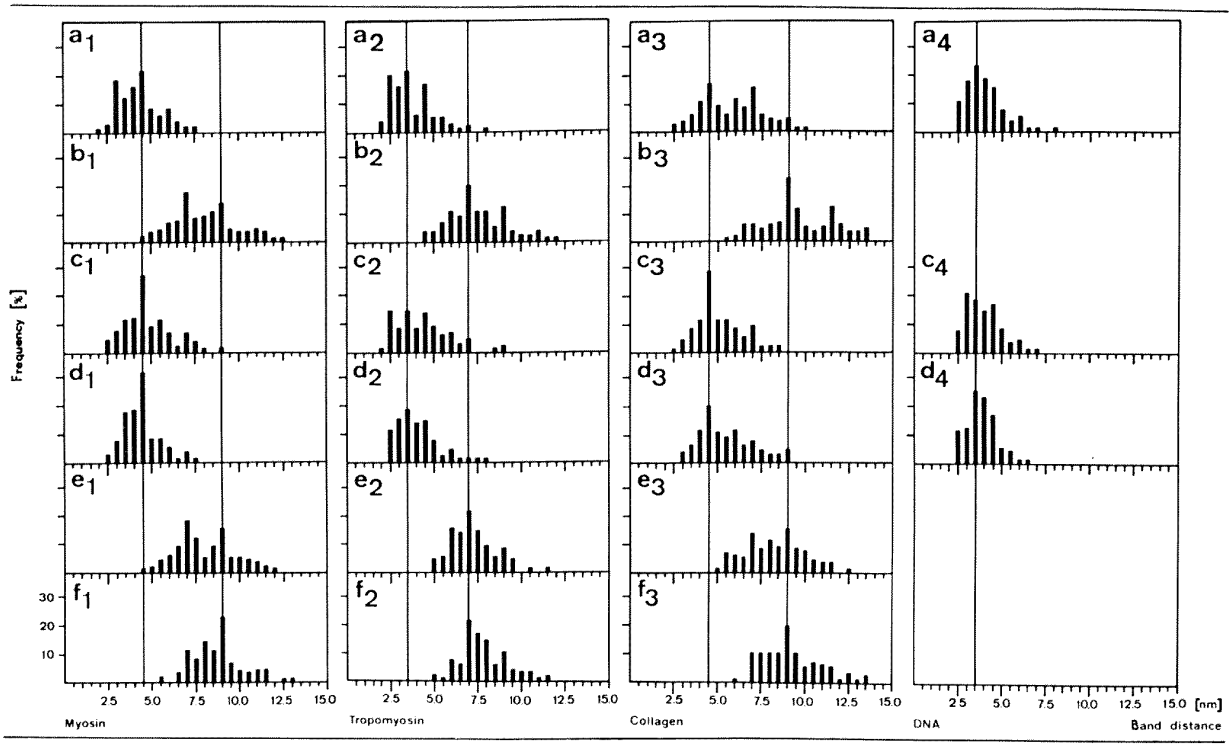

Fig. 4. Class distributions of distances between all bands and their nearest neighbours on myosin (a_1–f_1), tropomyosin (a_2–f_2), collagen (a_3–f_3) and DNA molecules (a_4, c_4, d_4) irrespective of their relative position on the molecules. The distances between bands were grouped into classes to the nearest multiple of 0.5 nm and plotted according to their frequency. The molecules were rotary shadowed with Ta/W at 6° (**a**), rotary shadowed with Ag at 6° (**b**), covered first with a 1.0 thin layer of C at 90° before rotary shadowing with Ta/W at 6° (**c**), rotary shadowed with C alone at 6° (**d**), unidirectionally shadowed with Ag at 6° (**e**), and covered first with a 1.0 nm thin layer of C at 90° before unidirectional shadowing with Ag (**f**). — Unfortunately, shadowing with Ag did not work for DNA molecules which were spread on mica, since the silver grains evaporated onto mica always coalesced to large silver "droplets", presumably formed as the replica was floated off onto water.

peating unit ≅3.0 nm, correspond to the 2.8 nm charge repeat on tropomyosin [17, 29, 30, 35, 48]; and 6.0 to 7.0 nm (Ta/W) and 11.5 nm (Ag), repeating units ≅6.0 nm and 12.0 nm, correspond to the charge repeat of 12.0 nm on collagen [1, 3, 14, 18, 19, 38, 52, 54, 55].

Thus, it was concluded that the latter values were due mainly to charge periodicities.

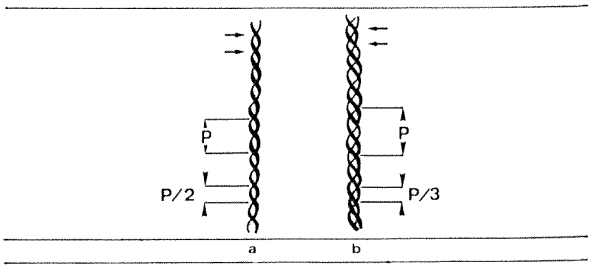

Fig. 5. Schematic drawing of double-coiled (**a**) and triple-coiled α-helices (**b**) with repeating units of pitch (P) and fractions thereof, P/2 and P/3, respectively. Grooves between the loops are indicated by *arrows*.

Discussion

Distinct banding patterns were visible on myosin, tropomyosin, collagen and DNA molecules examined after shadowing with heavy metals or with carbon alone. Since the calculated mean distances between the grains or bands on all molecules rotary shadowed with the same heavy metal were similar, it may be argued that the distances between these bands were due exclusively to physical parameters inherent in the heavy metals used for shadowing, e.g. grain size, charge, diffusion coefficients and other atomic properties. Due to the fact that the angle made to the source of evaporation by the molecules is different from that made by the background, one would expect there to be more heavy metal deposited on the molecules compared to the background, and consequently the distributions and sizes of grains on the molecules to be different from that on the background, but the same on all molecules. Since the sizes and distributions of grains in the background differ from that on the molecules examined (Tab. I), therefore, one might argue that physical parameters alone are causing the differences. However, since the patterns of grains also differ significantly among the molecules with

different relief and surface charge distributions, it is concluded that not only the above cited physical parameters (type of heavy metal, angle of evaporation, grain size, etc.) of the heavy metals, but also, and most importantly, the underlying molecules are directly involved in causing the different patterns. Since the different grain sizes and grain distances on the molecules, on the one hand, and in the background, on the other, were caused by physical parameters of the evaporated heavy metals, these parameters were assumed to be equal for all molecules and, therefore, could be neglected in the further examination. As presented in the results, the α-helical protein molecules displayed primarily a bimodal distribution of distances between heavy metal grains or bands. These distributions were non-random and differed with each molecule investigated. Our observations strongly suggest that diffusion, nucleation and coalescence of heavy metals were also different among the molecules examined, and, therefore, the differences in grain patterns were mainly ascribed to different physico-chemical properties of the underlying molecules, such as relief structures and surface charge distributions. Thus, the next step was to determine whether the banding pattern was most likely due to a repeat of surface charges or relief structures, or to both.

Since two most frequent distances between bands were obtained after rotary shadowing with both Ta/W and Ag, the latter distances being twice as large as those shadowed with Ta/W, both charge *and* relief repeat were assumed to cause these two predominant distances.

Experiments in which the molecules were first covered with a very thin carbon layer, before shadowing with heavy metals (Figs. 4c, f), or in which the molecules were shadowed with carbon alone (Fig. 4d), yielded only one of the two most frequent distances found after shadowing directly with heavy metals (Figs. 4a, b). This finding suggested that evaporation of C prior to evaporation of heavy metal and evaporation of C alone masked one of the periodicities. Since the remaining most frequent distance classes of 3.5 nm on tropomyosin and 3.5 nm on DNA after such treatments agreed well with repeat distances representing pitch values or fractions thereof, it was assumed that these remaining most frequent distances between bands corresponded to the relief periodicity. Moreover, the fact that the mean distance between Pt/C grains on single-stranded DNA (2.5–3.0 nm, detailed data not shown here) is smaller than the mean distance between grains on double-stranded DNA (~3.5 nm, Tab. II) strongly suggests that the latter value corresponds to a periodicity inherent in the coiled relief which lacks single-stranded DNA. Accordingly, the band distances that had decreased in frequency were assumed to be related to the surface charge periodicity. The remaining most frequently measured distances between bands on myosin were 4.0 to 4.5 nm (Ta/W, C) and 8.0 to 9.0 nm (Ag). These values were shown to be related to relief structures, to ¼ and ½ pitch of the α-helical double-coiled rod of myosin, respectively. The pitch was inferred for structural studies by theoretical means [26, 27]. For space filling structural models of the myosin rod a pitch value of 16.8 nm was used which corresponds to a value of 112 amino acid residues assuming a twist of 90° over each regular 28 residue zone [26, 27].

Double- and triple-coiled helices have deep grooves (Fig. 5) which are the features one would expect to see after shadowing and to give rise to a periodicity of P/N, where P is the pitch and N the number of strands. That is to say ½ pitch for the coiled-coils (myosin and tropomyosin) and ⅓ pitch for the triple-coiled collagen. Periodic distances of ¼ pitch or ⅙ pitch, which were measured after rotary shadowing with Ta/W and with C alone, would imply that the number of strands was 4 (in myosin and tropomyosin) or 6 (in collagen), or else, that evaporated material was found in the grooves at distances of ¼ or ⅙ pitch, respectively. This can be explained as the following: When triple- or double-coiled helices are rotary shadowed at the very low elevation angle used, these elongated molecules are hit by the evaporated material mostly on the sides, perpendicular to the long axis. The grains predominantly grow in the grooves which are lying on both sides, but are staggered, depending on the molecules, by repeating units of ¼ or ⅙ pitch along the axes (Fig. 5). This is the reason why after rotary shadowing one does not get signals at P/2 for the coiled-coils and at P/3 for collagen, but rather at P/4 and P/6. However, if the molecules are unidirectionally shadowed with Ta/W or C, distances between grains corresponding to ½ or ⅓ pitch would be expected. Experimentally, the measured distances between grains on unidirectionally shadowed molecules are indeed larger than ¼ or ⅙ pitch, but somewhat smaller than ½ or ⅓ pitch. The fact that they are not exactly ½ or ⅓ pitch can be explained by a diffusion capacity of Ta/W or C that is lower than the distances of ½ or ⅓ pitch.

The reason why greater distances are measured with Ag than with Ta/W, is probably due to the higher lateral mobility of the Ag atoms that coalesce to form larger grains at fewer sites compared with Ta/W. According to the position of Ta and W in the periodic table of elements, it is assumed that these heavy metals, if charged at all, are more likely to be positively charged, possibly decorating clusters of negative charges on the myosin rod. According to McLachlan and Karn [26, 27] clusters of amino acids with the same charge are evenly spaced every 28 residues, or 4.1 nm, along the entire length of the rod. The fact that, after pre-treatment of myosin with a thin layer of C followed by shadowing with Ta/W (Fig. 4c), distances between bands in the range of 3.0 nm, or, in the case of shadowing with Ag, in the range of 7.0 nm were attenuated in frequency, indicates that these periodicities are the result of decoration of these charged clusters assuming that C neutralizes surface charges. It also indicates that the difference between the predominant distance classes obtained with Ta/W of 3.0 nm (Fig. $4a_1$), and with Ag of 7.0 nm (Fig. $4b_1$), both of which are related to repetitive surface charges, was ~4.0 nm which corresponds exactly to the 4.0 to 4.1 nm repeat of 28 amino acids with alternating surface charges on myosin rod. The same holds true for values of 3.0 nm (on tropomyosin), and 6.0 nm, 11.0 to 12.0 nm (on collagen) for their respective charge periodicities.

The fact that one of these two most predominant distances between bands on myosin is attenuated or disap-

pears in frequency after treatment with C indicates that the repeat of surface charges (~4.0 nm) and the relief repeat of ¼ pitch, ~4.0 to 4.5 nm, although in the same range, are not in register.

The results presented in this paper indicate that positive "staining" with heavy metals that condense and coalesce to perpendicular bands along the lengths of the filamentous molecules examined can provide data on the underlying physico-chemical properties, such as the relief structure and distribution of surface charges. The capacity of heavy metals to portray relief as well as to decorate charged sites has to be taken into account as a general feature of the heavy metal shadowing procedure. Both, relief and surface charges influence the deposition and final location of heavy metal grains along such filamentous proteins. By evaporating first a very thin C layer prior to heavy metal shadowing or by evaporating C alone at 6°, the periodicities due to charge can be masked and the periodicities due to relief can clearly be demonstrated. These experiments strongly suggest that C is capable of covering and "neutralizing" some of the surface charges and to portray the relief structures quite perfectly.

Thus, the described examination of distances between heavy metal grains yielded a value of ~17.0 nm for the hitherto undetermined pitch of the double-coiled myosin rod and confirmed the existence of repeating positive and negative surface charges every 28 amino acids or 4.0 nm along the entire length of the myosin rod.

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