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BRAIN CLATHRIN

VISCOMETRIC AND TURBIDIMETRIC PROPERTIES OF ITS ULTRASTRUCTURAL ASSEMBLIES

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Summary

Clathrin was isolated in highly purified form from bovine brain preparations rich in coated vesicles and by some improvements of our previous procedures. At pH 7.5, clathrin's solution was viscous, but clear. At pH 6.5, clathrin's solution was less viscous, but turbid. By electron microscopy, clathrin's turbidity at pH 6.5 correlated with the presence of numerous basket-like lattices or cages; the higher viscosity observed at pH 7.5 correlated with a mixture of various polymeric forms of clathrin having linearly assembled filaments or filamentous bundles of cross-linked clathrin molecules. In vivo, clathrin's capacity for assembling or disassembling itself into baskets or cage-like structures is compatible with a mechanism that retrieves areas of the plasma membrane containing protein molecules, smaller stimulatory or inhibitory compounds bound on the external cell membrane surface.

Introduction

Clathrin is a 180 000 molecular weight polypeptide [1] and the major component of coated vesicles [2], i.e., those membrane bilayers bound and surrounded by polygonal lattices. Coated vesicles were postulated to represent transient cellular organelles of the membrane recycling process [3—5]. As a consequence of this process, many molecules, e.g., proteins, lipids, nucleotides,

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

neurotransmitters, bound to their respective receptor sites, are endocytosed within pinocytotic bristle-coated vesicles [3]. Recent improvements in clathrin's purification procedure [6—8], have provided not only a better understanding of clathrin's biochemical and biophysical properties but also have defined clathrin's specific involvement in the formation of coated organelles and their relationship to cellular functions.

We reported that purified clathrin in solution is capable of forming basket structures in vitro [6—8]. Woodward and Roth [9] and Keen et al. [10] reported similar findings. We found that the conversion of baskets into filaments was reversible: adjusting the pH of monodispersed clathrin molecules in solution from 7.5 to 6.5 caused clathrin to form baskets; readjusting the solution's pH back to 7.5 caused the baskets to disassemble and/or to reform into filamentous structures [8]. Thus, we postulated that clathrin may possess cellular functions other than the capacity to form clusters around vesicles. In this report, new properties of clathrin in solution are described.

Materials and Methods

Isolation of vesicles from bovine brain. Bovine brains were cleansed of meninges and the gray matter separated as described elsewhere [8]. Some modifications resulted in improved yield and purity and are described below. Unless specificed otherwise, all steps of the fractionation procedure were carried out at 4°C. For each preparation of clathrin, three beef brains were used to obtain a crude, coated-vesicle fraction. An extract obtained by 10—12 strokes of a Teflon-glass homogenizer, was centrifuged at 100 000 × *g* for 60 min. From the clear supernatant, crude clathrin was precipitated by 30% saturated $(NH_4)_2SO_4$ pelleted at 15 000 × *g* for 15 min, resuspended with and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, which could be stored frozen at −20°C in 10% sucrose for up to 4 weeks at a concentration of 3—6 mg/ml.

Further purification resulted when crude clathrin was dialyzed and loaded on a large Sepharose 4B column of 5 × 80 cm equilibrated with 20 mM Tris-HCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.2% sodium azide, 2 M urea buffer, pH 7.5. The fraction comprising a second peak was precipitated by 50% saturated $(NH_4)_2SO_4$, dissolved, dialyzed and rechromatographed on a similar Sepharose 4B column equilibrated with the same buffer, without urea. Next, the protein solution was dialyzed against two changes of 20 vols. of the latter buffer (without EDTA) clarified at 150 000 × *g* for 1 h and the supernatant used as the final clathrin preparation. Protein determinations were carried out by the method of Lowry et al. [11], using bovine serum albumin as the standard.

Sodium dodecyl sulfate gel electrophoresis. Slab gels containing a gradient of 7.5—20% polyacrylamide and 0.1% sodium dodecyl sulfate and cylindrical gels were used to verify the purity of clathrin. Before loading, the protein was made 1% in sodium dodecyl sulfate and reduced by 0.1% 2-mercaptoethanol before application to the gel.

Turbidimetric determinations. Clathrin's turbidity was measured at 425 nm using a Beckman model 25 recording spectrophotometer at room temperature. Clathrin in solution was pumped continuously through a microflow cuvette

using a peristaltic pump (Buchler Instruments) from a reservoir of approx. 3—4 ml wherein a combination electrode monitored pH changes. The pH was adjusted by addition of calibrated amounts of 0.4 M Mes buffer, pH 6.0, or 0.5 M Tris-HCl, pH 7.5. At selected pH values, samples were withdrawn for viscosity measurements, if indicated, or for electron microscopic analysis by negative staining in order to correlate turbidity changes with the ultrastructural organization of clathrin.

Viscometric determination. An Oswald viscometer with a capillary flow time of 335 s for buffer was used. Viscometric determinations were performed in a constant temperature bath at 25°C. At each pH change, a sample was removed for electron microscopy. Protein aggregates that occasionally formed as a result of the passage of clathrin through the capillary tube were removed by centrifugation at 37 000 $\times g$ for 10 min to prevent clogging of the viscometer. The values showing viscosity changes of clathrin as a function of concentration were obtained by gradual dilution and clarification by centrifugation at each determination point. The solution of clathrin used for this purpose was free of urea and filaments.

Electron microscopy. Negative staining was performed immediately after withdrawing a sample (10—15 μg of clathrin) from the viscometric or turbidimetric determination. The samples were placed on a Formvar carbon-coated grid, stained with 1% uranyl acetate, air dried and examined in a Jeol 100-B electron microscope at 80 kV.

Results

The purification of clathrin by two consecutive gel filtration chromatographic procedures on Sepharose 4B, the first using 2 M urea with Tris-HCl buffer at pH 7.5, and the second omitting urea and EDTA, resulted in the removal first, of small membrane fragments that emerged in the void volume, and second, of large aggregates and most of the accompanying proteins banded below clathrin's 180 000 molecular weight. The protein eluting in a second peak was devoid of membranes and consisted of a major polypeptide band with an electrophoretic mobility of 180 000 (Fig. 1a, left gel). Overloading amounts of clathrin (Fig. 1a, center and right gels) revealed minor amounts of a heavier polypeptide and at least two polypeptide bands below that of clathrin. The polypeptide composition of the protein peak eluted by the second column run is illustrated in Fig. 1b. Densitometric scan at 550 nm indicated that the concentration of contaminants represented 3—5% of the concentration of the major band.

The reduced specific viscosity determined on clathrin in solution and plotted as a function of its concentration is illustrated in Fig. 2 by the line through the open circles. Reference to the reduced specific viscosity curve at various concentrations of a given clathrin preparation established that the manifest changes in viscosity are achieved at a constant protein concentration by a change in pH. The results obtained by adjusting the pH of two other (separately prepared) clathrin solutions at various starting protein concentrations are illustrated in the same figure by the dark circles and dotted lines connecting the various pH values. In all instances, a drop in viscosity was

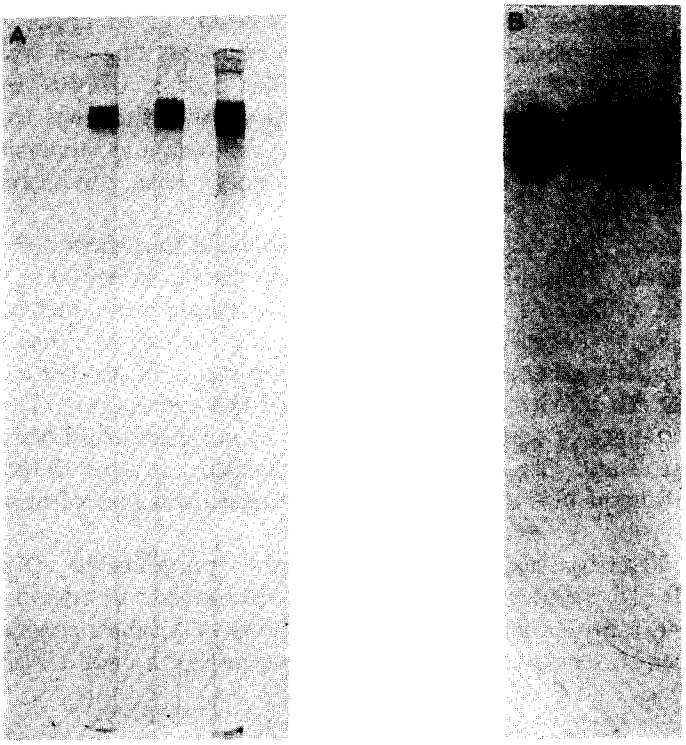

Fig. 1. (A) Polyacrylamide gel electrophoresis of purified bovine brain clathrin after the first chromatographic run on Sepharose 4B with a Tris/urea buffer. All gels contain 7.5% acrylamide and 15 μg (left gel), 25 μg (center gel) and 35 μg (right gel) of clathrin. (B) Slab gel 7.5—20% polyacrylamide electrophoresis in 0.1% sodium dodecyl sulfate of clathrin obtained after the second chromatographic run on Sepharose 4B using a Tris buffer without 2 M urea. Left lane, 35 μg; middle lane, 25 μg, and right lane, 15 μg clathrin.

observed when the solution's pH was decreased. Viscosity returned to the expected value after its pH was raised. Viscosity changes were evident as soon as the sample was transferred to the viscometer, usually 1—2 min after adjusting the pH.

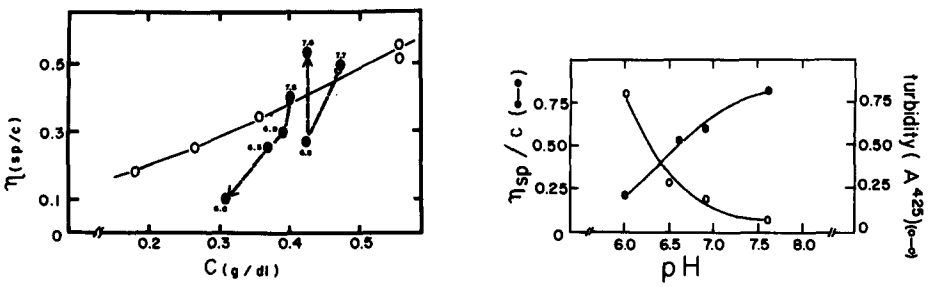

Fig. 2. Intrinsic viscosity of clathrin in buffer A without EDTA as a function of protein concentration. Numbers above and below dark circles indicate the pH values of various protein solutions that were subjected to pH changes and viscosity determinations.

Fig. 3. 4 mg/ml clathrin solution the turbidity and viscosity of which were determined simultaneously at different pH values.

When viscosity and turbidity were determined simultaneously on the same protein solution, a distinct reciprocal correlation between these two properties was obtained. Fig. 3 illustrates this phenomenon graphically. The protein solution at pH 7.6 showed no turbidity and moderate specific viscosity. When the pH was lowered, however, viscosity dropped and turbidity increased until pH 6.0 was reached. At this point, viscosity was at its minimum value and turbidity at its maximum. Also, as with viscosity, the effects of pH on turbidity were found to be reversible and almost instantaneous, remaining constant thereafter. Below pH 6.0, clathrin formed amorphous aggregates [9].

Clathrin was present in its disaggregated state when observed immediately after dialysis at pH 7.5 to remove urea from the protein. Clathrin was visualized as rods, many of them attached, giving the appearances of Y-shapes, Z-shapes, pentagons and hexagons. It was not clear if such arrangements were made by two or more parallel attached molecules. Few aggregates of clathrin were found under these conditions (Fig. 4). When the pH was lowered to 6.5, the clathrin solution became increasingly turbid. Turbidity was higher with increased concentrations of protein. By electron microscopy, the protein was

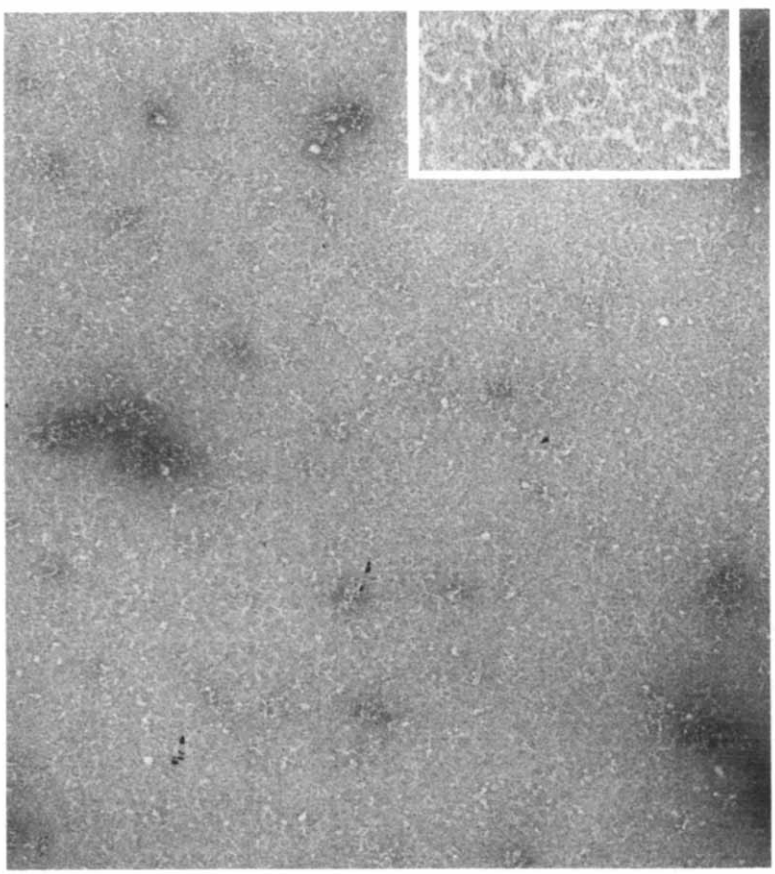

Fig. 4. Negatively stained clathrin monodispersed in solution. Clathrin seems to consists of elongated molecules dispersed or attached (X 72 000). Inset: detail of clathrin's molecular shapes (X 195 000).

present as well-defined basket-like structures. These basket- or cage-like structures were composed of pentagons and hexagons linked by their vertices (Fig. 5, inset); they resembled those originally seen surrounding vesicle membranes in the crude, coated vesicle fraction. Under our experimental conditions it was not possible to visualize baskets in the process of being assembled. The majority of baskets were present as individual units with little or no aggregation among them. A mixture of baskets and filaments was observed upon raising the pH to 7.5 (Fig. 6). After 15 min, no basket was seen. Instead, the field was filled with bundles of filaments apparently as a result of the linear arrangements of clathrin molecules (Fig. 7). Cross-linking of filaments within the bundles and between separate bundles was observed.

Filamentous bundles of clathrin also were formed upon passage of clathrin through the capillary tube. In several instances these bundles formed simply by allowing a clathrin solution to stand for a period of 4—6 h. If it was dialyzed against a Tris-HCl buffer (pH 7.5) which contained 2 M urea, the clathrin filamentous bundles disaggregated. After dialysis against a similar buffer but

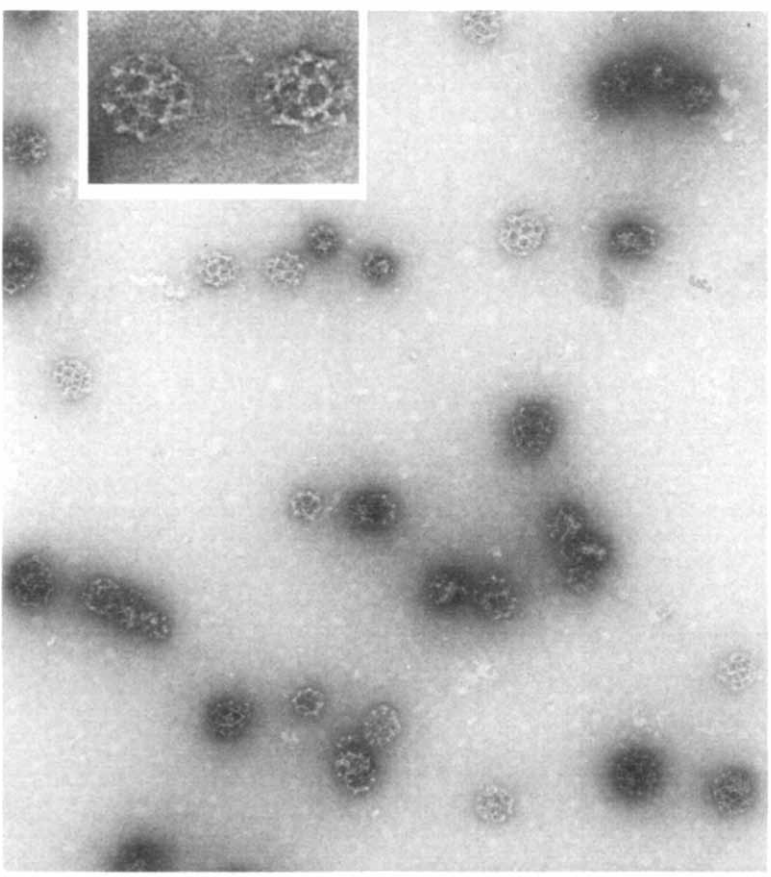

Fig. 5. Same solution as in Fig. 4, after adjusting its pH to 6.5. Numerous and uniform basket structures were found. Inset: larger detail of two baskets (× 150 000).

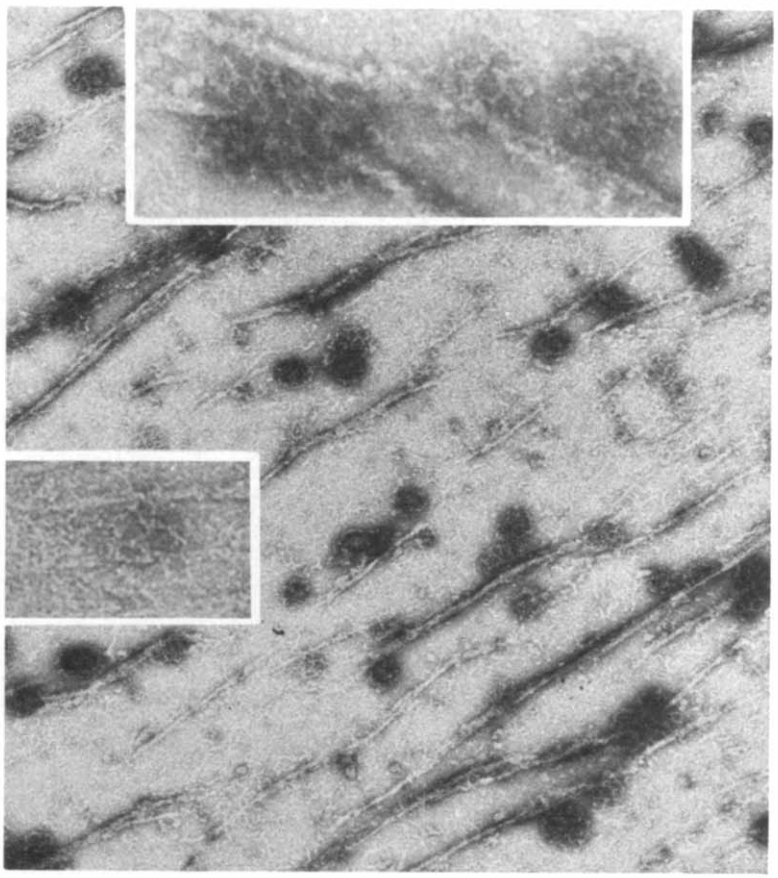

Fig. 6. Clathrin as in Fig. 5, after raising its pH to 7.5. Many filaments were found. Insets: molecules branched from linear structures (× 195 000).

without urea and with high-speed centrifugation (150 000 × g for 60 min), the supernatant was composed essentially of monodispersed clathrin. By altering the solution's pH, clathrin again formed baskets and/or filaments. These steps can be repeated several times with identical results.

Discussion

The finding that clathrin solutions exhibited intrinsic viscosity suggests that the asymmetrically shaped molecules, the axial ratio of which is approx. 2.7 (unpublished data), polymerized into reticular or linear structures as some other structural proteins do, e.g., tubulin, actin, myosin, tropomyosin. Our postulate was proved to be correct when the protein solutions were examined by electron microscopy with negative staining, and filaments or bundles of filaments were found. Following these considerations, a rapid drop in viscosity reduction of the pH from above 7.0 to 6.5 or 6.0 was expected. At this pH, the new conformation of the polypeptide molecules evidently favored their assembly into basket structures. This was confirmed by electron microscopy.

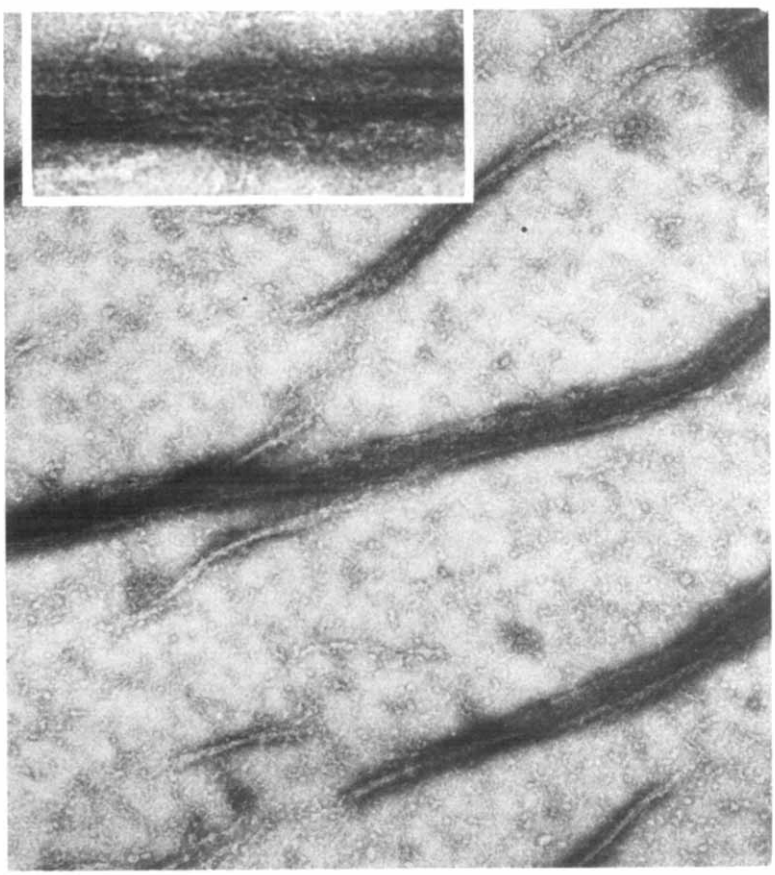

Fig. 7. Clathrin as in Fig. 6, 15 min after raising the pH to 7.5. Filamentous bundles of clathrin intra- and intercross-linked. Baskets were absent. Inset: a larger detail of clathrin forming a filamentous bundle (× 190000).

Apparently, when assembled in this manner, the clathrin molecules offered reduced viscosity because of less frictional resistance or diminished shear force during their passage through the viscometer's capillary tube.

Presently, we do not know what percentage of clathrin's reticular network closes to form sealed cages or baskets. It is likely that a considerable amount of clathrin remains assembled as units of pentagonal and hexagonal shapes joined as sheets lying flat on the Formvar layer of the copper grid away from detection. This undetected portion of clathrin may lack closure strength, i.e., the capacity to form closed baskets. It is possible that cofactors are needed for closure and that they have been dissociated during the purification of clathrin.

It is apparent from out results that the basket- or cage-like formations of clathrin produced a light-scattering effect with increased turbidity as the obvious result. Although turbidity increases and viscosity drops occurred rapidly, within 1—3 min, we were able to visualize what appeared to be intermediate stages in the reversible transformation of filaments into baskets. Apparently, these baskets formed in places where lateral attachments between

filaments existed. If so, a certain rearrangement of clathrin molecules upon alteration of the proton concentration could induce basket formation from loose lateral attachments of clathrin bundles.

Decrease in turbidity occurred with the simultaneous disappearance of baskets and the appearance of bundles of filaments. If clathrin is not subjected to passage through a capillary tube for the viscometric determinations, an adjustment of its pH to 7.5 will result in the formation of single filaments which are loosely spread. Apparently, the continuous passage of clathrin through the capillary tube induced the arrangement of loose filaments into thick bundles. Often filaments formed spontaneously by standing at room temperature or as a result of raising the pH of the protein solution from values below 7 to 7.5.

If extrapolation of clathrin properties determined in vitro apply to their presence and behavior in the cells' cytoplasms, then when the proper stimuli and proton environment are provided, clathrin may form baskets around a given membrane area, constricting it, thus inducing the formation of a coated vesicle. The coat of these vesicles seems sturdy for these functions, surviving the effects of high centrifugal forces, fixation and staining needed for their visualization at the electron microscope. A vesicle protected by a lattice may be resistant to stresses and premature fusion with the abundant cytoplasmic cisternae. A far-reaching consequence of clathrin's newly described properties is that this particular protein could interact in vivo with the actin cytoskeleton [8]. In this way, the coated vesicle may arrive at its proper destination, possibly near the nucleus where a neutral pH could disassemble the lattice permitting the vesicle to fuse. Undoubtedly, further work will be required to verify precisely the overall role of clathrin in the living cell.

Acknowledgements

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