Clathrin-associated proteins contain bound nucleotide

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Clathrin-associated proteins purified from bovine brain exhibited an ultraviolet spectrum with absorbance maximum at 256 nm and were found to contain tightly bound nucleotide. This nucleotide was identified as AMP and/or ADP by thin-layer and high-performance liquid chromatographic analyses. The phosphorylation state of the bound nucleotide varied with storage conditions, suggesting that exchange with ATP might occur while a molar ratio of two nucleotides per protein molecule is maintained. This nucleotide binding site may play a role in the functions of clathrin-associated proteins.

Clathrin-associated protein *Nucleotide* *UV spectrum* *Bovine* *High-performance liquid chromatography*

1. INTRODUCTION

The process of coated pit/vesicle formation is of major interest [1–3]. Understanding this process at the molecular level requires elucidation of how the lattice-forming protein, clathrin, interacts with itself and other proteins for coat assembly and disassembly [4–6]. Clathrin exists as a trimer in solution, tightly associated with polypeptides of about 36000 and 33000 M_r [7] which were isolated and partially characterized in our laboratories [8,9]. Clathrin-associated proteins (CAPs) demonstrated calcium-dependent affinity for calmodulin [8] and co-localized with clathrin at both the light and electron microscope levels utilizing specific antibodies to CAPs elicited in rabbits [9,10]. We here show that CAPs contain bound nucleotide. These data suggest that an energy-dependent mechanism modulated via CAPs may exist in the clathrin complex.

2. MATERIALS AND METHODS

Clathrin and CAPs were extracted from coated vesicles prepared by centrifugation on discontinuous sucrose gradients and the clathrin–CAPs complex was purified by column chromatography on Sepharose 4B columns as in [4,11]. The CAPs were prepared either directly from the crude clathrin extract or from purified clathrin as in [8].

The nucleotide content of clathrin and CAPs preparations was initially determined by thin-layer chromatography (TLC) of perchloric acid-treated samples using half-sized sheets of polyethyleneimine cellulose (Brinkmann Instruments) and solvent systems of 0.5 M LiCl containing 2 N formic acid or 0.25 M LiCl. The former system was used for the separation of mono-, di- and triphosphonucleosides and the latter for the separation of cyclic and non-cyclic monophosphonucleosides. To increase the sensitivity, subsequent analyses were performed on an Altex model 100 high-performance liquid chromatography (HPLC) using a Partisil SAX anion exchange column (25 × 4.5 mm). The instrument was run isocratically at a flow rate of 0.5 ml/min using 0.3 M KH_2PO_4 (pH 4.5) to detect nucleoside mono- and diphosphates and 0.75 M KH_2PO_4 (pH 4.5) to detect nucleoside triphosphates [12].

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3. RESULTS

3.1. *UV spectrum of purified CAPs*

The sodium dodecyl sulfate polyacrylamide gel electrophoretic profile of a number of CAPs preparations is illustrated in fig.1. The reproducibility of the procedure is evident in the gel patterns, which also illustrate the high degree of purity obtained. When such a preparation was scanned at about 0.1 mg/ml (determined by the Lowry procedure using bovine serum albumin as the standard) the UV absorbance spectrum showed a maximal absorbance at 255 nm, in contrast to clathrin, which had a maximum at 280 nm (fig.2). To determine if the spectral shift was due to the presence of nucleotide, the protein was denatured by addition of perchloric acid and the UV spectrum again determined; this indicated the presence of nucleotide and suggested that it consisted mainly of an adenine moiety.

3.2. *Detection of adenosine mono- and diphosphate*

Several CAP preparations were acidified with perchloric acid and the supernatants obtained after centrifugation in a Brinkman microfuge for 2 min

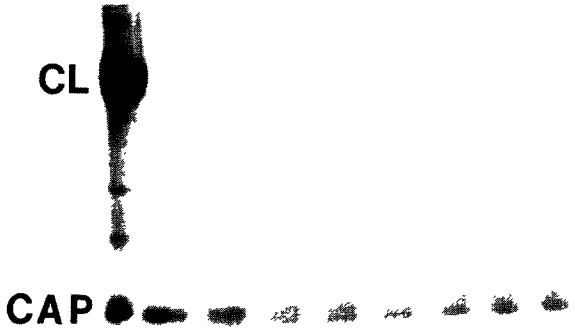

Fig.1. SDS–PAGE analysis of various protein preparations used. Lane 1, 75 μg of clathrin preparation; lanes 2–9, 15 μg of a number of different CAPs preparations, all of which showed high purity, although the CAPs were not always resolved as a doublet.

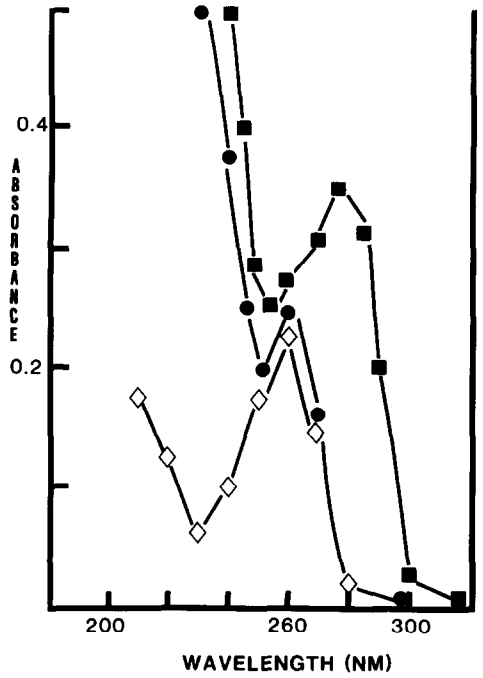

Fig.2. Comparison of the UV spectrum of clathrin and CAPs. The UV spectrum of a 0.6 mg/ml solution of clathrin in 20 mM Tris (pH 7.5) buffer containing 0.5 mM $MgCl_2$ and 7 mM 2-mercaptoethanol. The spectrum was obtained with a Beckman 25 kinetic spectrophotometer scanned at 20 nm/min. The UV spectrum of a 0.1 mg/ml solution of CAPs in the same buffer and run under identical conditions. The UV spectrum of the neutralized supernatant was obtained from perchloric acid treatment of this CAP solution. A similar spectrum is obtained if clathrin is subjected to perchloric acid treatment.

were neutralized by the addition of 5 N KOH and again centrifuged to remove insoluble $KClO_4$. The supernatant was spotted onto polyethyleneimine cellulose plates and developed in either 0.25 M LiCl or 0.5 M LiCl containing 2 N formic acid. These analyses showed that some preparations contained ADP, others AMP and in some instances a mixture of both. To exclude the possibility that the acid treatment and/or analysis time might have resulted in partial hydrolysis of the nucleotide a more quantitative and sensitive method of analysis was sought.

The high-performance liquid chromatographic analysis of protein extracts gave increased sensitivity using the following protocol for CAPs and

clathrin preparations. About 0.5 mg of CAPs or 3 mg clathrin in 0.5 ml of 20 mM Tris buffer (pH 7.5) was treated with 30 μl of concentrated perchloric acid, left on ice for 5 min, centrifuged and the supernatant containing the nucleotides was then extracted with 750 μl of a 3:1 Freon-Alamine mixture for 30 min on ice with occasional vortexing [13]. The suspension was centrifuged in a microfuge, the water (upper) layer was removed, neutralized with KOH and stored frozen until analyzed. The results of such analyses are given in table 1. When protein preparations were stored at 4°C and analyzed 7–14 days later the nucleotide composition shifted from ADP to a mixture of ADP and AMP or to AMP. Quantitation of the nucleotide present in several preparations resulted in a molar ratio between 1 and 2, suggesting that up to 2 mol of nucleotide are bound since the resin treatment is potentially capable of removing some bound nucleotide.

Table 1

Analysis of nucleotide content by HPLC

Sample	Nucleotide present	Total (nmol)	Molar ratio nucleotide/ protein
CAP_1	ADP	3.5 ± 0.2	1.3
CAP_2	ADP	5.2 ± 0.3	1.4
CAP_3	ADP	1.5 ± 0.2	1.8
CAP_4	AMP	5.1 ± 0.2	1.9
CAP_5	AMP	6.2 ± 0.3	2.1
3 Days storage at 4°C			
CAP_1	ADP, AMP	3.0 ± 0.2	1.3
CAP_2	ADP, AMP	4.7 ± 0.3	1.2
CAP_3	ADP, AMP	1.2 ± 0.2	1.6
7 Days storage at 4°C			
CAP_1	AMP	3.1 ± 0.2	1.3
CAP_2	AMP	4.7 ± 0.3	1.2
CAP_3	AMP	1.2 ± 0.2	1.6

Concentration of nucleotide was calculated from standard curve generated with known concentrations of AMP, ADP and ATP. The areas under peak for both known and unknown samples were integrated by triangulation. Protein concentration of each sample was determined before perchloric acid treatment, but after treatment with Dowex resin to ensure removal of unbound nucleotide. This treatment may remove some bound nucleotide, therefore true molar ratio is probably somewhat higher

4. DISCUSSION

Preparations of clathrin and CAPs have been shown to contain tightly bound nucleotide by 3 methods of analysis: UV spectroscopy, TLC and HPLC. The nucleoside phosphates identified have been ADP and AMP. Since storage of CAPs or clathrin at 4°C for several days resulted in a large decrease of ADP and an increase in levels of AMP, it appears that nucleotide hydrolysis occurred and possibly that ATP was, in fact, the original bound nucleotide. The nucleotide appears tightly bound and partially non-exchangeable because the protein preparation is performed without exogenous nucleotide. Chromatography on Sepharose 4B of the proteins should have removed any unbound nucleotide. In addition, all concentrated protein fractions underwent extensive dialysis before storage in the absence of exogenous nucleotide. As an additional precautionary step, these protein preparations were pretreated with Dowex-Ag 1 × 4 resin at pH 7.5 in a batch procedure immediately before analysis for nucleotide. The resin treatment was capable of decreasing 20-fold the concentration of free nucleotide present at one millimolar and therefore found effective in removing any free nucleotide still remaining in the protein preparation. Nevertheless, AMP was detected on clathrin and CAP preparations so far analyzed. On occasion, ADP was also detected, and several CAP preparations contained initially only this nucleotide. Within a few days of storage at 4°C, the ADP was converted to AMP. This observation suggests that extra precautions to stabilize the nucleotide present, such as inclusion of ATP during the purification procedure and more rapid storage of protein preparations in the freezer might result in the recovery of bound nucleotide as ATP.

Preliminary data suggest that 2 mol of nucleotides are bound (see table 1) and that 1 mol is exchangeable with exogenously added ATP (unpublished). These observations have important implications in the role of CAPs in the decoating process in lieu of the recent report of a cytosolic decoating factor which appears to become a functional ATPase only in the presence of the triskelion, possibly exerting its activity upon or in

concert with the CAPs [14,15]; a possibility supported by the requirement for intact CAPs to allow in vitro assembly of triskelions into baskets [16,17].

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