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Limited Proteolytic Digestion of Coated Vesicle Assembly Polypeptides Abolishes Reassembly Activity

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We have previously identified a fraction containing several assembly polypeptides (AP) that promotes reassembly of clathrin into vesicle-free coat structures [Zaremba S, Keen JH: J Cell Biol 97:1339, 1983]. The AP are prepared from purified bovine brain-coated vesicles by extraction with 0.5 M TRIS-HCl followed by Sepharose CL-4B column chromatography. Centrifugation in sucrose gradients under nonassembly conditions supports earlier observations suggesting that four active polypeptides in the AP preparation, of M_r ~110,000, 100,000, 50,000, and 16,500 are present in a discrete complex that is incorporated as a unit into reassembled coats. The 16,500-dalton polypeptide does not coelectrophorese with authentic bovine brain calmodulin and does not exhibit calmodulin's Ca^{2+}-induced shift in electrophoretic mobility. When the partially purified AP fraction was digested with elastase, the M_r ~110,000 and 100,000 polypeptides were rapidly degraded with little or no effect on the M_r ~50,000 and 16,500 bands. This treatment abolished the in vitro coat-forming ability of the AP fraction and the loss of activity closely parallels the loss of the M_r ~100,000 band. Disappearance of the M_r ~110,000 and 100,000 bands is accompanied by the generation of new bands at M_r ~76,000 and 65,000. When the elastase-treated AP is examined by sucrose gradient sedimentation in nonassembly buffers, the new bands continue to cosediment with the M_r ~50,000 and 16,500 polypeptides. This indicates that the elastase digestion has cleaved off a fragment of the M_r ~110,000 and 100,000 bands, leaving behind a truncated, inactive AP complex. A protein kinase activity has been detected in coated vesicle preparations that utilizes the 50,000-dalton AP as its preferred substrate [Keen JH, Zaremba S: J Cell Biol 97:174a, 1983]. Elastase treatment does not abolish this activity, indicating that the kinase by itself is not sufficient for maintaining reassembly activity.

Key words: coated vesicles, clathrin, assembly polypeptides, coat reassembly, elastase, protein kinase

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Coated membranes are ubiquitous, subcellular organelles observed in eukaryotic cells. They have been implicated in a variety of processes, including receptor-mediated endocytosis [1,2], intracellular transport or processing of proteins [3,4], and membrane recycling [5,6]. It has been hypothesized that all of these processes involve rapid coating and uncoating of local regions of membranes.

The major protein of coated vesicles, clathrin, [7] is composed of a heavy chain of M_r ~180,000 and a doublet of light chains in the M_r ~30,000–35,000 range in a tightly bound complex [8–10]. Clathrin can be extracted and purified and, under certain conditions, can reassemble into latticework coats that appear identical to those of coated membranes in vivo [11–14]. However, in an earlier report [15] we showed that in the buffer used for isolation of stable coated vesicles, the formation of clathrin coats required the presence of a protein fraction that contained assembly polypeptides (AP).

The AP fraction was obtained by extraction of coated vesicles with TRIS-HCl and separated from clathrin by chromatography on columns of Sepharose CL-4B. Electrophoresis on polyacrylamide-sodium dodecyl sulfate gels revealed a number of polypeptides in the AP fractions. Three of these, at M_r ~110,000, 100,000, and 50,000, are incorporated into the reassembled coats in stoichiometric amounts. The information necessary for correct reassembly seems to reside in these polypeptides since coat formation persisted after several cycles of disassembly and reassembly using only clathrin and AP [15]. The actual molecular roles of the AP polypeptides are unknown. Recently we have shown [16; manuscript in preparation] that the M_r ~50,000 assembly polypeptide is the phosphorylated substrate detected in coated vesicle preparations by others [17–19] and that the protein kinase activity cochromatographed with the AP.

In the present report we describe further features of AP structure and function. A 16,500 M_r AP polypeptide has been observed that is incorporated into reassembled coats along with the three AP polypeptides described earlier [15]. AP polypeptides are bound together in a complex even under nonassembly conditions. In addition, elastase digestion of the AP complex results in a loss of reassembly activity, concomitant with a specific cleavage of the M_r ~110,000 and 100,000 AP polypeptides. Such treatment, however, does not abolish the aforementioned protein kinase activity, indicating that phosphorylation of the M_r ~50,000 polypeptide is, by itself, not sufficient to induce reassembly.

MATERIALS AND METHODS

Coated vesicles were isolated and clathrin and AP were purified as described previously [13]. Reassembly was quantitated as described previously [15]. Protein was determined by the use of a Coomassie G-250 dye binding assay [20].

Phosphorylation was performed with coated vesicles in 0.1 M sodium (2 [N-morpholino]ethanesulfonate) (MES), 1 mM ethylene glycol-bis-(p-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA), 0.5 mM $MgCl_2$, 0.02% sodium azide, pH 6.5 (buffer A) by addition of $\gamma[^{32}P]$- adenosine-5′-triphosphate (ATP) to a final concentration of 1.0 μM. The $[^{32}P]$-ATP stock reagent was made by dilution of $[^{32}P]$-ATP with unlabeled ATP and was at a concentration of 7.64 μM and specific activity of 331 Ci/mmol. Coated vesicles were incubated with $[^{32}P]$-ATP for 10 min at 0°C. Phosphorylation was terminated by boiling with electrophoresis buffer unless otherwise noted.

Electrophoresis was performed in slab gels of 5–15% gradient in acrylamide unless otherwise noted [21]. Where necessary, samples were concentrated by precipitation with ice-cold 10% trichloroacetic acid in the presence of 0.1% deoxycholate. Gels were stained with Coomassie blue, destained, and scanned by densitometry as described previously [15]; destained gels were dried and exposed to Kodak XAR-5 film for autoradiography.

Incubation with elastase as described in the text was terminated by boiling with electrophoresis buffer or by addition of 0.2 M phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO) to a final concentration of 1 mM. When the time course of digestion was monitored, aliquots for reassembly assays were stopped by addition of human α_2- macroglobulin to a final concentration of 1.3 μM. Control experiments confirmed that such treatment inactivated elastase within 10 sec of addition (data not shown).

Sucrose gradient sedimentation of unassembled control and elastase-treated AP was performed at 4°C in a Beckman SW40 rotor centrifuged 50 hr at 38,000 rpm. Gradients were 10–30% sucrose (w/w) in buffer B (buffer A:1.0 M TRIS-HCl, pH 7.0 [1:1, v/v]) and bovine serum albumin (4.6 S), human immunoglobulin G (7 S), and catalase (11.2 S) were included as sedimentation markers. After fractionation into 0.92-ml fractions, protein was assayed by fluorescence [15].

The materials used in coated-vesicle isolation, in clathrin and AP purification, and in electrophoresis were obtained as described previously [15]. Bovine serum

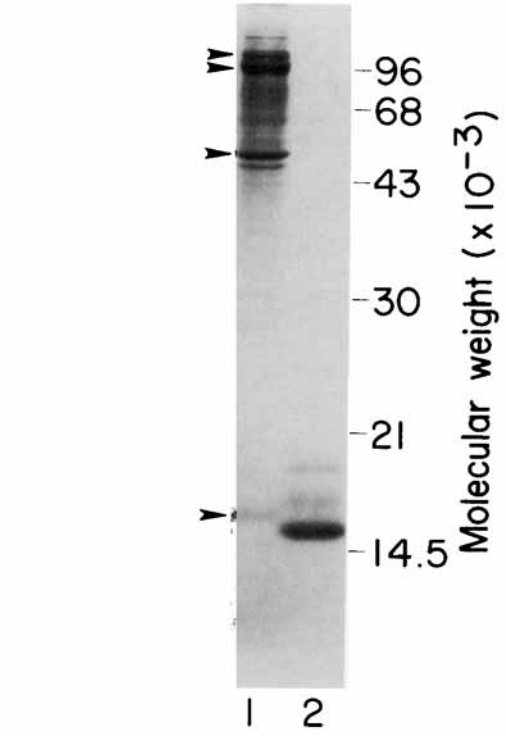

Fig. 1. Comparison of AP M_r ~ 16,500 polypeptide with bovine brain calmodulin by SDS-polyacrylamide (12.5%) gel electrophoresis. Lane 1) 62 μg AP; lane 2) 11 μg bovine brain calmodulin. Arrowheads indicate the position of assembly polypeptides.

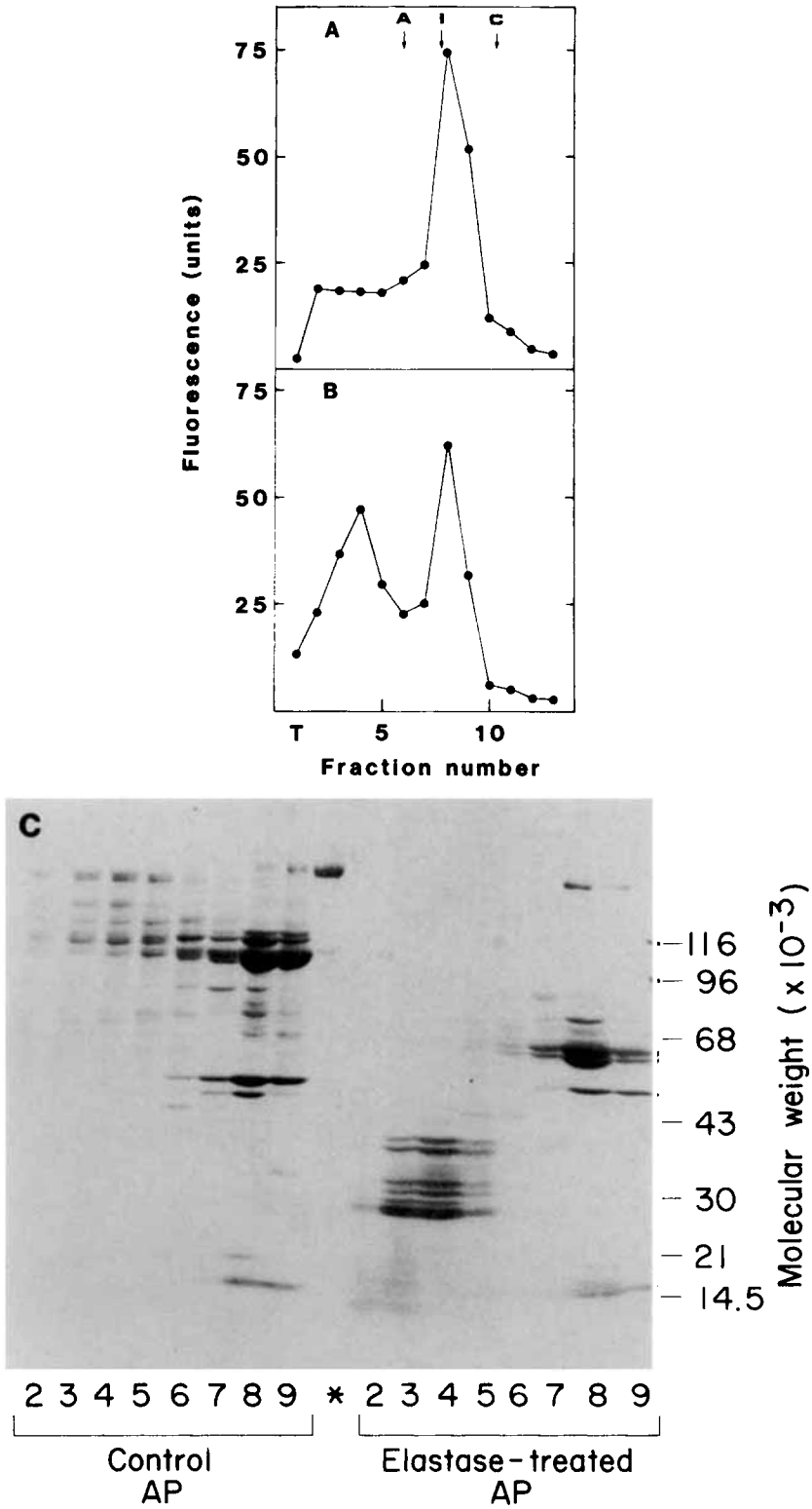
A
A
I
C
75
50
25
B
75
50
25
Fluorescence (units)
T
5
10
Fraction number
C
116
96
68
43
30
21
14.5
Molecular weight (x 10⁻³)
2 3 4 5 6 7 8 9 * 2 3 4 5 6 7 8 9
Control AP
Elastase-treated AP

Fig. 2.

albumin, immunoglobulin G, PMSF, and ATP were supplied by Sigma Chemical Company (St. Louis, MO). Bovine brain calmodulin was a generous gift of C. Klee of the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health. Elastase and human α_2-macroglobulin were products of Boehringer-Mannheim (Indianapolis, IN). Catalase was provided by Pharmacia P-L Biochemicals (Piscataway, NJ) and the Coomassie G-250 dye-binding protein reagent was from Pierce (Rockford, IL). γ [^{32}P]-labeled ATP (4,500 Ci/mol) was obtained from ICN (Irvine, CA). DMSO was certified, spectranalyzed grade (Fisher Scientific, Pittsburgh, PA). All other chemicals were reagent grade and deionized water was used in all procedures.

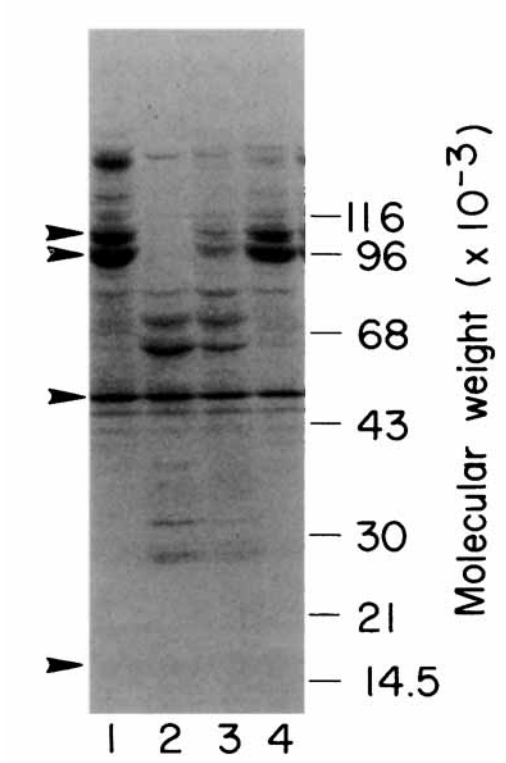

Fig. 3. Elastase digestion of AP fraction. Fractions of AP from Sepharose CL-4B column (0.31 mg/ml final concentration) were incubated for 10 min at room temperature with the following additions: lane 1) buffer B; lane 2) 5 μg/ml elastase in buffer B; lane 3) 0.5 μg/ml elastase; lane 4) 0.05 μg/ml elastase. Incubations were stopped by boiling in electrophoresis buffer. Samples were analyzed on a 5–20% polyacrylamide gel. Arrowheads indicate the position of assembly polypeptides.

Fig. 2. Upper: Sucrose gradient sedimentation of control and elastase-treated AP. Fractions of AP (1.66 mg/ml) in buffer B were incubated 1 hr at room temperature with buffer B (A) or 0.05 mg/ml elastase (B). Reactions were stopped with 1 mM PMSF. Samples were subjected to sucrose gradient centrifugation in buffer B, fractionated and assayed for protein by fluorescence. Positions of bovine serum albumin (A), immunoglobulin (I), and catalase (C) sedimentation markers are indicated. Lower: Indicated fractions from gradients were concentrated and electrophoresed on 5–15% acrylamide gradient gel. Lane marked with asterisk (*) contained purified, reassembled 250s to identify the AP polypeptides involved in reassembly.

RESULTS

Previous studies have shown that proteins released from coated vesicles can be fractionated on gel filtration columns separating clathrin from other proteins [13]. One minor protein peak was absolutely required for in vitro clathrin reassembly in the vesicle isolation buffer. Three polypeptides from this peak, termed *assembly polypeptides* (AP), were shown to be specifically incorporated into reassembled coats in a stoichiometric manner [15]. These polypeptides were identified on gels as having molecular weights of 110,000, 100,000, and 50,000. Using gels capable of resolving low molecular weight species, it was seen that reassembled coats isolated on sucrose gradients (data not shown) also contained an M_r ~16,500 polypeptide. This band is also detectable in AP fractions obtained by gel filtration of coated vesicle extracts (Fig. 1, lane 1) used as starting material for coat reassembly experiments. The 16,500 M_r species does not coelectrophorese with purified bovine brain calmodulin (Fig. 1, lane 2), nor does it exhibit the characteristic Ca^{2+}-EDTA electrophoretic mobility shift demonstrable with the latter (data not shown).

In addition to eluting in a single peak on gel filtration and being incorporated in constant stoichiometry in reassembled coat structures [15], we now report that the four assembly polypeptides cosediment on sucrose gradients under nonassembly conditions (Fig. 2A,C left). By comparison with sedimentation standards, the protein peak on sucrose gradients that contains the AP polypeptides corresponded to a sedimentation coefficient ($S_{20,w}$) of 7–8 S.

In the course of screening the AP fraction for sensitivity to selected proteases, it was found that elastase rapidly digested the 110,000- and 100,000- dalton AP bands while leaving the 50,000- and 16,500-dalton bands unchanged (Fig. 3). Two new

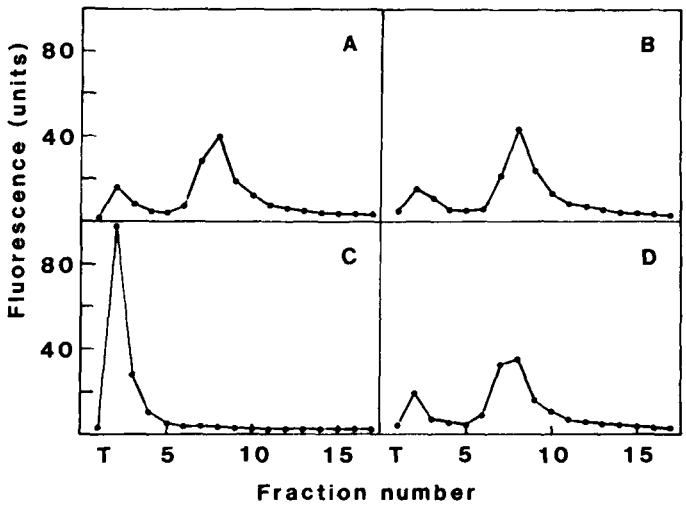

Fig. 4. Effect of elastase digestion of AP on reassembly activity. Reassembly was assessed by sedimentation in sucrose gradients in buffer A. AP (0.42 mg/ml) was incubated for 1 hr at room temperature with buffer B alone (A); buffer B followed by addition of 1 mM PMSF (B); elastase (6.7 μg/ml) in buffer B, reaction terminated with PMSF (C); PMSF-treated elastase (6.7 μg/ml) reaction terminated with PMSF (D). After incubations, clathrin was added (0.30 mg/ml) followed by dialysis against reassembly buffer and sedimentation.

polypeptide bands appeared in response to elastase treatment at molecular weights of 76,000 and 65,000. Their staining intensity on gels suggested that they were fragments of the 110,000- and 100,000-dalton AP bands. After elastase treatment of AP, it was found that the newly formed 76,000- and 65,00-dalton polypeptides still cosedimented with the 50,000- and 16,500-dalton polypeptides, indicating that the new bands were still associated in a complex (Fig. 2B,C right). The sedimentation position of this truncated form of AP was indistinguishable from that of native AP on our sucrose gradients.

To determine whether elastase-digested AP was still capable of reassembly it was incubated with clathrin and analyzed for reassembly as indicated in Figure 4. The digested AP was totally incapable of reassembly (Fig. 4C). Parallel controls showed that the loss of activity was not due to the PMSF (Fig. 4B) and could be prevented by prior incubation of elastase with PMSF (Fig. 4D). The latter observation ruled out any nonproteolytic contaminant in the elastase preparations as the factor responsible for destruction of reassembly activity.

Examination of the reassembly mixtures on dodecyl sulfate-acrylamide gels (Fig. 5) provided an internal control for the specificity of the digestion. Of the polypeptide bands known to be involved in reassembly, only the 110,000- and

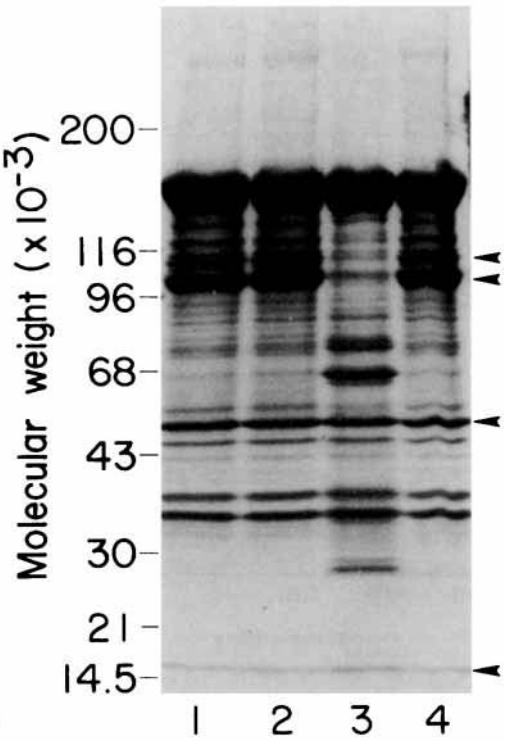

Fig. 5. Effect of elastase digestion of AP on reassembly activity: analysis by gel electrophoresis. Aliquots (60 μg protein) of the reactions described in Figure 4 after termination of incubation, addition of clathrin, and dialysis against buffer A were electrophoresed on a 5–15% gradient acrylamide gel. Lanes 1-4 correspond to the reactions described in panels A–D (Fig. 4), respectively. Arrowheads indicate the position of assembly polypeptides.

100,000-dalton species were cleaved (Fig. 5, lane 3). Incubation of AP with prepoisoned elastase, which protected the reassembly activity (Fig. 4D) also prevented the destruction of these two polypeptides (Fig. 5, lane 4). Clathrin light chains are known to be extremely sensitive to elastase [10,22]. In the present experiment it could be seen that elastase digestion was terminated prior to the addition of clathrin, since the light chains remained undigested throughout the subsequent dialysis (Fig. 5, lane 3).

In a subsequent digestion experiment, aliquots were taken at intermediate time points and examined for reassembly activity and polypeptide composition. When these two parameters were plotted as a function of digestion time, a close correlation was observed between the reassembly activity and the levels of the remaining 100,000-dalton polypeptide as measured by densitometry (Fig. 6). It was observed that at intermediate time points in the digestion a polypeptide of 87,000 daltons appeared and was then itself degraded. The time course for the disappearance of this band was much slower than that of the loss of reassembly activity.

Previous investigators have shown that coated-vesicle preparations contain a protein kinase activity that phosphorylates an $M_r \sim 50,000$ polypeptide [17–19] which has been shown to be immunologically related to microtubule-associated τ protein. We have shown [16] that this phosphoprotein is the $M_r \sim 50,000$ polypeptide involved in AP-mediated coat formation. When elastase-treated coated vesicles were incubated with $\gamma[^{32}P]$-ATP, protein kinase activity was seen to be elastase-resistant (Fig. 7, lane 3). No intact $M_r \sim 110,000$ and 100,000 AP bands remained after such treatment

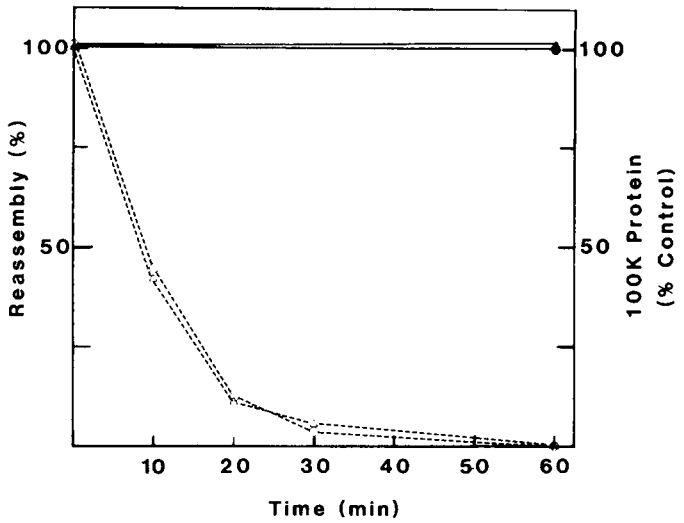

Fig. 6. Relationship between loss of reassembly activity and loss of AP $M_r \sim 100,000$ polypeptide. AP (0.42 mg/ml) was incubated for the indicated times with buffer B (solid symbols or lines) or elastase (6.7 μg/ml, dashed lines or open symbols). At each point one aliquot was boiled for electrophoresis and the amount of the 100 K dalton assembly polypeptide, determined relative to the undegraded 50 K assembly polypeptide in the same sample, was quantitated by densitometry (triangles). Digestion of a second aliquot (75 μg) at each time point was terminated by addition of α_2 macroglobulin, followed by addition of clathrin (65 μg), dialysis against buffer A, and assessment of reassembly (circles) by gradient ultracentrifugation. All values are expressed as percentage of control at zero time.

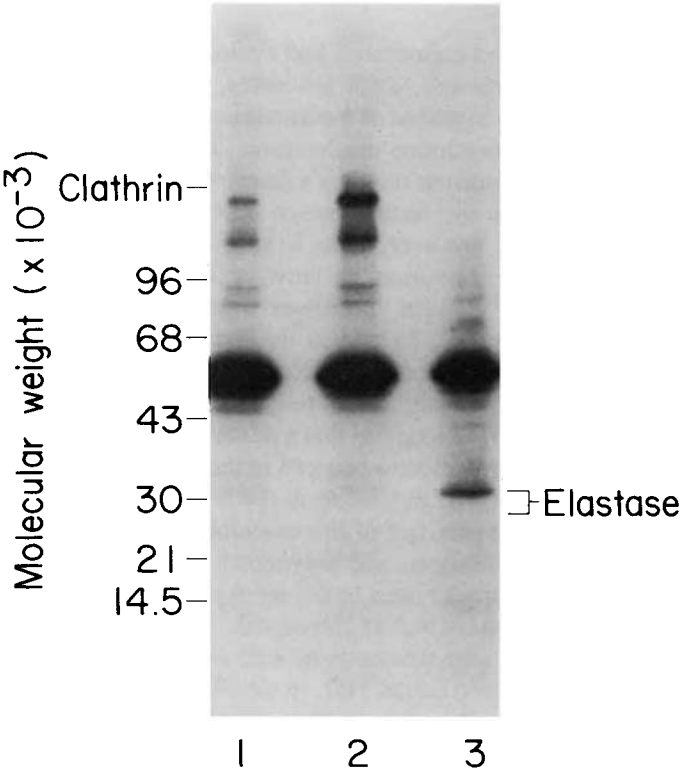

Fig. 7. Effect of elastase digestion of coated vesicles on protein kinase activity. Purified coated vesicles (0.77 mg/ml) were incubated for 1 hr at room temperature with buffer A (lane 1), 3 μg/ml PMSF-treated elastase in buffer A (lane 2), or 3 μg/ml elastase in buffer A (lane 3). Reactions were terminated with PMSF followed by addition of γ[^{32}P]-ATP. After 10 min at 0°C samples were boiled and electrophoresed and the dried gel was subjected to autoradiography. The label *elastase* indicates the electrophoretic migration positions of major polypeptides in commercial elastase preparations, as detected by protein staining of the gel.

(data not shown), indicating that neither intact protein was required for phosphorylation. Since phosphorylation was maintained under conditions that have been shown to abolish reassembly, it can be concluded that phosphorylation of the M_r ~50,000 polypeptide is, by itself, not sufficient for coat assembly.

In addition to the major M_r ~50,000 phosphoprotein (pp50), phosphorylation of elastase-treated coated vesicles results in the formation of a 27,000-dalton (pp27) phosphoprotein (Fig. 7, lane 3). The new phosphoprotein did not coelectrophorese with any major band in the elastase preparations nor did it appear after phosphorylation of samples containing PMSF-inactivated elastase (Fig. 7, lane 2). Therefore, pp27 must represent a new substrate for the kinase generated by elastase digestion of coated vesicle preparations. It is not certain whether pp27 is generated from pp50 or from some other elastase-sensitive polypeptide. However, it appears likely that the new phosphoprotein is derived from pp50 since less [^{32}P] was incorporated into the AP M_r ~50,000 polypeptides in elastase-treated coated vesicles than in control preparations (compare Fig. 6, lanes 1 and 3).

DISCUSSION

The participation of coated membranes and coated pits in a variety of cellular processes has been well-documented. Such processes, including receptor-mediated endocytosis, transport of newly synthesized macromolecules, and retrieval of synaptic membranes, are all dynamic membrane mechanisms. Therefore, a complete understanding of coated-membrane function requires a description of the molecular contacts (and enzymology) employed in the formation and dissociation of coat structures in vivo. On the molecular level, however, little is known about the interactions that determine coat structure and formation in vitro or in vivo. The major protein component of isolated coats, clathrin, possesses a unique three-legged structure (triskelion) that is thought to reflect its role as the basic subunit in the coat lattice. Although purified clathrin can reassemble into coat structures in vitro, it does so only under restrictive conditions (low salt, pH <6.7, millimolar Ca^{2+} concentrations) [10–14]. In an earlier report [15] we showed that a minor protein fraction from coated-vesicle extracts enabled coat formation to proceed in the buffer used to isolate stable-coated vesicles, and at cellular pH, as well as in the nonphysiological buffers noted above. The coats formed in the presence of this assembly polypeptide fraction have a unique size, sedimentation coefficient, and polypeptide composition. In the present report, we describe further characteristics of the AP fraction.

It was observed that an $M_r \sim 16{,}500$ polypeptide from the AP fraction was also incorporated into reassembled coat structures as well as the $M_r \sim 110{,}000$, 100,000, and 50,000 polypeptides described earlier [15]. In view of the many reports describing the functional association of calmodulin with coated membranes [23,24] and its binding to purified coated vesicles [25–27] it was of interest to find out whether the $M_r \sim 16{,}500$ polypeptide was identical to calmodulin. Our experiments indicated that the AP polypeptide did not coelectrophorese with bovine brain calmodulin nor did it demonstrate the typical conformational change from Ca^{2+}- to EDTA-containing buffers detected as a shift in electrophoretic mobility.

We have also presented evidence suggesting that these four AP polypeptides are present in a complex under nonassembly conditions prior to reassembly into coats. When centrifuged on a sucrose gradient in nonassembly buffers, these AP polypeptides cosedimented in one peak to a position corresponding to an $S_{20,w} \simeq 7$ S. The AP complex sedimented at a slightly slower rate than clathrin triskelions ($S_{20,w} =$ 8.1-8-4) [8,14]. This finding is in agreement with other reports that the $M_r \sim 100{,}000$ and 50,000 polypeptides of coated-vesicle extracts cochromatographed on gel permeation chromatography columns [9,19] and on hydroxylapatite columns [28] and with recent observations that these polypeptides are present in constant stoichiometry in highly purified preparations of the AP [29].

In the present report we have also shown that elastase digestion of column-purified AP selectively degrades the largest polypeptides present, the M_r 110,000 and 100,000 bands. Two new species are generated at $M_r \sim 76{,}000$ and 65,000. We believe the latter are fragments of the degraded AP bands since the only polypeptides of sufficient size and staining intensity to generate the M_r 76,000 and 65,000 proteins are the AP M_r 110,000 and 100,000 polypeptides. We have noted that microheterogeneity exists in the $M_r \sim 110{,}000$–100,000 region in the active AP complex [29], a finding also observed by others [28]. As a result, we cannot yet assign exact precursor-product relationships between the degraded AP polypeptides and the elas-

tase-generated fragments. When elastase-treated AP was sedimented on a sucrose gradient in nonassembly buffers, the new M_r 76,000 and 65,000 polypeptides cosedimented with intact M_r 50,000 and 16,500 polypeptides. This is interpreted to mean that separate peptide fragments are cleaved from the M_r 110,000 and 100,000 bands, leaving behind a truncated AP complex whose $S_{20,w}$ does not differ substantially from that of control AP.

The striking result of elastase treatment of AP is the loss of its ability to mediate coat reassembly. This loss of activity correlates well with the destruction of the M_r 100,000 polypeptide and suggests the existence of a domain of the M_r 100,000 polypeptide that binds to clathrin triskelions. Such a binding role for at least one of the AP polypeptides has been tentatively proposed as a result of studies that have measured the binding of purified triskelions to stripped vesicles [30]. It was noted that the specific high-affinity binding was reduced by proteolytic treatment of the stripped vesicles and that such treatment resulted in the disappearance of an M_r 100,000 polypeptide, among others. The extraction procedure used in those studies [30] was such that the polypeptides we now refer to as AP were not removed from the vesicles. Those authors also make the observation that when stripped vesicles were treated with elastase at low concentrations, the M_r 100,000 polypeptides are lost more rapidly than the M_r 50,000 protein bnads.

Alternatively, the clathrin binding domain may be elsewhere on the M_r 100,000 polypeptide, or even on another AP polypeptide, and rendered ineffective through some overall conformational change. The roles of the AP in general, and the M_r 100,000 polypeptide in particular, in coat reassembly and in anchoring of triskelions to a coated membrane are not mutually exclusive.

Elastase treatment of coated vesicle proteins also separates the reassembly process from the coated vesicle protein kinase activity. In purified coated vesicles, unfractionated extracts, and column-purified AP, the preferred substrate for the kinase is the M_r 50,000 polypeptide, pp50 [16]. The protein kinase activity was unaffected by elastase digestion and after such treatment pp50, the major phosphoprotein, was still the preferred substrate. Thus, intact $M_r \sim 100,000$ polypeptide is not necessary for kinase activity and the phosphorylation of pp50, while possibly necessary, is not by itself sufficient for AP to induce coat reassembly.

The role of the AP complex in in vitro coat formation has been demonstrated in this and previous reports [13,15] through the application of a number of biochemical methodologies. We have suggested that AP-mediated reassembly may be significant in vivo owing to its functioning under approximately physiological conditions. Based on the similarity in size between the in vitro AP-mediated coats and coated membrane profiles in Golgi stacks in vivo, we have raised the possibility that AP-mediated coats may represent a subset of coated membranes specifically associated with the Golgi region. Further studies are in progress to explore this hypothesis and to examine further the role of the AP in coated membrane structure and function.

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