The multimeric clathrin assembly proteins AP-1 and AP-2 with molecular masses of ~270 kDa and the monomeric clathrin assembly proteins AP-3 and auxilin with molecular masses of ~90 kDa catalyze the assembly of clathrin into artificial clathrin baskets under physiological conditions. We have now identified a much smaller ~20-kDa clathrin assembly protein in 0.5 M Tris, pH 7.0, extracts of bovine brain coated vesicles and purified it to near homogeneity. A polyclonal antibody against this protein did not cross-react with any of the other assembly proteins, and sequencing data suggest that this new protein is similar or identical to myelin basic protein (MBP). At a molar ratio of 3 molecules per clathrin triskelion, MBP catalyzes polymerization of clathrin into artificial baskets that appear structurally similar to the baskets assembled by the other assembly proteins. In addition, like the other baskets, the clathrin-MBP baskets are uncoated by hsp70. MBP represents a significant fraction of the total assembly protein activity present in 0.5 M Tris, pH 7.0, extracts of coated vesicles. It is not clear if it acts as an assembly protein in vivo, but because it is well characterized and easily available, MBP will be a useful protein to investigate the mechanism of clathrin assembly and disassembly in vitro.

Clathrin-coated vesicles are organelles involved in a number of cellular transport processes including receptor-mediated endocytosis, transfer of proteins from the trans-Golgi network to a pre-lysosomal compartment (reviewed in Ref. 1 and 2), and the recycling of synaptic vesicles at nerve terminals (3, 4). The main constituent of coated vesicles is clathrin, a triskelion composed of three ~190-kDa subunits and three ~23-27-kDa light chains (5, 6). In addition to clathrin, a number of proteins referred to as assembly proteins or adaptins copurify with coated vesicles (reviewed in Ref. 7). Under physiological conditions, these proteins induce polymerization of clathrin into artificial clathrin baskets that resemble coated vesicles (8). In addition, several of these proteins have been shown to bind to the cytoplasmic tails of receptors that are concentrated in clathrin-coated pits (9). To date, four different assembly proteins have been discovered. AP-1, AP-2, and AP-3 are multimere subunit complexes (10, 11) of M1, ~270,000; the former is localized to coated vesicles derived from the trans-Golgi apparatus and the latter to coated vesicles derived from the plasma membrane (12, 13). Two other assembly proteins, AP-3 (also referred to as AP-3) (14, 15) and auxilin (16) are monomeric, with M1 values of ~90,000. These proteins have been isolated only from neuronal cells and may be involved in the recycling of synaptic vesicles (16).

In vitro, both coated vesicles and clathrin baskets assembled from purified clathrin and assembly proteins are uncoated by hsp70 in an ATP dependent reaction (17, 18, 19). We recently found that, in addition to hsp70, a 100-kDa protein cofactor which appears to be auxilin is required for uncoating (20, 21, 36). Auxilin is heat sensitive, but while purifying auxilin we obtained evidence for a ~50-kDa heat-stable protein cofactor which, like auxilin, supported uncoating by hsp70. While attempting to purify this latter cofactor, we found that a protein fraction with a M1 of less than 50,000 was present in the 0.5 M Tris, pH 7.0, extracts of bovine brain coated vesicles which, rather than supporting uncoating by hsp70, actually suppressed it. Further examination of this fraction revealed it to contain a protein with strong clathrin polymerizing activity. We now report the purification of this 20-kDa protein and identify it as myelin basic protein (MBP).

### Experimental Procedures

#### Materials

- Superose-6 was obtained from Pharmacia Biotech Inc.
- Hydroxylapatite was from Calbiochem.
- Myelin basic protein (MBP) was from Calbiochem.
- The polyacrylamide gradient (4–20%) mini gels were from Integrated Separation Systems and the M1 standards used in the gels were from Bio-Rad.
- Endoproteinase Lys-C was from Boehringer Mannheim.
- Buffers—Buffer A contained 0.1 M MES, 0.5 mM MgCl2, 1 mM EGTA, 0.02% NaN3, pH 6.5. Buffer B contained 0.5 M Tris-HCl, 2 mM dithiothreitol, 1 mM EDTA, pH 7.0. Buffer C consisted of 20 mM imidazole, 2 mM magnesium acetate, 10 mM ammonium sulfate, 25 mM KCl, and 1 mM dithiothreitol, pH 7.0.

#### Preparation of Coated Vesicles, Clathrin, Assembly Proteins, Hsp70, and 100-kDa Cofactor

Coated vesicles were prepared according to the method of Prasad and Keen (22) which yielded a material rich in clathrin and assembly proteins similar to those obtained by using other published procedures. Clathrin, assembly proteins, and 100-kDa cofactor protein were obtained from the coated vesicles as reported previously (20).

Hsp70 was prepared as described previously (17) employing a method which is similar to the method of Schlossman et al. (18).

#### Clathrin Assembly Assay

Assembly assays (7) were performed by combining clathrin with either high molecular weight assembly proteins or ~20-kDa protein in Buffer B followed by overnight dialysis against Buffer A. The resulting solutions were then centrifuged in a TL-100 ultracentrifuge at 400,000 × g for 6 min. The amount of clathrin assembled was quantified by SDS-gel electrophoretic analysis of the solutions before and after centrifugation. The composition of the assembled baskets was evaluated by suspending the pellets in Buffer A and analyzing by SDS-gel electrophoresis. An aliquot of the sample was also negatively stained with 1% uranyl acetate and viewed in a Philips 410...
RESULTS

Superose-6 column chromatography of a 0.5 M Tris, pH 7.0, extract of coated vesicles yielded the characteristic elution profile reported previously (7) with a major peak of clathrin followed by a broad peak containing assembly proteins (Fig. 1A). A small peak prior to clathrin contained aggregated clathrin and a small peak following the assembly protein peak contained several uncharacterized proteins that were previously ignored (Fig. 1B). The ~100-kDa protein cofactor subsequently identified as auxilin, which was found to be essential for the uncoating of clathrin baskets by hsp70, was purified from the descending portion of the assembly protein peak. At the end of the assembly protein peak we observed the presence of a heat-stable protein cofactor with a purity of ~55,000–60,000 which could substitute for auxilin in supporting the uncoating reaction. In the course of characterizing this protein, we found that some fractions in the minor peak that followed the assembly protein peak strongly suppressed the uncoating activity of hsp70, suggesting that they might be inducing the polymerization of clathrin. Preliminary work suggested that they were causing clathrin polymerization under the same conditions as the other assembly proteins which induce the formation of clathrin baskets.

Purification of the Low Molecular Weight Assembly Protein—Recently, Lindner and Ungewickell (24) showed that clathrin assembly proteins can be efficiently purified by a cycle of clathrin basket formation, sedimentation of the baskets, and then refractionation of the dissolved baskets on a gel filtration column. Following this method, we combined clathrin from the major peak of the Superose-6 column with the low molecular weight fractions which caused polymerization of the clathrin. When this mixture was dialyzed against 0.1 M MES, pH 6.5, approximately half of the total clathrin polymerized. The polymerized clathrin was then sedimented, the pellet redissolved in 0.5 M Tris, pH 7.0, and rechromatographed on the Superose-6 column. The elution profile and SDS-gel electrophoretic pattern of the fractions obtained are shown in Fig. 2, A and B, respectively. Note that the polymerized clathrin apparently contained no high molecular weight assembly proteins, only relatively low molecular weight proteins (fractions 70–85) suggesting that one or more of these proteins was responsible for polymerizing the clathrin.

Each fraction of the redissolved polymerized clathrin eluted from the Superose-6 column chromatography was tested for its
through fractions contained predominantly a protein of 0.5 M Tris, pH 7.0. Fig. 3 shows the SDS-gel electrophoretic separation further on a hydroxylapatite column, equilibrated in 0.5 M Tris, pH 7.0; concentrated by ammonium sulfate precipitation and gel filtered on a Superose-6 column. Fractions containing clathrin and molecular weight standards are in the extreme left lane. Flow-through refers to purified 20-kD protein. High salt, refers to the protein bound to the column and eluted using 0.5 M phosphate, pH 7.0.

Identification of the 20-kDa Protein—Fig. 2. A, The M_r ~ 20,000 containing fraction induces clathrin to form sedimentable baskets. Fractions containing clathrin and M_r ~ 20,000 in Fig. 1 (74–81) were pooled and dialyzed against coated vesicle isolation buffer (Buffer A). Baskets formed were pelleted by centrifugation at 40,000 rpm at 4 °C for 2 h in a Ti 45 rotor and dissociated in 0.5 M Tris, pH 7.0; concentrated by ammonium sulfate precipitation and gel filtered on a Superose-6 column. B, Superose-6 column fractions of the dissociated baskets were analyzed by SDS-PAGE. Numbers above the lanes correspond to the column fractions shown above. The molecular weight standards are in the extreme left lane. T represents the total protein loaded on the column. Note the complete absence of known assembly proteins and enrichment of M_r ~ 20,000 in the assembly protein peak from the dissociated clathrin baskets.

ability to polymerize fresh clathrin. The fractions that had the highest activity (fractions 74–81) were then pooled and fractionated further on a hydroxylapatite column, equilibrated in 0.5 M Tris, pH 7.0. Fig. 3 shows the SDS-gel electrophoretic pattern of both the flow-through fractions and the proteins that are eluted with 0.5 M sodium phosphate, pH 7.0. The flow-through fractions contained predominantly a protein of M_r ~ 20,000 and examination of the assembly activity of these fractions suggested that they accounted for ~80% of the total clathrin assembly activity. The lower molecular weight bands are probably digestion products of the 20-kDa protein since their amount increases during the hydroxylapatite chromatography. Therefore, it appears that this 20-kDa protein was able to strongly induce clathrin polymerization. Table I summarizes the purification of this protein; approximately 40% of the protein was recovered with a 6-fold increase in specific activity.

Identification of the 20-kDa Protein—To determine whether the 20-kDa protein was a proteolytic digestion product of one of the higher molecular weight assembly proteins, a polyclonal antibody was raised against the 20-kDa protein and its immunoreactivity tested against the other assembly protein fractions. Fig. 4 shows that this antibody only recognized the 20-kDa protein on immunoblots and did not cross-react with a pool of mixed assembly proteins (lane 1), AP_180 containing fractions (lane 2), or AP-2 purified by hydroxylapatite chromatography (lane 3). These data suggest that the 20-kDa protein was not a proteolytic digestion product of one of the higher molecular weight assembly proteins. To confirm this point we tested whether the 20-kDa protein had any sequence homology to the other assembly proteins. The protein was digested with endoproteinase Lys-C and four pure peptides ranging from 9 to 17 amino acids were sequenced. Surprisingly, all four peptides showed 100% identity to MBP (25, 26), suggesting that the 20-kDa protein was MBP (Fig. 5). Furthermore, this protein was strongly immunoreactive with an antibody raised against the 20-kDa protein from the MBP-clathrin baskets. Therefore, it appears that this 20-kDa protein was a proteolytic digestion product of one of the higher molecular weight assembly proteins. The protein was digested with endoproteinase Lys-C and four pure peptides ranging from 9 to 17 amino acids were sequenced. Surprisingly, all four peptides showed 100% identity to MBP (25, 26), suggesting that the 20-kDa protein was MBP. In agreement with this view, we found that the MBP-clathrin baskets contained clathrin as efficiently as the 20-kDa protein which we isolated. Therefore, we conclude that the 20-kDa assembly protein which we isolated was MBP.

Properties of Clathrin Baskets Prepared with MBP—MBP is a very basic protein (27). Therefore, it was important to determine if the polymerization of clathrin induced by MBP was a specific effect. To investigate this question we first determined the ratio of MBP to clathrin required for polymerization. Various amounts of MBP were combined with a constant amount of clathrin and dialyzed against 0.1 M MES, pH 6.5, for 15 h. An aliquot of each sample was then centrifuged, and the supernatant assayed for unpolymerized clathrin using SDS-gel electrophoresis. Fig. 6A shows that as the amount of MBP was increased, increasing amounts of clathrin sedimented. As has been observed with other assembly proteins, the plot of clathrin polymerized versus 20-kDa protein added was sigmoidal (Fig. 6B). The midpoint of the plot suggested that half-maximal assembly required about 3 molecules of 20-kDa protein per triskelion. In agreement with this stoichiometry, examination of a pellet of the polymerized clathrin by SDS-gel electrophoresis (Fig. 6A, lane 12) showed that each triskelion was associated with about 3 MBP molecules. Therefore the ratio of MBP required for clathrin assembly is similar to that observed with AP-2 and auxilin where three molecules are needed to assemble each triskelion in contrast to AP_180, where only one molecule is required per clathrin assembled (15, 16, 21). We next used electron microscopy to determine the structure of the clathrin polymerized by MBP. Negative staining revealed characteristic basket-like structures resembling the structures seen with AP_180 (16) or AP-2 (10). The MBP-clathrin baskets were pre-
dominantly 85–90 nm diameter (Fig. 7). Their size was somewhat more heterogeneous than occurs with AP180-clathrin baskets or with AP-2-clathrin baskets. Similar size heterogeneity was recently observed for clathrin baskets assembled with auxilin (16).

We next tested the ability of the MBP-clathrin baskets to be uncoated by hsp70. As noted above, we recently determined that a 100-kDa protein cofactor, probably auxilin, is required for the uncoating of clathrin baskets assembled by AP-2 (20) or AP180 (21, 36). Fig. 8 shows that a similar effect occurs with clathrin baskets assembled by MBP; like the AP-2-clathrin and the AP180-clathrin baskets, the MBP-clathrin baskets were uncoated by hsp70 in a cofactor dependent manner. Furthermore, the time course of uncoating was similar to that observed with coated vesicles, AP-2-clathrin baskets, and AP180-clathrin baskets (data not shown). Therefore, the MBP-clathrin baskets appear to be similar to the other clathrin substrates in their ability to be uncoated by hsp70.

Since MBP is a basic protein, we were interested in determining whether other basic proteins could also induce clathrin basket formation. Table II shows that, of the five proteins

| Step | Protein Assembly activity | Specific activity | Yield |
|------|--------------------------|------------------|-------|
| 1. High M, assembly protein fractions from Superose-6 chromatography of coated vesicle extract. | 25 | 55.0 | 2.2 |
| 2. 20-kDa protein containing fractions from Superose-6 chromatography of coated vesicle extract. | 11.7 | 45.0 | 3.8 |
| 3. 20-kDa protein containing fractions from Superose-6 chromatography of dissociated clathrin 20-kDa protein baskets. | 3.1 | 23.3 | 7.5 |
| 4. Hydroxylapatite chromatography | 1.1 | 18.7 | 17.0 |

**TABLE I**

Activity of assembly proteins

Assembly activity was derived from the amount of assembly protein required to half-polymerize 0.8 μM clathrin. Polymerization was carried out by combining clathrin with varying amounts of assembly protein and dialyzing against coated vesicle isolation buffer. Note that the assembly activity of the ~20 kDa containing fractions is about 45% of the total assembly protein activity present in all the fractions of the column.
tested, three had no effect on the clathrin. The other two proteins, histone I and the polylysines, did affect the clathrin. However, with these proteins we could not detect polymerization of the clathrin into baskets. Rather, even at very low ratios of these proteins to clathrin, the clathrin precipitated in proportion to the amount of basic protein added and electron microscopy of the precipitates revealed no basket-like structures (data not shown).

DISCUSSION

Like the previously discovered higher molecular weight clathrin assembly proteins, AP-1, AP-2, AP180, and auxilin, MBP copurifies with coated vesicles and interacts with clathrin in vitro under physiological conditions inducing it to polymerize into baskets. Furthermore, like the plasma membrane-associated assembly protein, AP-2 (and perhaps the trans-Golgi membrane associated assembly protein, AP-1), MBP catalyzes the polymerization of clathrin with a defined stoichiometry of 3 MBP molecules per clathrin triskelion. Therefore, MBP is acting like a clathrin assembly protein.

MBP accounts for a little less than half of the total clathrin-assembly promoting activity in bovine brain coated vesicles isolated by conventional differential gradient centrifugation procedures. However, this does not mean that, in vivo, MBP makes up half of the assembly proteins present in coated vesicles. Since MBP is associated with myelin (27), the percentage of MBP present in our preparation of clathrin-coated vesicles may reflect the amount of white matter present in the bovine brain starting material from which we isolate the vesicles rather than the amount of MBP directly involved as a clathrin assembly protein.

Since MBP is a strongly basic protein, the question arises as to whether its ability to polymerize clathrin is a nonspecific effect. It has previously been reported that several basic compounds enhance the rate of clathrin polymerization (28). However, these studies were carried out before assembly proteins were discovered (8) and therefore the clathrin used may have been contaminated with assembly proteins. In addition, turbidity was used to follow polymerization and therefore it was not clear if the clathrin was actually forming baskets or was simply being precipitated by the basic proteins tested. In our experi-

Fig. 8. Cofactor dependence of the uncoating of clathrin-MBP baskets by hsp70. Clathrin-MBP baskets (0.2 μM clathrin) were incubated with hsp-70 (0.3 μM) in presence of 20 μM ATP and varying concentrations of 100-kDa cofactor for 15 min and the uncoating activity was determined. Note that in the absence of cofactor very little uncoating occurs as observed previously with clathrin-AP-2 baskets (20) or clathrin-AP180 baskets (21).
The amount of endocytosis occurs in oligodendrocytes because of these coated vesicles may be involved in the insertion or deletion of clathrin-coated vesicles in one or both of these processes. However, as (32, 33). It is therefore possible that MBP forms a bridge associated with microtubules in oligodendrocyte cell cultures of the brain(31) which may be related to the observation that MBP is present in the oligodendrocytes which form myelin (29). Although it is clear that changes in MBP cause pathological changes in the function of myelin, the exact function of MBP in the formation and properties of myelin are not known (30). Recently it has been reported that, like other proteins, e.g. STOP 220, MBP stabilizes microtubules derived from bovine brain (31) which may be related to the observation that MBP is associated with microtubules in oligodendrocyte cell cultures (32, 33). It is therefore possible that MBP forms a bridge between clathrin-coated vesicles and microtubules. However, as yet, there is no direct evidence that MBP is associated with clathrin in vivo.


Clathrin-coated vesicles have been observed in specialized regions of the axon, at the site of adhesion between the axon and its myelinating process; it has been suggested that these coated vesicles may be involved in the insertion or deletion of the junctional membrane (34). In addition, a large amount of endocytosis occurs in oligodendrocytes because of their uptake of iron by the transferrin pathway (35). It is possible that MBP may be involved in the formation of clathrin-coated vesicles in one or both of these processes. In any case, whether or not MBP interacts with clathrin in vivo, its easy availability and small size should make it very useful in studying the mechanism of the assembly of clathrin into clathrin baskets and the disassembly of these baskets by hsp70.

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TABLE II

| Protein    | pH | Effect                        |
|------------|----|-------------------------------|
| RNase      | 9.6| No effect                     |
| Lysozyme   | 11 | No effect                     |
| Cytochrome c| 10.6| No effect                    |
| Histone I  | >10| Clathrin precipitated*        |
| Polylysine ~8 kDa | >10| Clathrin precipitated*        |
| Polylysine ~25 kDa | >10| Clathrin precipitated*        |

*Precipitated clathrin is distinguishable from polymerized clathrin baskets by low speed centrifugation in an Eppendorf centrifuge. In such a centrifugation, clathrin baskets do not sediment but remain as a suspension.
