Self-Association of the Plasma Membrane-associated Clathrin Assembly Protein AP-2*

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A self-association reaction involving the plasma membrane-associated clathrin assembly protein AP-2 has been detected by incubating AP-2 alone under solution conditions that would favor the assembly of complete coat structures if clathrin were present. Self-association was rapid, unaffected by nonionic detergents, readily reversible, and gave rise to sedimentable aggregates. Only the AP subtype AP-2 exhibited self-association: the structurally or functionally related assembly proteins AP-1 and AP-3 and unrelated proteins neither self-associated nor were incorporated into the AP-2 aggregate. AP-2 interactions responsible for self-association were of high affinity, with an apparent Kd of approximately 10^-8 M. By proteolytic dissection, the self-association domain was localized to the core of the molecule containing the intact 50- and 16-kDa polypeptides in association with the truncated 60-66-kDa moieties of the parent α/β polypeptides. Self-association of the intact AP-2 molecule was pH-dependent, exhibiting an apparent pKd ~ 7.4. While it is unlikely that the large AP-2 aggregates formed in solution are themselves biologically relevant structures, the AP-2 interactions involved in their formation have properties consistent with their occurrence in intact cells and thus may be important in cellular functions of the plasma membrane-localized assembly protein.

Clathrin-coated pits and vesicles are ubiquitous cellular structures that are involved in both the endocytosis of various extracellular macromolecules and in Golgi sorting and transport (1-4). The clathrin coats of these in structures contain two major components. The first of these is clathrin which is found in its unassembled (dissociated) form as a three-legged triskelion-shaped structure (5, 6). These clathrin triskelions represent the major structural units of the coat and are responsible for its characteristic polygonal lattice morphology (3, 5). The second component of the coat is a group of proteins referred to as clathrin assembly proteins (AP)† by virtue of their ability to promote the assembly of clathrin into coat structures in vitro (7, 8). In bovine brain three such complexes of AP, designated AP-1, AP-2, and AP-180 (9-12), have been identified. Immunofluorescence localization studies (13, 14) have indicated that the AP-2 subtype is associated with coated pits of the plasma membrane whereas AP-1 is present in the Golgi apparatus.

Clathrin coats are peripherally associated with the cytoplasmic surfaces of membranes and can be stripped from isolated coated vesicles under a number of conditions (15-17) including 2 M urea, high concentrations of protonated amines at neutral pH (0.5 M Tris-HCl, pH 7.0), and high pH buffers (usually 10 mM Tris-HCl, pH 8.5). These treatments disrupt the interactions of the coat proteins with both the membrane and each other and therefore give rise to individual free clathrin triskelions and AP molecules. They are also non-denaturing and hence produce free coat components that are structurally and functionally intact. Mixtures of purified clathrin and AP in 0.5 M Tris-HCl will assemble into spherical coat structures resembling the coats found on coated vesicles in their polygonal lattice morphology when the Tris-HCl is removed by dialysis (7, 8).

In this study we show that when AP is treated alone under such assembly conditions, a self-association reaction is revealed. Further investigation demonstrates that this reaction is unique to the AP-2 subtype and has characteristics consistent with its occurrence in intact cells. The nature and properties of this self-association reaction are described and its potential relevance to AP-2 functions in cells is discussed.

EXPERIMENTAL PROCEDURES

Materials—AP was isolated by Superose 6 B gel filtration as described previously (7, 11). In most of the experiments, material corresponding to fractions 36-38 in Fig. 2 of Ref. 11 were used. For experiments with pure AP-2 and partially purified AP-1, fractions corresponding to 38-40 in Fig. 2 of Ref. 11 were pooled and fractionated by clathrin-Sepharose chromatography as described elsewhere (11). For experiments involving AP 180, fractions corresponding to 35 and 36 in Fig. 2 of Ref. 11 were used. Trypsin (1-tosylamido-2-phenylethyl chloromethyl ketone-treated, 241 units/mg) was from Worthington (Freehold, NJ).

Methods—AP self-association experiments were carried out either by dialysis of AP (0.5 M Tris-HCl) against 0.1 M sodium MES, pH 6.5 (buffer A) or by diluting concentrated samples of AP (in 10 mM Tris-HCl, pH 8.5) with buffer A. Dialysis experiments were carried out by placing samples of AP (100 µg/ml in 0.7 ml of 0.5 M Tris-HCl, pH 7.0) in collodion dialysis bags (UH100/25, Schleicher and Schuell) and dialyzing against 300 ml of buffer A at 4 °C. For dilution experiments AP was first precipitated with 50% saturated ammonium sulfate, resuspended in a small volume of 10 mM Tris-HCl, pH 8.5 (0.3 ml), and dialyzed against this buffer overnight to remove residual ammonium sulfate. Concentrated samples of AP prepared in this way (typically 1-2 mg/ml) showed little evidence of aggregation (2% of what is seen in buffer A). Aggregation experiments were carried out by diluting these samples with buffer A (at 4 °C) such that the final AP concentration was typically 100 µg/ml and the Tris-HCl concen-

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‡The abbreviations used are: AP, clathrin assembly protein; MES, 2-[N-morpholino]ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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tration (<1 mM) was low enough to not interfere with aggregation or alter the pH of the diluent. The formation of AP aggregates was monitored as a function of time by measuring the absorbance at 350 nm (A350) due to light scatter of the turbid sample. Alternatively, the final extent of self-association (measured 30 min after dilution) was quantitated by pelleting the aggregates by centrifugation for 2 min at 10,000 × g in an Eppendorf Microfuge. Equal amounts of the pellet fractions and the supernatant material (the latter precipitated with ice-cold 10% trichloroacetic acid) were electrophoresed on 5–15% sodium dodecyl sulfate-polyacrylamide gels and stained with Coomasie Blue as described previously (11).

Proteolysis of AP was carried out by incubating AP (1.76 mg/ml in 10 mM Tris-HCl, pH 8.5) with trypsin (200:1, wt/wt) for 30 min at 22 °C. The reaction was terminated by the addition of soybean trypsin inhibitor to a 2-fold molar excess with respect to trypsin. Quantitative densitometry of Coomasie Blue-stained gels was performed on a Hoefer GS-300 Densitometer with Hoefer GS-360 Data System software (Hoefer Scientific Instruments, San Francisco, CA). Protein concentrations were determined by sample absorbance at 280 nm using an extinction coefficient of 8.7 for AP-2 (11).

RESULTS

Upon removal of Tris-HCl from AP solutions by dialysis, samples became visibly turbid, and the AP protein could be sedimented by brief (2 min) centrifugation at 10,000 × g, indicating the presence of large protein aggregates (similar to Fig. 1, lane 3). Thin-section electron microscopy revealed irregular structures with dimensions on the order of 0.5–5 μm (not shown). Aggregation occurred slowly over a long time course (t½ ~ 1 h) consistent with the slow rate of dialysis of Tris-HCl from the sample. Aggregate formation under these conditions was independent of temperature within the range of 0–25 °C and was unaffected by the nonionic detergents Triton X-100 or Tween 20 (both at 1%, v/v), indicating that hydrophobic interactions are not crucial in the formation of AP aggregates.

To begin to assess the specificity of this self-association reaction, we allowed AP to self-associate in the presence of various unrelated proteins (soybean trypsin inhibitor, serum albumin, transferrin, and ovalbumin) and found that none of these added proteins either coaggregated with the AP or affected the latter's association (data not shown). As a more stringent test of the specificity of AP-AP interactions responsible for aggregate formation, we compared the ability of purified AP-2 and partially purified AP-1 (Fig. 1, lanes 1 and 2) to undergo self-association. When diluted into buffer A, the AP-2 fraction quantitatively self-associated (Fig. 1, lane 3). However, under similar conditions essentially all of the AP-1 remains in the supernatant; a small amount of contaminating AP-2 present in the AP-1 sample can be detected in the pellet lane (lane 4). These results were unchanged when a recombined mixture of both fractions was examined (lane 5).

The AP 180 (which we elsewhere show is identical to the 155K protein (18) and NP185 (19) and have termed AP-3)2 also possesses clathrin assembly activity, but differs from the AP-1 and AP-2 in that it consists of only a single subunit (10, 12). To determine if the AP-3 undergoes self-association, a fraction containing both it and AP-2 was examined (Fig. 1, lanes 6). While the vast majority of the AP-2 in this preparation was in the pellet fraction, the opposite was true for the AP-3. A small amount of contaminating clathrin heavy chain as well as an unidentified 150-kDa protein were quantitatively sedimented with the AP-2 aggregate; preliminary immunological studies reveal the latter to be a unique coated-vesicle protein.3 Collectively, these results are consistent with the conclusion that the AP-2 self-association process occurs through specific AP-2-AP-2 interactions, in that other proteins that are both structurally (AP-1) or functionally related (both AP-1 and AP-3) do not partake in the interaction.

To measure the association of AP molecules over short time courses, we developed an assay based on dilution of AP in buffer A and quantitation of the resultant aggregation by light scatter, as A350 (see "Methods"). As shown in Fig. 2A, immediately upon dilution there was a large increase in sample absorbance that occurred at a rate too rapid to be directly measured. This was followed by a more gradual increase which reached a maximum after 10 min and was stable for 30 min. When the nature and concentration of the salt was varied, it was found that substitution of MES by acetate or HEPES gave identical results, with maximal self-association observed throughout the range of 20–120 mM in each case (data not shown). Tris-HCl (Fig. 2A), as well as other chloride salts (NaCl or KCl) produced a concentration-dependent inhibition of aggregation. These results suggest variable sensitivity to different anions but indifference to monovalent cations.

To confirm that sample absorbance was indeed an accurate quantitative measure of the amount of self-associated AP, the samples shown in Fig. 2A were subjected to brief centrifugation and the resultant pellets and supernatants were analyzed by electrophoresis (Fig. 2B). When these results were quantitated by densitometry (Fig. 2C) identical profiles were obtained for bands corresponding to either the 50- or the major 100-kDa subunits of the AP, indicating that the AP sediments as a complex under these conditions. The coincidence of these curves with that of AP aggregation measured by light scatter (A350) demonstrates that A350 is an accurate quantitative measure of the amount of self-associated AP in solution. As non-AP-2 protein does not aggregate (Fig. 1), there is some variability in the light scatter signal which depends on the purity of the preparation. In addition, we have also observed a progressive loss of self-association on storage.

AP-2 aggregation was complete in solutions containing 100 μg/ml protein (Fig. 2B). The light scatter assay indicated that self-association was linear, and therefore also complete, over the concentration range of 25–250 μg/ml AP (Fig. 3) and could be detected at concentrations as low as 4 μg/ml (Fig. 3, inset). Whether the curvature in the concentration dependence detected in this region indeed reflects the absence of a

3 T. Pleasure, unpublished observations.
Sharp critical concentration cannot be rigorously concluded because of potential limitations in the assay. However, the apparent lower limit of complete self-association at 25 μg/ml or ~75 nm corresponds to a dissociation constant of approximately 10^{-8} M. This is supported by the extrapolated intercept on the abscissa which yields a value of 6 μg/ml or 17.5 nm for the equilibrium concentration of free monomer.

AP self-association was maximal at pH 6.5 and decreased sharply with increasing pH (Fig. 4). The effect of pH on the AP concentration dependence of self-association (Fig. 4, inset) is to alter the slope and is therefore consistent with the titration of an active species rather than a pH-dependent reduction in the affinity of AP-AP interactions or increase in the critical concentration (34). Hence, the steepness of the curve in Fig. 4, which agrees well with a titration curve calculated for a single species (Fig. 4, dashed line), is consistent with the existence of a discrete site on AP-2 that is required for the reaction; presumably the interacting sites that are involved in the aggregation reaction are also present on the AP-2 molecule. The pK, derived from the titration curve, 7.4, indicates that self-association is most sensitive within the range in which cytoplasmic pH is known to vary (pH 7.0-7.4 (29)).

to determine if the AP-AP interactions leading to self-association are reversible we first prepared AP aggregates by dilution with 0.1 M sodium MES solutions at either pH 6.5 or 7.5 and measured their formation by A_{350} (Fig. 5). The aggregated protein in the pH 6.5 sample was separated by centrifugation and resuspended in 10 mM Tris-Cl, pH 8.5. The low A_{350} of the resulting solution (details in figure legend) indicated that the AP had indeed dissociated. When this sample was again induced to reassociate at either pH 6.5 or 7.5, the extent of self-association was quantitatively equivalent to that observed in the first round of aggregation (Fig. 5).

A domain structure of AP-2 has been elucidated by a combination of proteolytic studies and electron microscopy. Deep-etch electron microscopy of pure AP-2 reveals a tripartite structure consisting of a large central domain with two smaller appendages (21). Treatment of AP-2 with elastase or trypsin results in the generation of two separable products previously referred to (23) as heavy and light mero-AP. The larger of these two fragments, heavy mero-AP, corresponds to the large central domain of the native protein and is a complex of the intact 50- and 16-kDa subunits (22, 23). The term light mero-AP describes the 30-36-kDa fragments derived from the 100-kDa subunits, and corresponds to the smaller appendages.

FIG. 2. Quantitation of AP self-association. Concentrated samples of AP (1.84 mg/ml) in 10 mM Tris-Cl, pH 8.5, were diluted 15-fold with buffer A containing various concentrations of Tris-Cl, pH 6.5. A, sample absorbance was measured at various times after dilution. The final Tris-Cl concentrations were 0.3 (○), 25.3 (□), 50.3 (△), and 100.3 mM (●). The zero time value reflects dilution into an equivalent volume of 10 mM Tris-Cl, pH 8.5. B, the amount of self-associated AP in the samples was quantitated by centrifugation (see “Methods”). The lanes correspond to supernatant (s) and pellet (p) fractions of samples treated in the presence of 0.3 (lane 1), 25.3 (lane 2), 50.3 (lane 3), and 100.3 mM (lane 4) Tris-Cl. The positions of the AP-2 subunits are indicated (right). C, the percentage of sedimented AP (both the 100-kDa (○) and 50-kDa (□) subunits) in the samples was quantitated by densitometry and is plotted as a function of the Tris-Cl concentration. Also shown is the amount of self-associated AP as determined by A_{350} (Fig. 1A), relative to the control maintained in 0.3 mM Tris-Cl (●).

FIG. 3. AP self-association as a function of AP concentration. Samples of AP were diluted with buffer A to the indicated final concentrations and their A_{350} was measured after 30 min. The A_{350} of unaggregated protein, assessed by dilution into an equivalent volume of 10 mM Tris, pH 8.5, was negligible (<5%). The inset is an expansion of lowest concentration range.

FIG. 4. Effect of pH on AP self-association. AP self-association was induced by diluting concentrated samples of AP with 0.1 M sodium MES of varying pH (final [AP] = 155 μg/ml) and after 30-min aggregation was measured by sample A_{350}. Plotted in the figure is the percentage of the total extent of aggregation of a given sample (measured by sample absorbance) with respect to what is seen at pH 6.5 where essentially 100% of the protein is aggregated. Also shown in the figure is a calculated titration curve (dashed line) for a weak acid with a pK, of 7.38, plotted as the percentage in the protonated form. The inset shows the extent of self-association as a function of AP concentration at pH 6.5 (○) and at pH 7.2 (△).
the pellet was resuspended in the original volume of 10 mM Tris-HCl, pH 8.5. This sample was again diluted with 0.1 M sodium MES, pH 3.0 min the pH 6.5 sample was centrifuged (2 min at 10,000 g) and the pellet was resuspended in the original volume of 10 mM Tris-HCl, pH 8.5. This sample was again diluted with 0.1 M sodium MES, pH 6.5 (○) or 7.2 (□) such that the final concentration of AP was again 153 μg/ml. The value at time zero represents the A500 obtained when an equivalent sample was appropriately diluted with 10 mM Tris, pH 8.5, and gives an indication of the extent of aggregation before dilution with MES.

![Graph](image)

**Fig. 5.** Reversibility of AP aggregation. AP (1.16 mg/ml in 10 mM Tris-HCl, pH 8.5) was diluted 7.6-fold in 0.1 M sodium MES, pH 6.5 (○) or pH 7.2 (□) and the sample A500 was monitored as a function of time. The final AP concentration was 153 μg/ml. After 30 min the pH 6.5 sample was centrifuged (2 min at 10,000 g) and the pellet was resuspended in the original volume of 10 mM Tris-HCl, pH 8.5. This sample was again diluted with 0.1 M sodium MES, pH 6.5 (○) or 7.2 (□) such that the final concentration of AP was again 153 μg/ml. The value at time zero represents the A500 obtained when an equivalent sample was appropriately diluted with 10 mM Tris, pH 8.5, and gives an indication of the extent of aggregation before dilution with MES.

![Diagram](image)

**Fig. 6.** Identification of the AP-2 domain responsible for self-association. Intact AP (lane 1) and trypsin-treated AP (lane 2) were diluted with buffer A (132 μg/ml final), incubated 30 min, centrifuged, and analysed by gel electrophoresis. With proteolyzed AP-2 only the 16-, 50-, and 60-66-kDa polypeptides (designated heavy mero-AP (HM-AP)) self-associate, whereas the 30–40-kDa fragments (light mero-AP (LM-AP)) remain in the supernatant fraction (s).

determine which of these domains is responsible for the self-association interactions, proteolyzed AP was diluted into buffer A. A rapid increase in sample turbidity indicated that the proteolyzed sample retained its self-association activity (data not shown). On centrifugation, the heavy mero-AP pellet while the light mero-AP remained exclusively in the supernatant (Fig. 6), indicating that the site or sites on AP-2 responsible for self-association are located within its large central domain.

**DISCUSSION**

We have found that molecules of AP-2 can undergo an extensive self-association reaction that can be detected quantitatively by measuring sample absorbance due to turbidity. While it is unlikely that the resultant large aggregates formed in vitro are themselves biologically relevant structures, the AP-2 self-association process is consistent with the existence of discrete, specific, and high affinity interactions. Self-association occurs rapidly, on relatively mild changes in pH and buffer conditions, and is readily and rapidly reversible. It is not driven by exposure of buried hydrophobic domains such as might accompany gross denaturation, as nonionic detergents have no discernible effect on the self-association process. The specificity of the interactions are further supported by the observations that of the three structurally and functionally related bovine brain assembly proteins, only the AP-2 undergoes self-association (Fig. 5).

Several of our observations indicate that AP-2 self-association may occur within the intact cell. The reaction occurs in vitro under conditions that approximate cytoplasmic pH; its pKₐ (7.4) suggests that self-association could be effectively modulated by small shifts in intracellular pH. Furthermore, the dissociation constant that is inferred from the concentration dependence of self-association (<10⁻⁹ M) reflects a high affinity interaction. This affinity is equal or greater than that of other protein-protein interactions of physiological significance such as tubulin (33) or actin (36) polymerization, or the interactions of profilin with actin (24) or red blood cell band 4.1 with glycophorin (25). Although direct quantitation of AP-2 in cells or tissues has not been reported, by comparison with known clathrin levels (26) the whole cell concentration of AP-2 in brain can be estimated to be approximately 30–60 μg/ml or 80–160 nM.4 These concentrations are well within the range in which AP-2 self-association has been observed in the experiments reported here. Moreover, cellular AP-2 has been localized to plasma membrane (14, 20) and endosome surfaces (31). In this context, local concentration effects due to membrane binding have been shown to greatly amplify protein polymerization events, potentially by orders of magnitude (27), suggesting that the concentrations required to observe self-association in solution may represent an extreme upper limit.

Under the assay conditions used here, AP-1 and AP-3 are capable of assembling clathrin into coat structures but do not exhibit self-association. With respect to AP-2 in particular, in the accompanying paper (35) we show that molecules containing polyphosphate groups block self-association at concentrations that do not significantly affect AP-2-mediated coat assembly in solution. These findings indicate that self-association is not a general requirement for the in vitro assembly of clathrin lattices in solution. However, to the extent that the mechanism of lattice assembly on membrane surfaces may differ fundamentally from the solution process, it may be premature to completely rule out a role for the AP-2 interactions in the assembly of clathrin coat structures within the intact cell.

An alternative view is that self-association is a function of AP-2 independent of clathrin assembly. The observation that AP-2 can bind the cytoplasmic domain of several transmembrane receptors (29, 30) suggests that self-association may contribute to receptor clustering at the cell surface, giving rise to the aggregated receptor intermediates seen early in the endocytic pathway. AP-2-induced receptor clustering might also occur on the surface of endosomes, where AP-2 has recently been localized (31), perhaps driving the segregation of receptors from released ligand seen within the tubulovesicular regions (32). These possibilities suggest that the novel self-association reaction described here may play important roles in AP-2 functions within cells.

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**REFERENCES**


4 This assumes that the Clathrin/AP ratio in cells is similar to that present in isolated coated vesicles (2:1, Ref. 11).


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