

Oligomerization of pro-opiomelanocortin is independent of pH, calcium and the sorting signal for the regulated secretory pathway

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Abstract Studies indicate that pro-opiomelanocortin (POMC) is sorted to the regulated secretory pathway by binding to a sorting receptor identified as membrane-bound carboxypeptidase E (CPE) [Cool et al. (1997) *Cell* 88, 73–83]. The efficiency of this sorting mechanism could be enhanced if POMC molecules were to self-associate to form oligomers, prior or subsequent to binding to CPE. Using cross-linking and gel filtration techniques, we demonstrated that POMC forms oligomers at both neutral and acidic pHs and calcium was not necessary. Δ N-POMC, which lacks the N-terminal sorting signal for the regulated secretory pathway, also formed similar oligomers, indicating that the sorting and oligomerization domains are different. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Pro-opiomelanocortin; Oligomerization; Aggregation; Prohormone sorting

1. Introduction

Endocrine cells possess a distinct regulated secretory pathway (RSP), in addition to a constitutive secretory pathway present in all cells [2,3]. Proteins, such as prohormones, upon synthesis at the endoplasmic reticulum (ER) are routed to the *trans*-Golgi network (TGN) where they are sorted and packaged into RSP secretory granules. The prohormones are processed within these organelles to biologically active hormones, which are released upon stimulation [4,5]. The hallmark of the RSP mature secretory granules is a dense core, observed at the electron microscopic level, due to protein condensates within these organelles. This condensation or aggregation facilitates stability and storage of high concentrations of RSP proteins in the mature secretory granule. In vitro precipitation experiments [6–10] have shown that at an acidic pH and high calcium concentration, RSP proteins such as chromogranin A, B [7,11], pro-opiomelanocortin (POMC) [8] and prohormone processing enzymes, such as PC2 and carboxypeptidase E (CPE) [9,12,13], form homo- or heterotypic aggregates that were sedimented by centrifugation. No such aggregation occurred at neutral pH. In contrast, oligomerization is a biophysical phenomenon that occurs earlier in the RSP and involves a smaller number of protein molecules, generally homotypically and reversibly bound together [14–

17]. This phenomenon may occur at neutral and/or acidic pH and could therefore take place within the ER, Golgi and the TGN, which has an acidic environment of pH 6.2 [18]. It may also serve as nucleation cores for higher order aggregation/condensation as the pH decreases further and the Ca^{2+} concentration increases in the immature and mature granules. For some proteins, oligomerization is essential for folding in the ER (for review, see [19]). In the case of RSP proteins such as prohormones, oligomerization may play an important role in sorting to the RSP by facilitating concentration and segregation at the TGN.

The prohormone, POMC (adrenocorticotropin hormone (ACTH)/endorphin precursor), can be sorted to the RSP at the TGN by binding to a sorting receptor, membrane CPE, via its di-sulfide stabilized amphipathic loop or sorting signal located at the N-terminus of the molecule [20–23]. The sorting efficiency may well be enhanced if POMC molecules were to bind the receptor as an oligomer. In this study, we investigated whether POMC could oligomerize under various conditions. We show that POMC forms oligomers independent of calcium, at both acidic and neutral pHs. Moreover, Δ N-POMC (lacking the sorting signal at the N-terminus) was also capable of forming oligomers under similar conditions, indicating that the RSP sorting signal at the N-terminus of POMC is not essential for this oligomerization phenomenon.

2. Materials and methods

2.1. Expression of POMC proteins in *Sf9* cells

POMC or Δ N-POMC (with residues 2–26 deleted) cDNAs were subcloned into the pAcGP67 baculovirus transfer vector downstream of the strong signal peptide of the acidic glycoprotein, gp67. The pAcGP67 plasmids containing the POMC inserts were co-transfected with the BaculoGold viral DNA (Pharming, San Diego, CA, USA) into *Spodoptera frugiperda* (*Sf9*) cells. Recombinant viral particles were identified by plaque assay and harvested for high titer stock generation.

Sf9 cells were grown to ~90% confluency in TNM-FH media (purchased from Pharmingen, San Diego, CA, USA) containing 10% fetal bovine serum (FBS) and supplemented with penicillin and streptomycin. The cells were then infected with the recombinant baculovirus (multiplicity of infection = 10). At 48 h post-infection, the media were changed with fresh media and the cells continued to grow for another 18 h. At 66 h post-infection, the cells were washed three times with *Sf9* Grace medium lacking the amino acids L-lysine and L-arginine (Grace K^-/R^-). The cells were then incubated for 1 h in these same media after which it was replaced with fresh Grace K^-/R^- media that were supplemented with 5 μM L-lysine and 5 μM L-arginine (Sigma, St. Louis, MO, USA). After incubation for a further 4 h, the media were collected, centrifuged at 1000 $\times g$ for 3 min to remove floating cells and frozen at -20°C until used in chemical cross-linking experiments. This procedure of POMC expression yielded media which contained a highly pure preparation of POMC as determined

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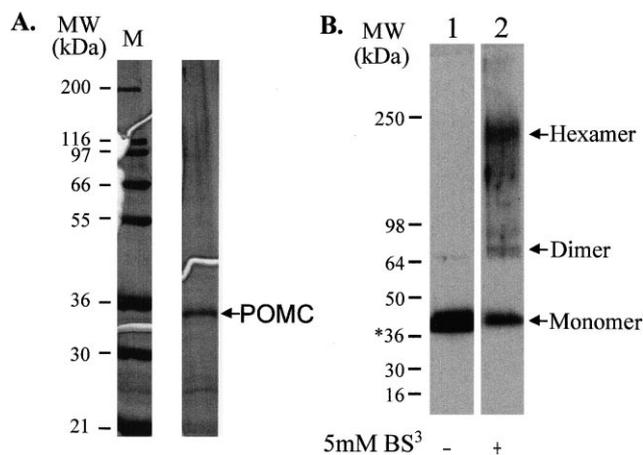


Fig. 1. Western blot analysis of BS³ cross-linked, oligomerized POMC. A: SDS-PAGE/silver stain analysis of highly pure POMC from *Sf9* cell medium used in cross-linking experiments. POMC and the molecular weight markers (Mark12[™], Novex, CA, USA) are indicated. B: Western blot of POMC after incubation for 15 min at pH 7.4 and then cross-linked (lane 2) or not (lane 1) with BS³. POMC protein was detected with anti-ACTH antibody (DP4). The prestained molecular weight markers (SeeBlue[™], Novex, CA, USA) and the oligomeric forms of POMC are indicated. *The 36 kDa SeeBlue[™] marker migrates at a lower apparent molecular mass than expected.

by silver stain analysis using the SilverSnap[™] kit (Pierce, Rockford, IL, USA) (Fig. 1A). This highly pure POMC was used in the cross-linking experiments.

For gel filtration studies, it was found necessary to use a less pure preparation of POMC to avoid the non-specific sticking of the POMC to the Superdex beads of the column which resulted in the complete loss of POMC signal during the runs. Consequently, POMC and Δ N-POMC were expressed as described above, however at 60 h post-infection, the medium was collected after centrifugation and stored at -20°C until used for gel filtration experiments. This medium was analyzed by Western blotting and Coomassie blue staining and found to contain POMC, as well as serum proteins that had been supplemented in the media as FBS. Others have shown that constitutively secreted serum proteins, such as bovine serum albumin (BSA) and immunoglobulin, do not aggregate with proteins of the RSP, such as POMC [8]. Therefore, analysis of the oligomeric states of POMC in this medium is predicted to represent valid homotypic oligomers of POMC and is used as supporting evidence for the results of the cross-linking experiments with the highly pure POMC.

2.2. Cross-linking of POMCs

POMC or Δ N-POMC (~ 50 ng/ml final concentration for each) was adjusted to pH 7.4 with $10\times$ phosphate-buffered saline (PBS) and incubated for 15 min at 22°C . Then the incubate was made to 5 mM BS³ (bis(sulfosuccinimidyl)suberate) (Pierce, Rockford, IL, USA) for 30 min at room temperature. The samples were separated on a denaturing 8–16% polyacrylamide tris-glycine gel under reducing conditions and transferred to a nitrocellulose membrane (Novex, San Diego, CA, USA) in a tris-glycine buffer containing 20% methanol. Standard immunoblotting procedures were then carried out using ACTH specific antisera (DP4) generated in our laboratory. Detection of the immunoreactive protein was by enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

2.3. Gel filtration analysis of POMC and Δ N-POMC

500 μl of *Sf9* media containing either POMC or Δ N-POMC (both at ~ 0.3 $\mu\text{g}/\text{ml}$) were desalted with water, lyophilized and reconstituted in 150 mM phosphate buffer at pH 7.4, 6.2 and 5.5. After incubation at 22°C for 15 min, the samples were centrifuged at $1000\times g$ for 5 min to remove any precipitates and the supernatant then loaded onto a Superdex G-200 HR 10/30 gel filtration column (Pharmacia) using the Fast Protein Liquid Chromatography system (FPLC), at 4°C . The flow rate was 0.5 ml/min and 0.5 ml fractions were collected. Fractions were immediately passed through a nitro-

cellulose membrane using the BioDot apparatus from Bio-Rad. The membranes were blocked with 5% non-fat milk in PBS for 1 h and then incubated with DP4 antiserum (1/2500). After ECL, the resulting film was scanned on a Microtech II scanner using the Adobe Photoshop 4.0 software, followed by quantification using the NIH-image v1.57 software. Media from *Sf9* cells infected with the empty baculovirus vector were also tested in a similar manner to demonstrate specificity of the antiserum (data not shown). Prior to each run, the column was cleaned with 10 ml of 30% acetonitrile and then non-specific sites blocked by applying 500 μg BSA through the column and then equilibrated with three column lengths of the pH-specified phosphate buffer. The column was previously calibrated with Dextran Blue 2000 (V_0), immunoglobulin (150 kDa), BSA (136 kDa dimer, 68 kDa monomer), ovalbumin (43 kDa), chymotrypsin (25 kDa), ribonuclease (13 kDa) and tryptophan (0.5 kDa). The calibration curve obtained with these standards for this column was calculated as $y = 5.83 - 2.98x$, where $x = K_D$ and $y = \log(\text{MW})$, $r^2 = 0.99$.

2.4. Precipitation assay of POMC and Δ N-POMC

To test the aggregation properties of POMC and Δ N-POMC with another prohormone of the RSP, an in vitro aggregation assay was performed similar to those previously described [12]. POMC and Δ N-POMC were expressed in approximately equal concentrations in *Sf9* cells and harvested in Grace medium as described above. 10 μl of either POMC or Δ N-POMC media were incubated in the presence or absence of purified rat proenkephalin² (0.3 mg/ml) in 5 mM HEPES, pH 7.5, 0.02% Triton X-100 in a volume of 100 μl . The samples were then made to 20 mM CaCl₂ and acidified with 0.5 M sodium acetate, pH 5.8. After incubation at 22°C for 30 min, the samples were centrifuged at $15000\times g$ for 20 min. The supernatants were removed and the pellets were reconstituted in 110 μl of 5 mM HEPES, pH 7.5. 20% of the supernatants and 20% of the reconstituted pellets were run on a 12% tris-glycine gel and analyzed by Western blot for POMC immunoreactivity.

3. Results

To determine whether POMC undergoes oligomerization, cross-linking experiments were carried out on a preparation of highly pure POMC, the purity of which is demonstrated in Fig. 1A by silver stain analysis. Fig. 1B, lane 2, shows a Western blot of POMC that had undergone cross-linking with BS³ following a 15 min incubation at 22°C in a pH 7.4 buffer. In addition to the staining of monomeric and dimeric POMC, a high molecular mass oligomer of POMC was also observed. Based on the equation of the standard curve obtained for this gel, the calculated apparent molecular mass of this band was 221 kDa, consistent with the size of a POMC hexamer (based on the monomeric size of 35 kDa indicated in Fig. 1A, hexamer = 210 kDa). These results indicate that POMC forms hexamers at pH 7.4. Cross-linking under acidic conditions is inefficient due to increased hydrolysis of the cross-linker. Therefore, FPLC using gel filtration chromatography was employed to determine the oligomerization states of POMC at acidic pHs. Fig. 2 shows the FPLC elution profiles of POMC incubated at 22°C for 15 min at pH 7.4, 6.2 and 5.5. Similar elution profiles showing two peaks in the included volume of the G-200 Superdex column were obtained for all the pHs. Based on the equation of the standard curve obtained for this column (see Section 2), the apparent molecular masses of these peaks were 218 kDa (peak I) and 410 kDa (peak II). These apparent molecular masses are consistent with the size of POMC hexamers and dodecamers, re-

² CHO cells expressing rat proenkephalin were a generous gift from Dr. Iris Lindberg, St. Louis, MO, USA. The procedure for the purification of proenkephalin from the conditioned medium was exactly as described in [1].

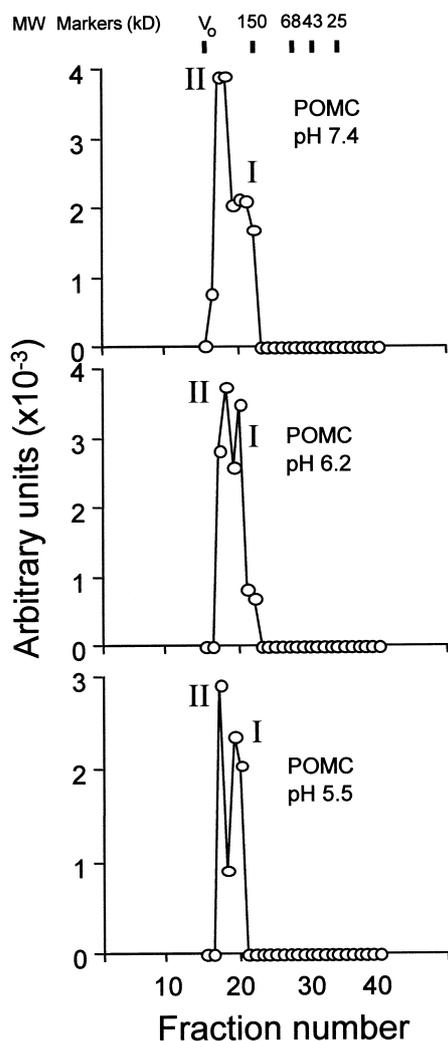


Fig. 2. Gel filtration analysis of POMC at various pHs. *Sf9* cell medium containing POMC (see Section 2) was incubated with phosphate buffers at various pHs for 15 min at 22°C and loaded onto a Superdex G-200 column. Detection of POMC was carried out using dot immunoblotting with anti-ACTH antibody (DP4). The molecular weight markers are shown on the top of the profile. Note the same profile of oligomerized POMC at all pHs. The calculated molecular masses of each peak from extrapolation of the standard curve were 218 kDa (peak I) and 410 kDa (peak II), respectively.

spectively (hexamer = 210 kDa, dodecamer = 420 kDa), and demonstrates that POMC can form these oligomers at both neutral and acidic pHs. It is important to note that the resolving range of this column in the area where the larger POMC oligomer (peak II) eluted is limited and so accurate molecular mass determination of this peak is not possible. However, as a tool to study oligomerization, this column suffices to demonstrate pH independent oligomerization of POMC. Taken together with the cross-linking data, it is possible that hexamers of POMC (Fig. 1B) could oligomerize to form dodecamers or higher order multimers. Additional gel filtration runs were performed in the presence of 10 mM Ca^{2+} or 10 mM EDTA with similar results indicating that oligomerization was independent of Ca^{2+} (data not shown). The protein profile by the Bio-Rad protein assay from the gel filtration fractions identified the major serum proteins in fractions not coincident with the POMC signal (data not shown).

In order to determine if oligomerization of POMC was dependent on its RSP sorting signal domain at the N-terminus, $\Delta\text{N-POMC}$ was studied. Fig. 3A, lane 2, shows a Western blot of $\Delta\text{N-POMC}$ incubated at pH 7.4 for 15 min and then cross-linked with BS^3 , in an identical manner to that performed on POMC. Similar to POMC, $\Delta\text{N-POMC}$ was cross-linked to form dimers and hexamers, while in the absence of cross-linker, a strong band of monomeric $\Delta\text{N-POMC}$ is seen as well as a minor component of dimeric $\Delta\text{N-POMC}$ (lane 1). The ability of $\Delta\text{N-POMC}$ to oligomerize was confirmed by FPLC. The elution profile of $\Delta\text{N-POMC}$ at pH 6.2 (Fig. 3B) showed a peak of $\Delta\text{N-POMC}$ eluting in the same range as that described for POMC oligomers in addition to a peak that is consistent with the size of monomeric $\Delta\text{N-POMC}$ (fractions 34–35). These results indicate that $\Delta\text{N-POMC}$ can oligomerize at neutral and acidic pHs and that the oligomerization domain is different from the sorting signal domain.

Furthermore, in an *in vitro* aggregation assay in the presence of 20 mM Ca^{2+} at pH 5.8, both POMC and $\Delta\text{N-POMC}$ did not form any detectable precipitates when incubated in the absence of proenkephalin (Fig. 4), presumably because their concentrations were too low. However, in the presence of proenkephalin, which normally partially precipitates under these conditions (Cawley et al., unpublished data), significant amounts of both POMC and $\Delta\text{N-POMC}$ were recovered in the pellet (Fig. 4). Based on densitometric scanning of Western blots of the POMC and $\Delta\text{N-POMC}$, the % recovery of POMC and $\Delta\text{N-POMC}$ in the pellets were 46% and 47%, respectively, indicating similar abilities of POMC and $\Delta\text{N-POMC}$ to aggregate *in vitro*.

4. Discussion

POMC interacts with membrane CPE via its N-terminal sorting signal [21] and it is this interaction that mediates the sorting of POMC to the RSP. Other prohormones that have similar structural motifs encoded in their tertiary and quaternary structures, such as proenkephalin and proinsulin, are predicted to utilize CPE in a similar manner [23]. The stoichiometry of binding between POMC and CPE raises questions of sorting efficiency *in vivo*. Is there enough CPE to handle its high concentration of prohormone cargo? Can the efficiency be increased by binding multimeric complexes? To address the first question, it can be estimated, based on the B_{MAX} for N-POMC^{1-26} binding to CPE [24] in bovine pituitary intermediate lobe secretory vesicle (ILSV) membranes and conversion for total protein, that there are $\sim 0.1\text{--}0.2 \times 10^{-12}$ mol membrane-associated CPE/ μg total protein. For comparison, the concentration of POMC in purified ILSV was previously estimated as $\sim 3.9 \times 10^{-12}$ mol POMC/ μg total protein [25]. Similar results were obtained from purified chromaffin granules where quantification of proenkephalin (which shares the structural motif found in the N-POMC sorting signal [23]) and CPE by radioimmunoassay yielded $\sim 0.2 \times 10^{-12}$ mol proenkephalin and $\sim 0.2 \times 10^{-12}$ mol CPE/ μg total protein, respectively³. Both sets of results demonstrate that within experimental and estimation error, the concentration of CPE is significantly higher than expected, reaching equimolar levels

³ Vivian Hook, UCSD, San Diego, CA, USA, personal communication.

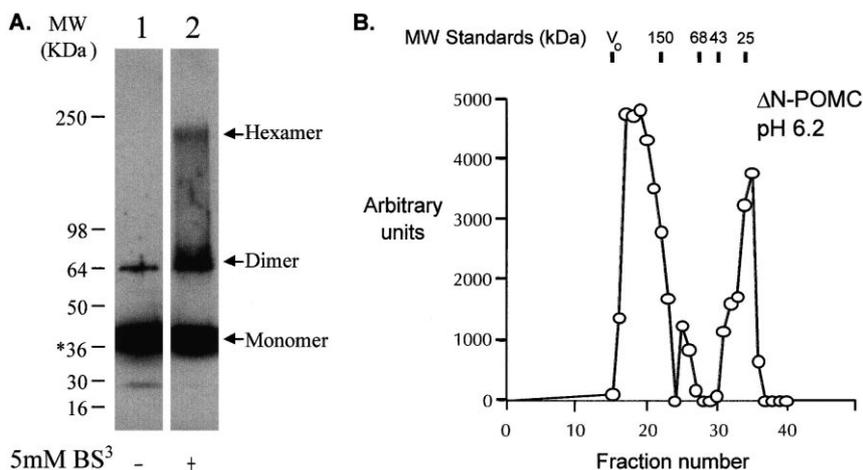


Fig. 3. A: Western blot of Δ N-POMC after incubation for 15 min at pH 7.4 and then cross-linked (lane 2) or not (lane 1) with BS^3 . Δ N-POMC protein was detected with anti-ACTH antibody (DP4). The prestained molecular weight markers (SeeBlue[®], Novex, CA, USA) and the oligomeric forms of POMC are indicated. *The 36 kDa SeeBlue[®] marker migrates at a lower apparent molecular mass than expected. B: Gel filtration analysis of Δ N-POMC at pH 6.2. Gel filtration and detection procedures are described in Fig. 2. Note the presence of a peak in the same range as that obtained for POMC in addition to an apparent monomer of Δ N-POMC. Due to saturation of the film, the relative amount of monomer appears greater than it actually is. We estimate that $< 30\%$ of the total Δ N-POMC is in this form and it could be much less since we cannot determine the extent of cross-reactivity of the oligomeric form versus the monomeric form.

with its proposed cargo, proenkephalin, in chromaffin granules. This renders a 1:1 binding between proenkephalin and CPE a possibility in this tissue. In ILSV, POMC is in excess over CPE with ratios of 1:19–1:39. With hexamerization of POMC, CPE would be capable of binding $\sim 25\%$ of the POMC. However, this is presumed to be a minimum binding since our studies here show the existence of higher order multimers of POMC (Fig. 2) which may also bind CPE. Ultimately, CPE sorting of POMC may be complemented by other mechanisms such as aggregation or other receptors and thus efficient sorting to the RSP will likely involve a combination of mechanisms depending on the tissue studied.

To address whether POMC can exist in multimeric states and thereby enhance the efficiency of sorting by binding to CPE as a multimer, we have demonstrated using cross-linking and gel filtration experiments that POMC can self-associate into homo-oligomers, that are at least hexameric in size, at neutral (pH 7.4) and acidic pHs (pH 6.2 and 5.5) (Fig. 2). Moreover, Ca^{2+} did not appear to be required (data not shown), suggesting that oligomerization of POMC could begin as early as in the ER, similar to that proposed for the dimerization of chromogranin A and B [16]. Similar peaks at steady state in the gel filtration profiles were also observed for Δ N-POMC (Fig. 3B), indicating that oligomerization of POMC could occur in the absence of its N-terminus. In addition, chemical cross-linking studies show that both POMC (Fig. 1B, lane 2) and Δ N-POMC (Fig. 3A, lane 2) could be cross-linked into homo-dimers and hexamers, providing evidence again that the N-terminus of POMC is not essential for oligomerization. Higher order oligomerization of POMC, however, may be stabilized by its N-terminus as evidenced by the presence of monomeric Δ N-POMC on gel filtration columns and the lower cross-linking efficiency of Δ N-POMC in the cross-linking experiments.

Previously, expression of transfected Δ N-POMC in Neuro2a cells resulted in this mutant not being sorted to the RSP as evidenced by its lack of punctate immunostaining characteristic of regulated secretory granules, and its lack of

stimulated release in secretion experiments [20]. This classical characteristic of RSP mis-sorting demonstrated by the POMC mutant was predicted to be caused by its inability to bind to CPE *in vivo*, and is consistent with our previous *in vitro* data showing that Δ N-POMC did not bind CPE in ILSV membranes whereas full length POMC did. Alternatively, the mis-sorting of Δ N-POMC in these cells could have been the result of poor aggregation. Our current data do not support this since we have shown that Δ N-POMC can predominantly form higher order oligomers at steady state (Fig. 3B) and can be cross-linked into hexamers at pH 7.4 (Fig. 3A). More importantly, Δ N-POMC appears to aggregate with similar properties to POMC as revealed by *in vitro* aggregation studies (Fig. 4). This suggests that the mis-sorting of Δ N-POMC in Neuro2a cells observed previously [20] was not likely to be as a result of aberrant aggregation. Interestingly, since Neuro2a cells synthesize, store and secrete proenkephalin in a regulated manner [26,27] and to which Δ N-POMC could aggregate in

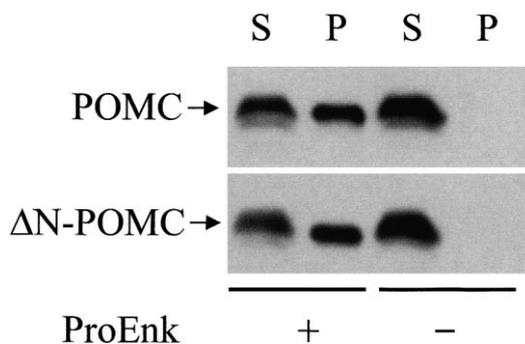


Fig. 4. Aggregation assay of POMC and Δ N-POMC. POMC or Δ N-POMC, incubated in the absence (–) or presence (+) of purified proenkephalin, was centrifuged at $15000\times g$ for 20 min. 20% of the recovered supernatants (S) and pellets (P) was assayed for POMC immunoreactivity by Western blot. Note the similar recovery of both POMC (46%) and Δ N-POMC (47%) in the aggregated pellet in the presence of proenkephalin only.

vitro as we have shown (Fig. 4), one would have expected that if heterotypic aggregation played a role in sorting POMC to the RSP, the Δ N-POMC should have been sorted to the RSP with the endogenous proenkephalin. Since this was not observed, we can conclude that the mutant POMC and the endogenous proenkephalin did not significantly interact in vivo to effect sorting and indicates an almost exclusive reliance on CPE for sorting POMC in these cells.

Hexamerization and oligomerization of POMC at steady state, as seen in the cross-linking and gel filtration experiments, Figs. 1B and 2, respectively, would indicate that in its oligomeric state, the N-terminal of POMC is free to effect an interaction with CPE as observed previously in binding studies under similar conditions [21]. Our data support this since the N-terminal does not appear to play a significant role in oligomerization (Fig. 3), yet it is required for binding to CPE [21]. Further support of this comes from the cross-linking of hexamers of proinsulin to membrane CPE in bovine ILSV membranes [24], indicating that hexamerization does not preclude interaction with the sorting receptor. Thus, oligomerization of prohormones may be a phenomenon that occurs as part of the sorting process, to facilitate concentration and segregation from constitutive proteins, at the TGN. Formation of smaller oligomers, rather than large insoluble aggregate complexes, to facilitate sorting at the TGN renders the prohormones accessible to processing enzymes [28]. Undoubtedly, the processed hormones could also form hexamers, e.g. insulin [29], which then further condense with other RSP proteins, e.g. the chromogranins, giving rise to mature dense-core secretory granules.

In conclusion, we propose a model in which POMC at least hexamerizes as early as in the ER. The hexamer with the sorting signal containing N-terminal domains exposed traverses to the TGN and binds the receptor, membrane CPE, at the acidic pH. Higher order aggregation is initiated to facilitate further concentration and segregation. The CPE/prohormone complex is packaged into the immature secretory granule and processing begins and/or continues. The processed hormones then condense further with other secretory granule proteins to form a dense core within the mature secretory granule.

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