Differential aggregation properties of secretory proteins that are stored in exocrine secretory granules of the pancreas and parotid glands

S. G. Venkatesh, 1 Darrin J Cowley, 1 and Sven-Ulrik Gorr 1,2

1 Department of Periodontics, Endodontics, and Dental Hygiene; and 2 Department of Biochemistry and Molecular Biology, University of Louisville Health Sciences Center, Louisville, Kentucky 40292

Submitted 5 August 2003; accepted in final form 12 October 2003

Venkatesh, S. G., Darrin J Cowley, and Sven-Ulrik Gorr. Differential aggregation properties of secretory proteins that are stored in exocrine secretory granules of the pancreas and parotid glands. Am J Physiol Cell Physiol 286: C365–C371, 2004. First published October 22, 2003; 10.1152/ajpcell.00338.2003.—Low-pH- and calcium-induced aggregation of regulated secretory proteins has been proposed to play a role in their retention and storage in secretory granules. However, this has not been tested for secretory proteins that are stored in the exocrine parotid secretory granules. Parotid granule matrix proteins were analyzed for aggregation in the presence or absence of calcium and in the pH range of 5.5 to 7.5. Amylase did not aggregate under these conditions, although <10% of parotid secretory protein (PSP) aggregated below pH 6.0. To test aggregation directly in isolated granules, rat parotid secretory granules were permeabilized with 0.1% saponin in the presence or absence of calcium and in the pH range of 5.0 to 8.4. In contrast to the low-pH-dependent retention of amylase in exocrine pancreatic granules, amylase was quantitatively released and most PSP was released from parotid granules under all conditions. Both proteins were completely released upon granule membrane solubilization. Thus neither amylase nor PSP show low-pH- or calcium-induced aggregation under physiological conditions in the exocrine parotid secretory granules.

EXOCRINE AND ENDOCRINE SECRETORY CELLS possess multiple post-Golgi pathways for protein secretion (27, 34). The constitutive pathway, common to all eukaryotic cells, directly secretes proteins in the absence of stimulation. In the constitutive-like secretory pathway, proteins initially enter immature secretory granules but are removed during granule maturation and are also secreted in the absence of stimulation (1). In the regulated secretory pathway [comprising a major and minor regulated pathway in salivary acinar cell (7)], proteins are stored at high concentration in secretory granules and released upon stimulation of the cells by secretagogues (6, 27, 34). The delivery of different proteins to specific post-Golgi vesicles and secretory granules involves processes collectively termed protein sorting or targeting (1, 36). Sorting presumably involves the selective entry of regulated secretory proteins into nascent secretory granules or the selective retention of these proteins in maturing secretory granules (36). The mechanisms of protein storage in secretory granules remain the subject of intense investigation.

Although individual differences exist (21), a common theme for protein storage in secretory granules is the ability of regulated secretory proteins to undergo calcium- and low-pH-induced aggregation. Such aggregation has been demonstrated for both endocrine (3, 16, 17, 19, 22, 26, 28, 32, 35, 37, 38, 41, 42) and exocrine (12, 13, 15, 30, 31, 43) secretory granule proteins. In many of these examples, aggregation is enhanced at the moderately acidic pH found in immature secretory granules (33).

Consistent with this role of aggregation in protein storage, endocrine adrenal secretory granules (39), and exocrine pancreatic secretory granules (30) require calcium for stability, suggesting that regulated secretory proteins form a calcium-dependent storage complex in these cell types. Unlike exocrine pancreatic granules, parotid secretory granules do not require calcium for stability, suggesting that parotid exocrine proteins do not form a calcium-dependent storage complex (14). Because both exocrine pancreatic and parotid secretory granules store amylase, this finding leads to the unanticipated hypothesis that different mechanisms are involved in amylase storage in the two types of exocrine secretory granules. However, it is not known whether parotid exocrine secretory proteins exhibit low-pH- and calcium-induced aggregation. Thus the goal of this study was to test whether the aggregation properties of parotid secretory proteins differ from those of exocrine pancreatic proteins. The results demonstrate that the regulated secretory proteins amylase and parotid secretory protein (PSP) do not show low-pH- and/or calcium-dependent aggregation in the parotid, but amylase aggregates in pancreatic samples. This suggests that aggregation is not necessary for sorting or retention of amylase and other regulated secretory proteins in parotid cells.

EXPERIMENTAL PROCEDURES

Pancreas

Isolation of pancreatic secretory granules. Animal use was approved by the Institutional Animal Care and Use Committee at the University of Louisville. Secretory granules were isolated from rat exocrine pancreas as described by Dartsch et al. (12). Briefly, male Sprague-Dawley rats (250–300 g) were fasted overnight, with water ad libitum, and euthanized by carbon dioxide asphyxiation. The pancreas was surgically removed and cleaned of fat and connective tissue and placed in ice-cold isolation buffer [0.25 M sucrose, 5 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.25, 0.1 mM MgSO4, 1 mM dithiothreitol, 0.1 mM PMSF, and 1 mM soybean trypsin inhibitor]. The glands were weighed and homogenized in five volumes of the isolation buffer. The homogenate was centrifuged at 500 g for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 2,000 g for 10 min at 4°C. The supernatant and the brownish layer of mitochondria on top of the pellet were discarded and the pellet was resuspended in 50 mM HEPES, pH 8.0, containing...
0.1 mM PMSF. The suspension was centrifuged at 2,000 g for 10 min, and the resulting pancreatic secretory granule pellet was resuspended in the same buffer until further use.

**In vitro aggregation of pancreatic granular proteins.** Pancreatic granules were lysed in 50 mM HEPES, pH 8.0, by freeze-thawing and the extracts were centrifuged at 16,000 g for 10 min to remove granule membranes. Further experiments were performed by using the soluble protein fraction present in the supernatant (granule extract). The amylase activity in the granule extract was quantified, and equal amylase activity was used for the aggregation assays. The aggregation assay was performed by using the buffer conditions as described by Dartsch et al. (12). Briefly, to obtain the aggregation buffer, equal volumes granule extract (50 mM HEPES, pH 8.0) and 100 mM MES buffer, either pH 4.7 or 6.25, were mixed to get a final pH of 5.9 and 7.5, respectively. (12). The calcium concentration in the final mix was 20 mM. The pH of the solution did not vary by more than 0.1 pH unit. Aggregation, in a total volume of 100 μl, was performed at room temperature for 1 h, and the mixture was then centrifuged for 30 min at 16,000 g for 30 min. The pellet was resuspended in 50 μl of distilled water, and the amylase activity in the pellet and supernatant fractions were quantified by the Phadebas amylase kit.

**Retention/aggregation experiments.** Saponin-permeabilized exocrine pancreatic granules were used to determine the retention of aggregated proteins. The granules were suspended in 50 mM HEPES, pH 8.0, and the amylase activity was quantified by the Phadebas amylase assay kit. Aliquots containing equal amylase activity were placed in separate tubes and the granules were centrifuged at 2,000 g for 10 min. The supernatant was discarded and the granules were resuspended in 40 μl of the appropriate aggregation buffer, pH 5.9 or 7.5, either in the presence or absence of 0.1% saponin and 1 mM calcium. Thus the granule volume was negligible compared with the volume of the resuspension buffer. The retention experiments were carried out for 1 h at room temperature. The suspension was then centrifuged at 16,000 g for 30 min. Amylase activity in the pellet and the supernatant was quantified by the Phadebas amylase kit.

**Parotid**

**Isolation of parotid secretory granules.** Secretory granules were isolated from the parotid glands of male Sprague-Dawley rats (Harlan, Indianapolis, IN), as described earlier (2, 44).

**In vitro aggregation of parotid granular proteins.** Parotid granules were lysed in 50 mM HEPES, pH 8.0, by freeze-thawing and centrifuged at 16,000 g for 10 min to remove granule membranes. Further experiments were performed by using the soluble protein fraction present in the supernatant (granule extract). The amylase activity in the granule extract was determined and equal amylase activity was used for the assay. Aggregation was performed under the same conditions as described above for the exocrine pancreas. The amylase activity in the aggregated and soluble protein fractions was quantified by the Phadebas amylase kit.

To confirm the lack of pH-dependent aggregation of parotid-regulated secretory proteins, aggregation of granule extracts was performed at pH 5.5–7.5. Twenty micrograms (total protein) of the parotid granule extract were suspended in a total volume of 35 μl of 50 mM HEPES or MES buffer of pH ranging from 5.5 to 7.5. Aggregation was performed at room temperature for 1 h. The samples were then centrifuged at 16,000 g for 30 min and the supernatants were collected. The pellets were washed with the respective buffers. The supernatants and the washed pellets were analyzed by SDS-PAGE.

To determine whether microaggregates of amylase or PSP were present under aggregating conditions, the in vitro aggregation was conducted as described for exocrine pancreatic granules (12). Rat parotid granules were lysed in 50 mM HEPES, pH 8.0, by freeze-thawing, followed by centrifugation at 100,000 g for 1 h at 4°C. The supernatant containing the soluble proteins (high-speed granule extract) was used for the assay. The protein concentration in the extract was determined using the Bio-Rad protein assay, and a final concentration (after pH adjustment) of 2 mg/ml was used in the aggregation assay. The aggregation assay was performed in either the presence or absence of 1 mM Ca^{2+} in the “aggregation buffer.” To 20 μl of sample, 20 μl of either 100 mM MES, pH 6.25, or 100 mM MES, pH 4.7, was added to yield the aggregation buffer with a final pH of 7.5 or 5.9, respectively (12). After incubation at room temperature for 2 h, the samples were centrifuged at 16,000 g for 30 min at 4°C. The pellets were resuspended in 50 mM HEPES, pH 8, and used for immunoblotting. The 16,000-g supernatant fractions from the aggregation reaction were centrifuged at 400,000 g for 2 h. The final supernatant, wash, and pellet fractions were analyzed by SDS-PAGE, followed by immunoblots.

In an alternate protocol, additional wash steps were introduced to distinguish between aggregated protein and protein sticking to the centrifuge tube after centrifugation. In this protocol, the pellets obtained after the 16,000-g and 400,000-g centrifugation steps were washed with the aggregation buffer and then centrifuged for 30 min at 16,000 g and 400,000 g for 2 h, respectively (the wash supernatants were retained for immunoblotting).

**Retention/aggregation experiments.** To test the retention of parotid proteins in saponin-permeabilized granules, parotid granules were suspended in the granule buffer (285 mM sucrose-20 mM HEPES buffer, pH 7.8), equal aliquots of this suspension were placed in individual microcentrifuge tubes, and the granules were pelleted by centrifuging at 2,000 rpm for 10 min. The supernatant was discarded and the granules were resuspended in 40 μl of the appropriate buffers. The granules were permeabilized with the detergent saponin (Sigma, St. Louis, MO). The appropriate concentration of saponin and the effect of calcium was determined by suspending the granule buffer, pH 6.8, with varying concentrations of saponin either in the presence or absence of 20 mM calcium. The release of the parotid granule proteins from permeabilized granules was compared with the release from exocrine pancreatic granules. For this, rat parotid granules were initially suspended in 50 mM HEPES, pH 8.0, and the amylase activity was quantified by the Phadebas amylase assay kit. Aliquots containing equal amylase activity were placed in separate tubes and the granules were centrifuged at 2,000 g for 10 min. The supernatant was discarded and the granules were resuspended in 40 μl of the appropriate aggregation buffer, pH 5.9 or 7.5, either in the presence or absence of 0.1% saponin and 1 mM calcium. Thus the granule volume was negligible compared with the volume of the resuspension buffer. The retention experiments were carried out for 1 h at room temperature. The suspension was then centrifuged at 16,000 g for 30 min. Amylase activity in the pellet and the supernatant was quantified by the Phadebas amylase kit.

The effect of calcium was determined by suspending the granule buffer, pH 6.8, with varying concentrations of saponin either in the presence or absence of 20 mM calcium. To determine whether pH affects the retention/aggregation of the regulated secretory proteins, the parotid secretory granules were suspended in granule buffer, pH 5.0 to 8.4, containing 0.1% saponin and 20 mM Ca^{2+}. The samples were equilibrated at room temperature for 1 h. The suspensions were then centrifuged at 16,000 g for 30 min. The retained (pellet) and released (supernatant) proteins were analyzed by SDS-PAGE and immunoblotting.

To preclude the effect of the buffer used in the retention experiments and to determine whether a more complex buffer containing salts affected the aggregation behavior of the granular contents, the granules were permeabilized under conditions that closely mimicked the in vivo milieu (2, 47). Parotid granules were suspended in either buffer I (285 mM sucrose-20 mM HEPES buffer, pH 6.8), buffer II (buffer I containing 20 mM MgCl{$_2$}, 50 mM KCl, and 28 mM NaH$_2$PO$_4$) or buffer III (buffer II containing 75 mM CaCl$_2$). The experiment was conducted in the above buffers either in the presence or absence of 0.1% saponin. The samples were equilibrated at room temperature for 1 h, followed by centrifugation at 16,000 g for 30 min. Amylase activity in the pellet and supernatant fractions was determined with the Phadebas kit. The presence of PSP in the fractions was determined by SDS-PAGE and Western blotting.
Effect of Triton on the aggregation of amylase and PSP in the rat parotid secretory granules. Rat parotid granules were suspended in granule buffer, pH 7.8. Equal aliquots (40 μl) of the suspension were placed in individual tubes and the granules were pelleted by centrifugation at 2,000 rpm for 10 min. The granule pellets were resuspended in granule buffer of pH from 5.0 to 8.4, containing 0.1% Triton X-100 and 20 mM CaCl2, and incubated for 1 h at room temperature. The samples were centrifuged at 16,000 g for 30 min and amylase and PSP were detected by immunoblotting.

Gel electrophoresis and immunoblotting. Protein samples were separated by SDS-PAGE on 10% or 15% gels (29). The protein bands were visualized by either staining with Coomassie brilliant blue (0.05% in 10% methanol:15% acetic acid:75% dH2O for 1 h, followed by destaining in methanol-acetic acid-water (10:5:85)) or silver staining using the Fastsilver staining kit (Genotech, St. Louis, MO). For immunoblotting, the unstained protein bands were transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 2% Tween 20 in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.4) containing 0.05% Tween 20 for 1 h and subsequently incubated either with antisera to rat PSP (a kind gift from Dr. William Ball, Howard University, Washington, DC) (1:10,000) or human amylase (Sigma Immunochemicals) (1:5,000) in Tris-buffered saline containing 0.05% Tween 20 for 1 h. The membranes were then washed and incubated in diluted (1:5,000) horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA) in Tris-buffered saline containing 0.05% Tween 20 for 1 h. Immunoblots were processed using Supersignal West Pico substrate (Pierce, Rockford, IL), and the chemiluminescence of the bands was visualized by exposure of the blot to Kodak X-omat film or scanning on a Kodak Image Station 440 CF (Kodak, Rochester, NY).

RESULTS

Amylase is stored in exocrine secretory granules of both the pancreas and parotid glands (4) (24). To test the aggregation properties of amylase from these granules, granule extracts were incubated under aggregating or nonaggregating conditions (Fig. 1). Pancreatic amylase exhibited significant aggregation, whereas parotid amylase did not aggregate under the identical conditions.

To test whether alternate conditions were needed to detect aggregation of parotid amylase, the aggregation experiments were performed in the pH range 7.5 to 5.5. Under these conditions, none of the parotid granule proteins underwent extensive pH-dependent aggregation. In particular, amylase did not aggregate in the pH range of 7.5 to 5.5, whereas acidic epididymal glycoprotein (AEG) and PSP exhibited some aggregation at pH 5.5 (Fig. 2).

To test the effect of calcium and higher centrifugal forces, high-speed parotid granule extracts were analyzed as described in one study of the exocrine pancreas (12). Although some amylase was detected in the pellet fraction after centrifugation at 400,000 g, this was removed by washing of the pellets and recentrifuging (Fig. 3). Thus amylase does not aggregate from parotid granule extracts. As above, PSP exhibited some aggregation, even after the wash step, but this was neither calcium nor pH dependent (Fig. 3).

A potential explanation for the lack of aggregation of parotid granule proteins is the low protein concentration in the granule extracts compared with that of the intact granules. Furthermore, it is possible that some factors necessary for parotid aggregation could be lost in the process of the preparation of the extracts. To overcome these potential problems, saponin-permeabilized vesicles have previously been used to more closely mimic the aggregation conditions in situ (9). To establish the optimal saponin concentration for these experiments, isolated granules were incubated with different saponin concentrations in the presence or absence of 20 mM calcium to mimic granule conditions (2, 25, 46). Figure 4 shows that amylase was quantitatively released from parotid granules treated with 0.05–0.5% saponin. PSP, on the other hand, was partially retained in secretory granules at saponin concentrations below 0.5%. This suggests that the lower saponin concentrations permeabilized but did not completely solubilize the secretory granules. Release and retention of amylase and PSP were not affected by calcium (Fig. 4).
Using these conditions, we directly compared the retention of amylase in permeabilized pancreatic and parotid secretory granules at pH 5.9 and 7.5 (12). Under nonaggregating conditions (pH 7.5), amylase was quantitatively released from both parotid and pancreatic granules (Fig. 5A). In contrast, under aggregating conditions (pH 5.9), amylase was partially retained in pancreatic granules, but not in parotid granules (Fig. 5A). Qualitatively similar results were obtained when the total protein patterns in the samples were analyzed by SDS-PAGE (not shown). Control granules, which were not permeabilized with saponin, retained amylase at both pH 5.9 and 7.5 (Fig. 4 and 5B).

To test whether calcium or varying the granule pH could increase the retention of parotid secretory granule proteins, parotid granules were permeabilized with 0.1% saponin in the pH range of 5.0 to 8.4 and in the presence of 20 mM Ca$^{2+}$. Amylase was quantitatively released from the permeabilized parotid granules at every pH, suggesting that the parotid enzyme does not aggregate under these conditions (Fig. 6). PSP again exhibited partial retention, but this effect was not pH dependent. Identical results were obtained when the experiments were performed in the absence of 20 mM CaCl$_2$ (not shown).

It has been suggested that the presence of “bridging” or “shielding” ions might aid in the aggregation of similarly charged protein molecules (47). To test whether the presence of salts in the buffers could influence the aggregation pattern of the parotid granule proteins, the parotid granules were perme-
suggesting that PSP does not form an insoluble complex.

Fig. 6. Effect of pH on the retention of amylase and PSP in permeabilized parotid secretory granules. Rat parotid secretory granules were isolated and incubated in granule buffer, of pH ranging from 5.0 to 8.4, in the presence of 20 mM Ca\(^{2+}\) and 0.1% saponin. After centrifugation, soluble (S) and retained protein (P) were detected by immunoblotting with antibodies to PSP and amylase. The position of the bands corresponding to amylase and PSP are indicated at right. The results are representative of 9 independent experiments.

Fig. 7. Effect of salts on the retention of secretory proteins in parotid secretory granules. Rat parotid granules were suspended in either buffer I containing 20 mM MgCl\(_2\), 50 mM KCl, 28 mM NaH\(_2\)PO\(_4\), or buffer II (buffer I containing 75 mM CaCl\(_2\)). The retention experiment was performed in the presence (+) or absence (−) of saponin. After centrifugation of the suspension, the amylase activity was quantified in the supernatant (S, soluble protein) and pellet (P, aggregated protein). The presence of PSP in the fractions was determined by immunoblotting. The data shown are of duplicate samples from a single experiment.

Fig. 8. Effect of Triton on the retention of secretory proteins in parotid secretory granules. Rat parotid granules were suspended in granule buffer, pH 5.0–8.4, containing 20 mM Ca\(^{2+}\) and 0.1% Triton. The suspension was centrifuged and the supernatant (S, soluble protein) and pellet (P, retained protein) fractions were analyzed by immunoblotting. The positions of the amylase and PSP bands are indicated at right. The results are from a single experiment.

Parotid secretory proteins differ in their aggregation properties from those of pancreatic exocrine proteins. Amylase is a marker for secretory granules in both the exocrine pancreas and parotid glands. Although closely related forms of amylase are stored in pancreatic and parotid secretory granules, these exhibit differential aggregation behavior (Fig. 5). Because we have previously shown that purified pancreatic amylase does not aggregate in vitro (23), it is likely that the protein mixture of pancreatic granules contributes to the aggregation of amylase in this tissue. The exact aggregation partners are not known, but it has previously been shown that chymotrypsinogen and trypsinogen interact with other regulated secretory proteins, including amylase, in pancreatic extracts (20). The differences in aggregation behavior of amylase from the two exocrine cell types are consistent with the previous findings that pancreatic and parotid exocrine secretory granules differ in their response to calcium depletion. Although pancreatic granules lyse in the absence of calcium (39), this is not the case for parotid granules (14), suggesting that parotid granules do not contain a calcium-dependent storage complex. Although our data strongly suggest that parotid amylase does not aggregate under conditions when pancreatic amylase aggregates, we cannot rule out the possibility that parotid amylase is retained in a complex in vivo that does not withstand the experimental procedures used in this study. However, the finding that parotid and pancreatic granules exhibit differential electron densities (40) supports the proposal that the in vivo aggregation properties are different in the two granule types.

In addition to the differences in amylase aggregation in pancreatic and parotid secretory granules, differences were observed between different parotid proteins. Thus amylase and PSP exhibited differences in their retention in permeabilized parotid secretory granules that may reflect differences observed in the sorting and storage of the proteins in parotid acinar cells (45). Whereas amylase was quantitatively released from permeabilized granules, PSP was partially retained in the absence or presence of calcium and in the pH range of 5.0 to 8.4. The presence of other ions in the buffer did not alter this result. The observation that PSP is partially retained in the saponin-treated granules suggests that the protein is not bound to cholesterol-rich patches of the granular membrane. The nature of this retention deserves further investigation.

Structures resembling condensed secretory proteins have been observed in the condensing vacuoles of parotid acinar cells (8). Our data now suggest that these structures are not stable after permeabilization of the granule membrane. This is consistent with the observed lysis of parotid granules in distilled water (2), which suggests that the granule cores are osmotically active. Thus, rather than an ionic core complex, we propose that a hydrated gel exists in parotid secretory granules. Formation of this hydrated gel presumably depends on sulfated proteoglycans that are stored in parotid secretory granules.
Indeed, we have recently shown that a sulfated proteoglycan, but not sulfation alone, is necessary for efficient storage of amylase and PSP in parotid secretory granules (44). Thus the glycosaminoglycan chains of the proteoglycan appear to play a direct role in protein storage. Consistent with this model, a subsequent report suggests that sulfated glycoproteins or proteoglycans may also contribute to the storage of secretory proteins in the exocrine pancreas (13). In contrast, sulfated proteoglycans do not play a role in the sorting and storage of regulated secretory proteins in endocrine cells (5, 18). Instead, calcium-induced aggregation and possibly other sorting mechanisms appear to operate in endocrine cells (10, 11, 41). Thus a mosaic of sorting mechanisms appears to operate in secretory cell types (21): endocrine cells use calcium- and low-pH-induced aggregation but not sulfated proteoglycans, exocrine parotid cells use sulfated proteoglycans but not calcium- or pH-induced aggregation, and exocrine pancreatic cells may use both mechanisms.

ACKNOWLEDGMENTS

Present address of D. J. Cowley: Diagnostic Division, Abbott Laboratories, 200 Abbott Park Rd., Abbott Park, IL 60064

GRANTS

This work was supported by National Institute of Dental Research Grant R01-DE-12205.

REFERENCES