IONIC INTERACTIONS BETWEEN BOVINE CHYMOTRYPSINOGEN A AND CHONDROITIN SULFATE A.B.C.

A Possible Model for Molecular Aggregation in Zymogen Granules

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ABSTRACT

The formation of large aggregates by ionic interactions between acidic glucosaminoglycans and cationic secretory proteins has been proposed as one of the critical steps in the concentration process in the condensing vacuoles of secretory cells. In this paper, this hypothesis was tested by studies on the interactions between bovine chymotrypsinogen A and chondroitin sulfate as a simplified model. Small amounts of chondroitin sulfate were found able to induce chymotrypsinogen precipitation. Like zymogen granules, the resulting aggregates were moderately sensitive to ionic strength and insensitive to osmolality. Moreover, their pH dependence was similar to that of isolated zymogen granules. When sulfated glucosaminoglycans isolated from the zymogen granules of the guinea pig pancreas were used instead of chondroitin sulfate, the same kind of interactions with chymotrypsinogen were obtained. Our data support the hypothesis that the strong ionic interactions between those sulfated glucosaminoglycans and cationic proteins could be responsible for the concentration process.

KEY WORDS chymotrypsinogen A · chondroitin sulfate · glucosaminoglycans · molecular aggregation · zymogen granule condensation · turbidity

A sulfated macromolecular fraction has recently been isolated from the zymogen granules of the guinea pig pancreas (17, 18). Preliminary results indicate that this fraction is composed of acidic glucosaminoglycans, principally heparan sulfate and chondroitin sulfate (18). Such compounds have been implicated in the process by which pancreatic acinar cells concentrate their secretory proteins in the condensing vacuoles of the Golgi complex (23, 16, 9). The formation of large aggregates by ionic interactions between those polyanions and cationic secretory proteins could reduce the osmotic activity within the vacuoles and lead to concentration of their content by water loss. In this paper, this hypothesis was tested by studies on the interactions between bovine chymotrypsinogen A (ChTg) and chondroitin sulfate (Ch-SO_4) as a simplified model. The results indicate that the ionic interactions of these compounds are strong enough to induce coprecipitation. Sulfated polyanions isolated from the zymogen granules of the guinea pig pancreas were found to interact with bovine ChTg in a manner comparable to that of Ch-SO_4.

MATERIALS AND METHODS

Bovine chymotrypsinogen A (Worthington Biochemical Corp., Freehold, N.J.) dissolved (1 mg/ml) in 2 mM Tris maleate buffer, pH 6, was mixed with solutions of

chondroitin sulfate A.B.C. (Sigma Chemical Co., St. Louis, Mo.) of increasing concentrations. The mixture was warmed up to 37°C for 15 min and left at room temperature for another 10 min. Aggregate formation was monitored by measuring the increase in turbidity at 430 nm (Phoenix light scattering photometer 2000 [Phoenix Precision Instrument Div., Virtis Co., Inc., Gardiner, N.Y.]). When abundant, the aggregates were centrifuged (10 min × 12,000 *g* at 25°C) and their amount was measured by spectrophotometric determination at 280 nm of the ChTg remaining in the supernate.

In control experiments, rat amylase (mixture of the two isomers prepared according to Marchis-Mouren et al. [13]), porcine amylase II (gift from Dr. Y. Mazzéi) and bovine ribonuclease A (Sigma Chemical Co.; type XI) were used under the same conditions as for ChTg.

Sulfated polyanions, prepared from guinea pig zymogen granunules as previously described (18), were resuspended in 2 mM Tris maleate buffer, pH 6. Increasing amounts of the ensuing solutions were mixed with ChTg (1 mg/ml) and aggregate formation was monitored by turbidimetry. ^{35}S-labeled compounds were mixed with 1 ml of ChTg solution (1 mg/ml) and were precipitated with different amounts of carrier chondroitin sulfate. After centrifugation, the supernate was removed, the pellet was gently rinsed with 0.5 ml of Tris maleate buffer, and ^{35}S radioactivity was determined in the supernate and washes. Pellets were dissolved in 20 μl of 0.1 N NaOH, the tube was rinsed twice with 0.5 ml of H_2O, and the two washes were counted together. Radioactivity was measured in scintillation fluid (1), using a Packard 300 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Chymotrypsinogen was activated by 2.5% bovine trypsin for 2 h at 3°C in 2 mM Tris maleate buffer, pH 9, and the resulting chymotrypsin activity was assayed according to Hummel (7).

RESULTS

Aggregation between ChTg and Ch-SO_4

When added to a solution of bovine chymotrypsinogen A (pI = 9.1), small amounts of chondroitin sulfate induced aggregate formation which was measured by the increase in turbidity of the solution (Fig. 1). This increase was measurable with Ch-SO_4 concentrations lower than 1 μg/ml (0.1% wt/wt of ChTg) (Fig. 1, inset). After centrifugation (10 min × 12,000 *g*), the aggregates were recovered as a pellet, and the amount of ChTg precipitated was measured by decrease in optical density (280 nm) of the supernate. This decrease was found to be linear as a function of the Ch-SO_4 concentration (Fig. 1). The turbidity of the corresponding supernate was measured and found equal to that of the initial ChTg solution, indicating that the aggregates were quantitatively recovered in the pellet.

Maximum precipitation of ChTg (95%) was

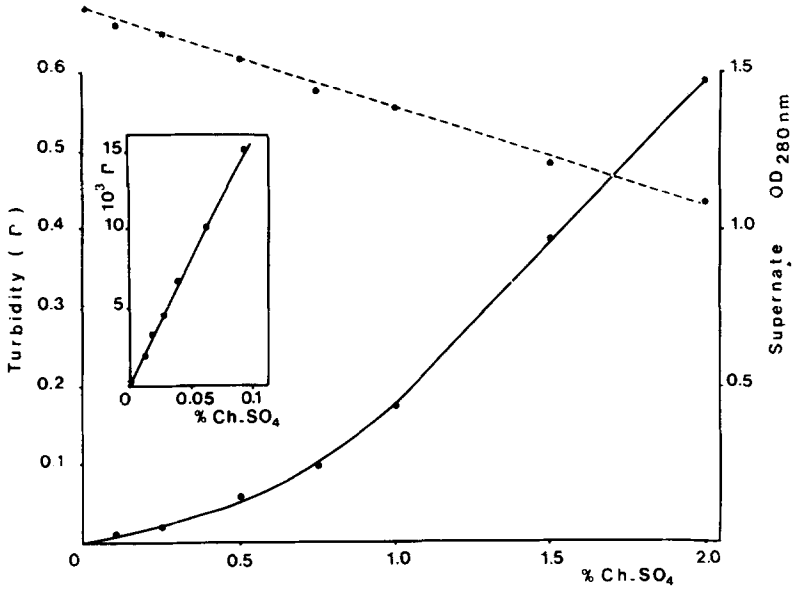

FIGURE 1 Aggregation of ChTg (1 mg/ml) by increasing amounts of Ch-SO_4. Solid line: turbidity at 430 nm of the mixture (final volume: 3 ml). Broken line: optical density at 280 nm of the solution after centrifugation (12,000 *g* × 10 min). Inset indicates turbidity at 430 nm for low concentration of Ch-SO_4. Concentration of Ch-SO_4 is expressed as percent weight of Ch-SO_4 to weight of ChTg.

obtained with 65 μg/ml Ch-SO_4 concentration (6.5% wt/wt of ChTg). Above this concentration, the fraction of ChTg precipitated decreased, and no precipitation was observed above 100 μg/ml of Ch-SO_4. The "zone of equivalence" for maximum aggregation appeared relatively restricted; as for lower Ch-SO_4 concentrations, turbidimetry measurements indicate the absence of small aggregates in the corresponding supernates.

Past the zone of equivalence, the turbidity of the supernate increased to reach a maximal value of 0.021 Γ. This indicates the presence of smaller aggregates that do not centrifuge under our conditions. Reduced aggregation was probably due to prevention, by Ch-SO_4 excess, of maximum cross-linking between Ch-SO_4 and ChTg. A similar interpretation has been given for resolubilization of the complex antibody-antigen.

Aggregation with Different Proteins

The two amylases of the rat (pI 8.7 and 8.9 [20]) and bovine ribonuclease A (pI 7.8 [14]) were also aggregated by Ch-SO_4 and the precipitation curves (not shown) were similar to those obtained with ChTg. However, maximum (95%) precipitations were obtained with Ch-SO_4 concentrations of 50 μg/ml for rat amylases (5% wt/wt of amylase) and 180 μg/ml for ribonuclease A (18% wt/wt of RNase). On the contrary, porcine amylase II (pI 5.4 [4]) did not precipitate with Ch-SO_4. These differences were expected because the interactions between the polyanion and the secretory proteins are believed to be essentially of ionic nature and thus depend on the isoelectric point of these proteins.

Effect of Ions

No information is available concerning ionic environment within condensing vacuoles. However, in guinea pig zymogen granules, calcium and magnesium have been estimated to ~37 and ~9 nmol/mg protein, respectively (5). Under our conditions (1 mg protein/ml), the concentrations of calcium and magnesium necessary to keep constant the Ca^{++}/protein and Mg^{++}/protein ratios (37 and 9 μM) were without effect on ChTg precipitation (not shown).

Increasing the ionic strength of the solution progressively prevented ChTg precipitation by Ch-SO_4 and abolished it completely at 70 mM NaCl (not shown). The aggregates were thus considered as the result of strong ionic interactions, given the very low concentration of ChTg in our experiments (4×10^{-5} M).

Calcium ions were found to compete with ChTg for binding Ch-SO_4. Within a range of 0.5–10 mM Ca^{++}, maximum precipitation was still obtained but with slightly higher Ch-SO_4 concentrations, disaggregation by excess of Ch-SO_4 was inhibited (Fig. 2). This inhibition indicates that calcium ions probably act as an extra cross-linking reagent. At the same ionic strength, the monovalent ion Na^+ was without effect on ChTg precipitation (Fig. 2).

Effect of Osmolality

Aggregates between ChTg and Ch-SO_4 were stable over a large range of osmolality. Maximum precipitation was still obtained in the presence of 1 M sucrose.

Effect of pH

Aggregate formation between ChTg (1 mg/ml) and Ch-SO_4 (1 μg/ml) was pH-dependent. They were stable below pH 8 and were dispersed drastically above this pH (Fig. 3*a*).

Effect of Precipitation on Potential Activity of ChTg

After precipitation with Ch-SO_4, the ChTg was resolubilized in Tris maleate buffer, pH 9, and activated. The resulting chymotrypsin activity was the same as in control experiments (without Ch-SO_4).

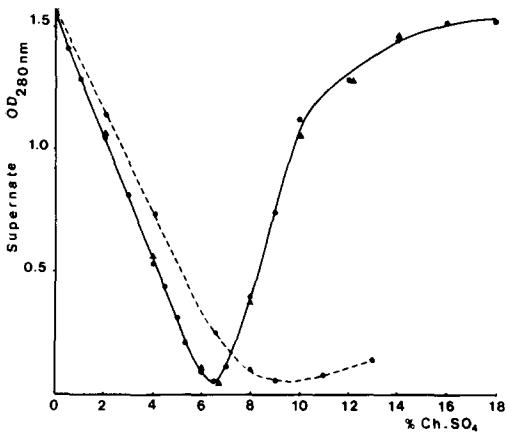

FIGURE 2 Influence of salts on ChTg aggregation by Ch-SO_4. Optical density at 280 nm of the mixture after centrifugation (12,000 *g* × 10 min). (-●-●-) without salts; (-▲-▲-) 1.5 mM NaCl; (--●--●--) 1.0 mM $CaCl_2$. Final volume: 1 ml. Concentration of Ch-SO_4 is expressed as percent weight of Ch-SO_4 to weight of ChTg.

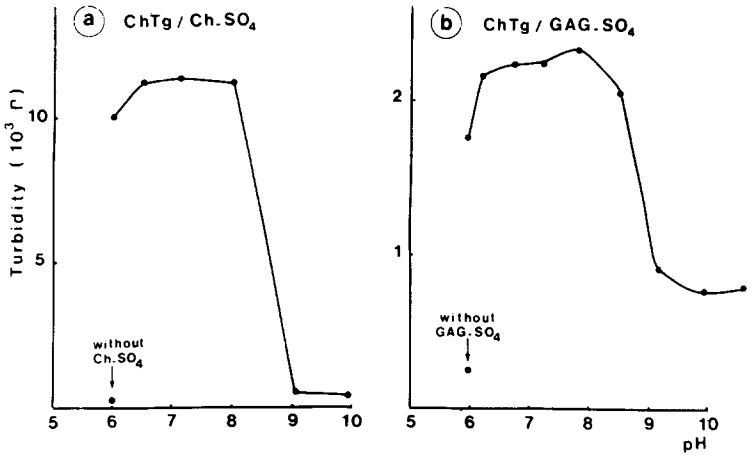

FIGURE 3 pH dependence of aggregate formation between ChTg (1 mg/ml) and (*a*) Ch-SO_4 (1 μg/ml) or (*b*) pancreatic sulfated glucosaminoglycan (GAG-SO_4). Final volume: 3 ml.

Aggregation with Purified Glucosaminoglycans from Guinea Pig Zymogen Granules

Increasing amounts[1] of the sulfated polyanions isolated from guinea pig pancreas zymogen granules mixed with ChTg solutions (1 mg/ml) induced increasing aggregate formation that was monitored by turbidimetry (Fig. 4). Their pH dependence was similar to that obtained for Ch-SO_4-induced aggregation (Fig. 3*b*). $^{35}SO_4^{-2}$-labeled pancreatic sulfated polyanions were used to verify whether the observed aggregation actually involved the sulfated compound and not a potential contaminant in the fraction. An aliquot of this fraction was mixed with ChTg (1 mg/ml) solutions and different amounts of Ch-SO_4 (Table I). The mixtures were then centrifuged and the radioactivity was determined in the pellets and in the supernates. When Ch-SO_4 concentration was below the equivalence point, ~100% of the radioactivity was found in the pellet; with higher concentration, partial solubilization was observed but to a lesser extent than for ChTg (as measured by optical density of the supernate) (Table I). No radioactivity was precipitated by buffer alone or by Ch-SO_4 without ChTg (controls). With ChTg (but without Ch-SO_4 carrier), 65% of the counts were found in the pellet.

[1] The amount of sulfated glucosaminoglycans isolated was too low to be assayed. Amounts refer to volume of the preparation or ^{35}S counts.

DISCUSSION

What we know about the mechanism by which pancreatic acinar cells concentrate and maintain concentrated their secretory proteins is limited to a few facts: the process is independent of protein synthesis and does not require energy (8). Recently, it was postulated that concentration results from a progressive reduction in osmotic activity within the condensing vacuoles, brought about by the formation of large aggregates, mostly by ionic interactions among secretory macromolecules (8).

To operate, such a system requires: (*a*) that one of the interacting species be generated (or become functional) only in the Golgi complex (as a soluble or membrane-associated molecule) and (*b*) that the aggregates be stable below pH 7.5 and disperse at higher pH to allow enzyme solubilization in pancreatic juice. Sulfated polyanions have been proposed as one of the critical interacting species as they satisfy the first requirement (23, 16, 9). Indeed, autoradiographic studies indicate convincingly that the primary site of SO_4^{-2} incorporation is the Golgi complex (3, 24, 18). Given their low pI (resulting from sulfation), such polyanions are expected to interact at neutral pH with cationic proteins, known to be an important fraction of the pancreatic secretion (in the guinea pig, ~60% have a pI > 8) (23, 21). In the present work, we used the interaction between ChTg and Ch-SO_4 as a simplified model. Our data indicate that the ionic interactions between those two compounds are strong enough to induce multimolecular aggregate formation and co-precipitation. Like zymo-

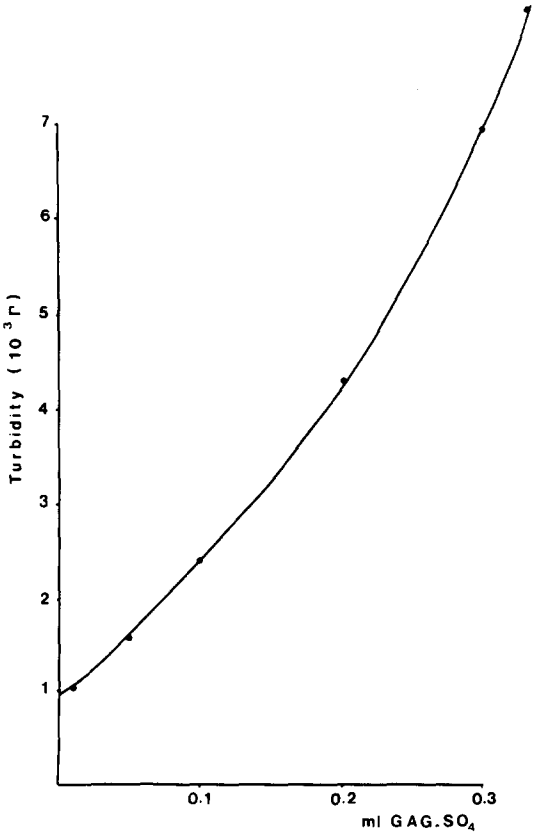

FIGURE 4 Aggregate formation between ChTg and different amounts of sulfated glucosaminoglycan isolated from guinea pig zymogen granules. Final volume: 3 ml.

gen granules, these aggregates are moderately sensitive to ionic strength and insensitive to osmolality of the solution. Moreover, our data indicate that the second requirement is satisfied in the simplified model ChTg-Ch-SO_4 inasmuch as the aggregates were completely dispersed when the pH was raised above pH 8. Our results may bear on the situation likely to prevail *in situ,* because Ch-SO_4 has been characterized together with heparan sulfate in guinea pig pancreas zymogen granules (18). The two compounds are chemically related, and circular dichroism studies indicate that both generate comparable ionic interactions with synthetic cationic polypeptides (22). Furthermore, we have detected aggregate formation between ChTg and purified pancreatic sulfated polyanions (with similar pH requirement) and our co-precipitation experiments indicate that the pancreatic compounds have a higher affinity for ChTg than CH-SO_4.

Of importance are our data concerning calcium affinity for Ch-SO_4 given the high concentration of this cation within zymogen granules and its preferential association with the membrane (5). Sulfated polyanions seem to be, at least in part, membrane associated (18) and therefore could constitute binding sites for Ca^{++}, for instance, with the SO_4^{-2} groups that cannot interact with cationic proteins for sterical reasons. Such binding would also cause a decrease in osmotic activity within condensing vacuoles and thus participate in the concentration process.

So far our data support the hypothesis that sulfated polyanions are involved in the concentration process. In our model experiment, they meet the two basic requirements of that hypothesis. However, in vivo the complete mixture of secretory proteins is already mixed in condensing vacuoles and most probably in the preceding compartments of the secretory pathway (11). Because some of them are neutral or acidic, besides the interaction of the nature discussed above we should also consider more complex protein-to-protein interactions. Sulfated polyanions represent a minor fraction of the guinea pig zymogen granules (<1%) and therefore are far from the conditions needed to obtain maximum precipitation in our model. Contrary to the assumption of Kronquist et al. (12), our data indicate that such a low level of sulfated glucosaminoglycans is com-

TABLE I

Co-Precipitation of Bovine ChTg with Ch-SO_4 and the Sulfated Glucosaminoglycan (GAG-SO_4) from Guinea Pig Zymogen Granules

Ch-SO_4 added	ChTg precipitated	GAG-$^{35}SO_4$ in the pellet
% of ChTg wt/wt	*% of total*	*% of total counts*
0	<5	65.6
2	30.2	98.7
4	68.5	97.1
6	82.0	99.4
6.5	90.0	98.3
8	87.2	96.6
10	70.0	84.6
12	42.9	63.1
14	24.3	31.7

The mixture contained in 1 ml of Tris maleate, pH 6.0: 1 mg of ChTg, 50 μl of GAG-$^{35}SO_4$ solution containing 1,500 cpm and various amounts of Ch-SO_4. When ChTg and ChTg + Ch-SO_4 were omitted, GAG-SO_4 precipitation was 0.5 and 2%, respectively.

patible with their involvement in the concentration process since such a process does not require that the sulfated polyanions interact with all cationic proteins under optimal conditions. Actually, the secretory proteins of the pancreas appear to precipitate easily out of solution in vivo as well as in vitro. Reaggregation of a zymogen granule lysate (pH 8.5) from rat pancreas has been obtained by lowering the pH to 5.5 (19). It is interesting to notice that reaggregation was less efficient when the membranes were removed inasmuch as it is known that such membranes are associated with an important fraction of sulfated polyanions (18, 12). *In situ,* the secretory proteins form intracisternal granules in the cisternal space of the rough endoplasmic reticulum when transport to the Golgi complex is slowed down by starvation (15) or blocked by cobalt poisoning (10). The secretory mixture appears close to aggregation. Hence, the very small amount of sulfated polyanions encountered in the Golgi complex may modify protein-to-protein interactions within the condensing vacuoles to a point at which aggregate formation becomes effective in the concentration process.

This process may be of broad significance because sulfated compounds have been found in many secretory cells (24, 2, 6). Moreover, secretory cells appear to use a variety of ionic interactions in the concentration process (stereospecific interactions, metal complexing, etc. cf. reference 9), in addition to the one we have been postulating. Such interactions are expected to lead to the same result.

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