REVERSIBLE AGGREGATION OF MUSCLE GLYCOGEN SYNTHETASE BY METABOLITES

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SUMMARY - Partially purified glycogen synthetase from rat skeletal muscle shows a single nearly symmetrical peak with a molecular weight of approximately 195,000, as judged by activity measurements in sucrose gradient centrifugations. In the presence of UDP-glucose and glucose-6-P this enzyme gives rise to a series of oligomers. The effect is specific, and it is shown that in order to obtain the glycogen synthetase polymerization the substrate site must be occupied, and that glucose-6-P greatly facilitates the process. ATP can reverse the aggregation. The results obtained are discussed in connection with the previously found allosteric behaviour of glycogen synthetase.

GS[1] (UDP-glucose: α-1,4 glucan α-4-glucosyltransferase, E.C. 2.4.1.11) from skeletal muscle is normally associated to glycogen (1, 2), but tetanic stimulation can release it from the polysaccharide (2, 3). Sucrose gradient centrifugation of crude homogenates obtained from muscle electrically stimulated have shown that this GS does not behave as a single, symmetrical peak, but rather has a complex pattern. On the other hand, it has been shown that muscle GS is an allosteric enzyme (4). The possibility of working with a glycogen-free GS, and the fact that the enzyme could be found under several aggregation states, prompted the investigation of the effect of various metabolites on the eventual distribution of GS molecular species.

MATERIALS AND METHODS - Enzyme Preparation. GS was prepared by a procedure similar to that used for phosphorylase-phosphatase (5). Muscles from the hind legs of male Wistar rats were homogenized (1:5) with 4 mM EDTA-4 mM KOH in a blendor for 3 min. The homogenate was centrifuged at 12,000 x *g* for 10 min, filtered through glass wool, and the supernatant fluid centrifuged at 105,000 x *g* for 60 min. The pellets were resuspended in 0.6 ml/g muscle of

[1] Abbreviations: GS, glycogen synthetase; MW, molecular weight.

50 mM Tris-HCl, pH 7.5, 40 mM mercaptoethanol, 1 mM EDTA (Buffer "A"). NaCl was also added to a final concentration of 0.1 M, and then, as an α-amylase source, 0.13 ml/g muscle of a 27,000 x g supernatant fluid of saliva. The preparation was incubated at 4° for approximately 20 hr, and was then centrifuged at 105,000 x g for 40 min. The clear supernatant fluid (from 30-40 g of muscles) was applied to a DEAE column (2 x 12 cm) equilibrated with buffer "A" containing 0.1 M NaCl, and the column was then washed with 600 ml of buffer "A" - 0.15 M NaCl at a flow rate of 0.3 ml/min. GS was eluted with buffer "A" - 0.35 M NaCl, and the most active fractions were pooled and passed through a G-25 Sephadex column (1 x 20 cm) equilibrated with buffer "A" in order to remove the NaCl. Sucrose was then added to a 5% (w/v) final concentration, the enzyme fractionated in aliquots, and frozen with liquid air. Recoveries with respect to the crude homogenate were 20-30%. The preparation is stable, and it is free of glycogen-phosphorylase and amylase. It has usually a 60% content of I-form, and the activity in the absence of glycogen, and under the standard assay conditions, is less than 2% of that obtained in its presence.

Sucrose-gradient Centrifugations. Sucrose solutions were made in 10 mM glycylglycine, pH 7.5, 50 mM NaF, 10 mM EDTA, 2 mM $MgSO_4$, and 20 mM mercaptoethanol. In addition, they contained the metabolites tested, as indicated in each experiment. The metabolites were also added to the enzyme, thus decreasing the concentration of sucrose present to 3.7%; 75 µl of this solution were layered on linear 8 to 15% (w/v) gradients (4.5 ml). Centrifugations were usually carried out at 60,000 rpm in a SW 65 K rotor (Spinco) for 3 hr at 2°. Gradients were fractionated into 40 fractions, and 25 µl aliquots used for enzymatic assays. The recovery of activity was usually 70-90%. MW were calculated according to Martin and Ames (6).

GS Assay. It was carried out by a procedure similar to that previously described (4). Glucose-6-P (10 mM) was included in all assays (60 min at 30°) in order to measure maximal activity.

RESULTS - Sucrose-gradient centrifugations of homogenates from resting muscles have shown that GS is associated to glycogen (100 S), and that electrical stimulation or epinephrine perfusion partially dissociates the enzyme from the polysaccharide, giving rise to a peak of approximately 11 S (2, 3). Sucrose-gradient centrifugations of a 105,000 x g supernatant fluid obtained from an homogenate of a 15 sec tetanically stimulated muscle have further indicated that GS, not only is dissociated from glycogen, but also that it has a complex pattern (Fig. 1), suggesting the existence of multiple states of aggregation. Even though the resolution is incomplete, two main species

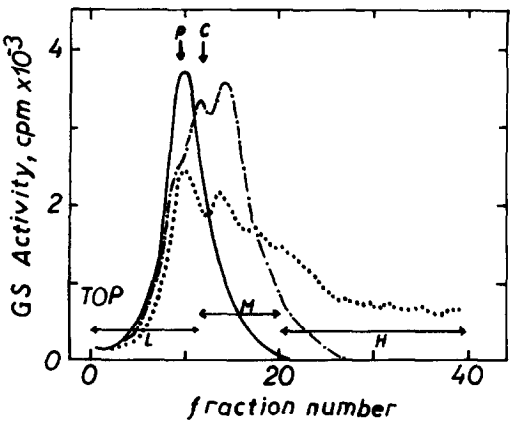

Fig. 1. Sucrose gradient sedimentation of GS. The crude enzyme (·-·-·) is a 105,000 x g supernatant fluid of an homogenate from muscles stimulated for 15 sec (2, 7). Purified GS is the preparation described under Methods, centrifuged in the absence (——) or presence (·····) of 0.5 mM UDP-glucose and 0.5 mM glucose-6-P. The arrows indicate the migration of phosphorylase b (P) and catalase (C), which were used as markers (8, 9).

with MW of approximately 270,000 and 370,000 seem to be present. Purification of GS gives rise to a single, nearly symmetrical peak of MW 195,000, as determined by comparison with catalase and phosphorylase b[2]. The slight asymmetry of the GS peak, which in few instances takes the shape of a shoul-

[2] The relationship between the patterns of purified and crude GS is not clear. In fact, the results of Fig. 3A (vide infra) suggest that the profile found with crude GS cannot be ascribed to the metabolites which might have been present in the homogenate supernatant fluid. Whether manipulation of GS during purification or other factors are responsible for the shift in MW is under current investigation.

der, is toward the region of higher MW indicating that the purified GS might undergo some aggregation. Addition of the substrate (UDP-glucose) and the positive effector (glucose-6-P) brings about a complete change in the sedimentation pattern. In fact, the profile obtained suggests that 30-40% of the species present have MW higher than the original one. The specific activities of each peak could not be determined because of the very low protein concentration used. No further changes in the profile were observed with higher concentrations of these metabolites.

The results of Fig. 2A show that glucose-6-P alone is unable to produce aggregation, and that UDP-glucose and UDP (a competitive inhibitor) can induce a slight aggregation, as indicated by a shoulder and a second peak, respectively. MW of this species is of approximately 340,000. However, neither metabolite seems to be able to produce species of higher MW, even though UDP-glucose or glucose-6-P were used at concentrations up to 5 mM. The substitution of either UDP-glucose or glucose-6-P by an analogue is indicated in Fig. 2B. As can be seen, UDP can replace UDP-glucose rather effectively, even though not completely. On the other hand, when glucose-1-P is substituted for glucose-6-P, the pattern is similar to that obtained with

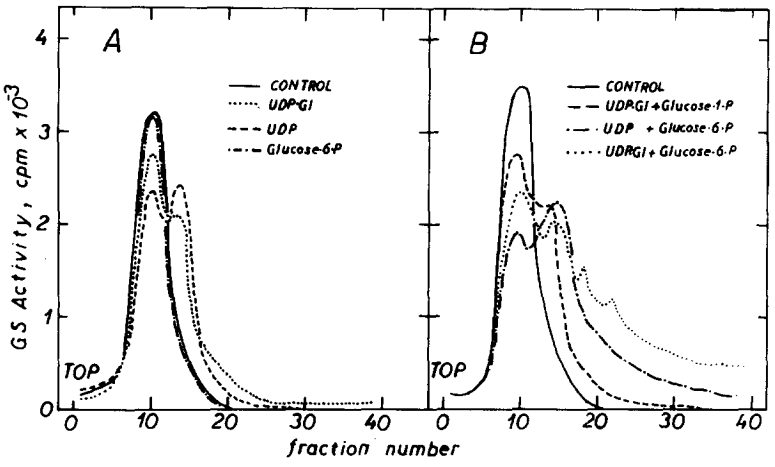

Fig. 2. The effect of various metabolites upon the sedimentation of GS in sucrose gradients. The experimental conditions are described under Methods. Metabolites were used at 0.5 mM concentration.

UDP-glucose alone. It should be pointed out that while UDP is a competitive inhibitor, glucose-1-P cannot replace glucose-6-P kinetically (4, 10). These experiments suggest that binding at the substrate site is necessary to obtain aggregation, but this cannot proceed to high MW species unless the positive effector site is also occupied.

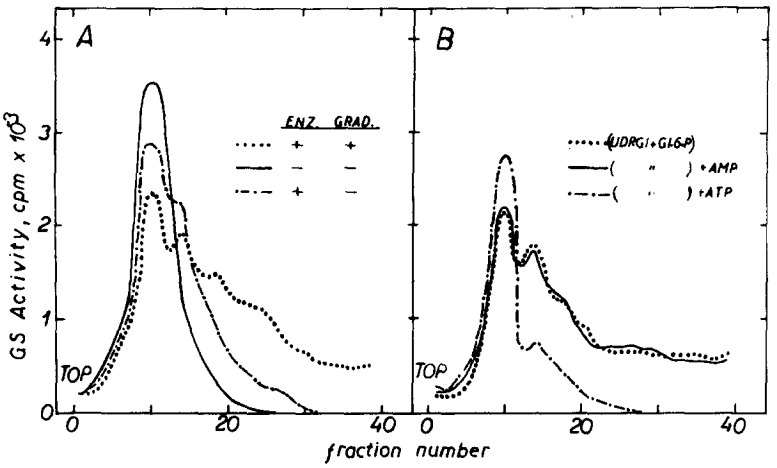

Fig. 3. The reversibility of the (UDP-glucose + glucose-6-P) effect upon the sedimentation of GS. (A) GS was centrifuged alone (——), or in the presence of the metabolites both in the sample layered on the gradient and in the gradient (•••••); and with the metabolites in the sample, but not in the gradient (•-•-•). (B) The inhibitors ATP and AMP were used at a 5 mM concentration. Other conditions are described under Methods.

The reversibility of the phenomenon just described was also investigated. The pattern of a GS centrifuged with UDP-glucose and glucose-6-P present only in the sample layered on the gradient is compared with those obtained when the metabolites were either present throughout, or not at all (Fig. 3A). It can be seen that when the metabolites are present only in the sample the pattern resembles that of the untreated enzyme. In addition, 0.6 M KCl can reverse the aggregation completely, even in the presence of UDP-glucose and glucose-6-P (not shown). The reversibility can also be achieved with ATP (Fig. 3B). In fact, when the nucleotide (5 mM) is included in the gradient most of the oligomers disappear. This effect cannot be elicited by 5'-AMP.

The GS inhibition by several nucleotides previously reported (4) was ob-

tained under different conditions than those used in the present study, hence, it was thought necessary to obtain the inhibition data under the same circumstances used for the sedimentation experiments. The results are summarized in Table I, together with the most pertinent data obtained by gradient centrifugation. It can be seen that, with the exception of UDP, the other nucleotides do not inhibit appreciably. While this result might seem to contradict the previous data (4) it should be pointed out that the GS used in this study has a relatively high content of I-form, and therefore, a small glucose-6-P concentration can reverse the inhibition. The percentage of oligomers distribution obtained by sedimentation under the various conditions is also indicated in Table I. It is apparent that there is no correlation between activity and, e.g., the amount of high MW species.

TABLE I - The Effect of Metabolites upon the Activity and Sedimentation of GS[a]

Additions[b]	% GS Activity	Species distribution (%)[c]		
		L	M	H
None	-	70	29	1
UDP-glucose + glucose-6-P	100	38	38	24
" + " + AMP	95	42	36	22
" + " + ATP	99	64	30	6
" + " + UDP	51	40	36	24

[a]The same purified GS (60% of I-form) was used for the sedimentation and kinetic experiments. The latter were performed with the buffer employed in the sucrose gradient.

[b]The concentrations used are: UDP-glucose, UDP and glucose-6-P, 0.5 mM; AMP and ATP, 5 mM.

[c]The percentages were calculated from the areas corresponding to the fractions which have been labelled in Fig. 1 as L, M, and H. They roughly represent the region of an eventual monomer, dimer-trimer, and higher oligomers, respectively.

DISCUSSION - Aggregation or disaggregation upon addition of substrate and/or effectors has been reported for a number of enzymes (11-20). The specificity and concentration of the metabolites which can produce GS aggregation and its reversion (Figs. 2 and 3) are within the levels found in muscle (7). It is

therefore tempting to speculate that they might play a role *in vivo*[3] and that this phenomenon is related to the allosteric behaviour of GS (4). The lack of correlation found under the present conditions between the kinetic and sedimentation data (Table 1) does not seem to substantiate this hypothesis. However, before dismissing it, a more exhaustive investigation is necessary, since - as pointed out by Stadtman (20) - the apparent inconsistencies between physical and kinetic data may reflect relative differences in the strength of subunit interactions. In fact, it is highly remarkable that ATP could reverse the aggregation, while AMP not (kinetically a strong and weak inhibitor, respectively).

The MW of the "untreated" purified enzyme (195,000) resembles that of phosphorylase *b* (8). Since it has been obtained at a rather low protein concentration it is possible that the actual value is e.g. twice, if synthetase can undergo dissociation with dilution like phosphorylase *a* (8, 21).

The possibility that the GS aggregation observed is due to polysaccharide synthesis seems rather unlikely since a) there is no increase in the amount of higher MW species on preincubation, while there is an increased incorporation into glycogen-like material (not shown), b) the aggregation occurs with UDP and glucose-6-P, that is, when there is no possibility of synthesis, and, c) the process is reversible under conditions (ATP or KCl) which do not affect the dissociation of GS from glycogen (2, 3).

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[3] GS is glycogen-bound *in vivo*, but this does not preclude that it might undergo changes in its state of aggregation on the glycogen surface.

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