PURIFICATION OF MUSCLE GLYCOGEN PARTICLES BY GLYCEROL-GRADIENT CENTRIFUGATION

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1. Introduction

In the recent years, several reports of the association of muscle glycogen with specific proteins have appeared [1–3]. This assembly has been a useful tool for the purification of glycogen metabolizing enzymes [4]. The observation that the catalytic and regulatory properties of the enzymes are different when they are attached in the particle or free in solution suggests furthermore that this assembly can be a physiologically important one. The regulation of phosphorylase kinase [5] and phosphatase [6] by calcium ions in solution and in the glycogen organelle is quite different, whereas at the level of phosphorylase [7] itself, of debranching enzyme [8] and of phosphatase a distinctive maximal catalytic activity of the enzymes has been reported. The nature of the stimulus which triggers this different behavior is still uncompletely understood although these properties have been allotted to protein–protein interactions, suggesting the importance of the supramolecular structure of the glycogen particle. In the present communication, we describe an improved purification procedure which allows to obtain in less than 6 h particles completely devoid of contaminating sarcoplasmic reticulum membranes and suitable for structural studies.

2. Methods

2.1. *Purification of glycogen particles*

The method of purification used is derived from the procedure described by Wanson and Drockmans [3]. All the steps are performed at 4°C.

One male rabbit is killed by bleeding after intravenous injection of 30 mg/kg of Nembutal. The muscles from the back are excised, ground, and homogenized with 2.5 vol. 30 mM phthalate potassium buffer, pH 6.05, in presence of 2 mM EDTA. The preparation is then centrifuged at 7500 rev/min in a JA10 Beckmann rotor. The supernatant is filtered through cheese cloth, adjusted at pH 6.05 with 1 N acetic acid and centrifuged 65 min at 200 000 × *g*. The pellet is taken up with 50 mM Tris–acetate buffer, pH 6.1, with 2 mM EDTA, with final vol. approx. 12 ml/100 g muscle. This fraction represents the classical muscle glycogen particle and is referred to as 'crude particles'.

The main differences in the present procedure with respect to the original one by Wanson and Drockmans [3] are inclusion of EDTA in the buffers, and decrease of the centrifugation time. These modifications are brought to avoid glycogen degradation by endogenous α-amylase.

2.2. *Glycerol gradient on the 'crude particles'*

Three ml of appropriately diluted particles are layered on the top of either a four-step glycerol gradient (10 ml 5% glycerol, 7 ml 10%, 7 ml 20%, 7 ml 30%) or a 10–30% glycerol gradient according to the method of Fritsch [9] which allows the determination of sedimentation coefficients. These gradients are done in Tris acetate 50 mM, EDTA 2 mM, pH 6.1, buffer.

These gradients are run 2 h at 4°C in a SW-27 Beckman rotor at 24 krev/min. The pellet, dissolved in Tris acetate buffer, and the 22–24 fractions

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obtained for each gradient, are assayed for glycogen and for the different enzyme activities.

2.3. *Assay of enzyme activities*

Phosphorylase b is assayed according to Helmreich and Cori [10], phosphorylase kinase according to Krebs et al. [11], glycogen synthase according to Thomas et al. [12], debranching enzyme (1–6 glucosidase) according to Cohen et al. [4], UDP glucose pyrophosphorylase according to Hansen et al. [13], orthonitrophenyl acetate esterase according to Beaufay et al. [14] and glycogen synthase kinase II according to Cohen et al. using phosvitin as a substrate [15]. Glycogen was titrated according to Montgomery [16] and proteins according to Lowry as modified by Stauffer [17].

2.4. *SDS–gel electrophoresis*

Sodium dodecyl sulfate gels are done according to Laemmli [18]. A ratio of biscrylamide upon acrylamide of 1/60 and a concentration of acrylamide of 7.5% is used to obtain a better resolution of the different proteins. The samples are precipitated with two volumes of 10% TCA, the pellet obtained after centrifugation is dissolved and neutralized in NaOH 0.2 N and diluted with Laemmli sample buffer, brought to 100°C during 3 min and deposited on the gel.

The gels are run 6 h at 70 V, and stained with Coomassie Brillant blue.

2.5. *Staining for electron microscopy*

Glycogen particles are observed in electron microscopy by negative staining. Grids are observed on a Siemens Elmiskop 101 electron microscope. Before putting a sample of the different fractions of the glycerol gradient on the grids, a rapid dialysis of these fractions against Tris acetate 50 mM, EDTA 2 mM, pH 6.1, is found necessary to avoid the interference of glycerol with the staining procedure.

3. Results

The recovery of the different enzymatic activities in 'crude particles' as compared with the activities of the enzymes present in muscle is seen in table 1. Clearly

Table 1
Recovery of enzyme activity in the glycogen particle (units/g wet wt)

R	Crude extracts	Particle	%
Phosphorylase[a]	40	17.5 ± 2.5	43.7
Phosphorylase[b] kinase	2.57	0.52 ± 0.063	20.2
Debranching[c] enzyme	0.032	0.0112 ± 0.0026	36
Glycogen[d] synthetase	1.6	0.57 ± 0.04	35.6
Phosphorylase[e] phosphatase	0.024	0.007	29.1
AMP deaminase[f]	160	0.87 ± 0.08	0.54
UDPG–pyrophosphorylase[g]	12.2	0.44	3.6

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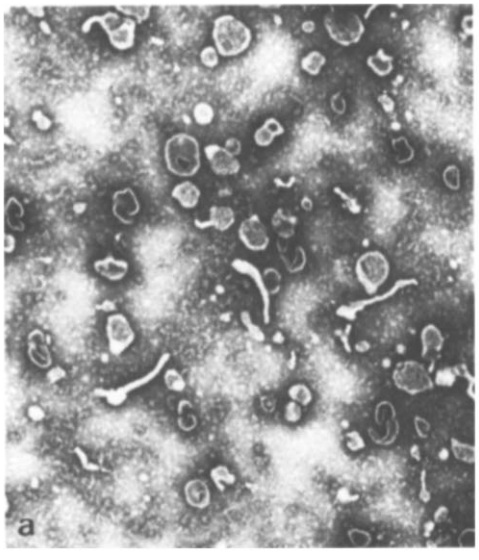
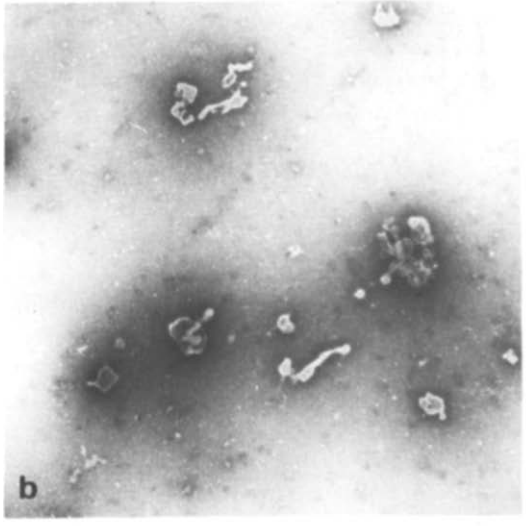
phosphorylase, phosphorylase kinase, phosphorylase phosphatase glycogen synthetase and debranching enzyme are specifically attached to the glycogen particles. A typical muscle enzyme has also been assayed, AMP deaminase: it is only weakly recovered in this glycogen fraction. Therefore, AMP deaminase appears to be a contaminant of this glycogen particle as well as UDPG pyrophosphorylase. These 'crude particles' are not completely purified, as shown by electron microscopy (fig.1A). Two distinct structures are seen: membranes and glycogen particles which present themselves as roundly shaped, irregular spheres of diameter comprised between 200 Å and 800 Å. These particles correspond to the β-type according to the nomenclature of Drockmans [19].

This pattern can only be observed on fresh preparations. After few hours, the particles are less distinct and they are progressively replaced by an amorphous mass.

At the bottom of the gradient is found a pellet, which is shown by electron microscopy (fig.1B) to be formed only with membranes devoid of glycogen particles. In this pellet only trace amounts of phosphorylase, phosphorylase kinase, glycogen synthetase and debranching enzyme are found. On the contrary 60% of a nitrophenyl acetate esterase which is known as a marker of sarcoplasmic reticulum is found in this pellet. The assay of the different enzymatic activities in the glycerol gradient shows a complex pattern (fig.2, 3A and 3B). However, it is possible to distinguish three fractions in this gradient.

The peaks of activities located at the top of the gradient are not associated with glycogen particles, since no particles can be detected by electron microscopy. Glycogen is also absent, and these





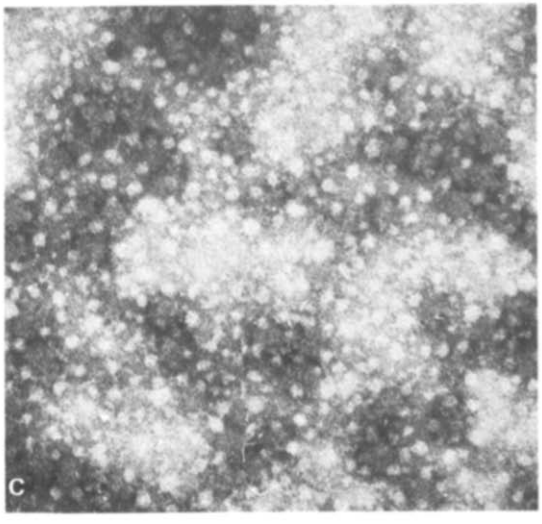


Fig.1. Study by negative staining in electron microscopy of the crude and purified glycogen particles. (1A) 'Crude particles' × 50 000. (1B) Glycerol gradient pellet × 50 000. (1C) Pure glycogen particles (corresponding to the light particle fraction of the gradient of fig.2) × 100 000.

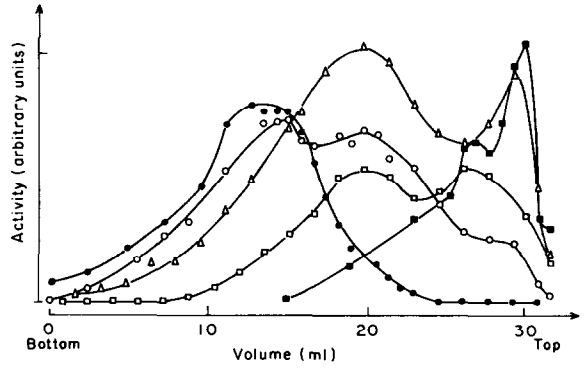


Fig.2. Glycerol gradient, 10–30%, of crude glycogen particles. (●—●—●) Glycogen synthetase activity. (○—○—○) Phosphatase activity. (△—△—△) Phosphorylase activity. (□—□—□) Phosvitin kinase activity. (■—■—■) UDPG–pyrophosphorylase activity. Conditions as described in Materials and methods.

peaks of activity probably correspond to the free proteins: one can notice that the peak of phosphorylase kinase migrates slightly deeper in the gradient than the phosphorylase b peak, a result which is in agreement with the known molecular weight of these proteins.

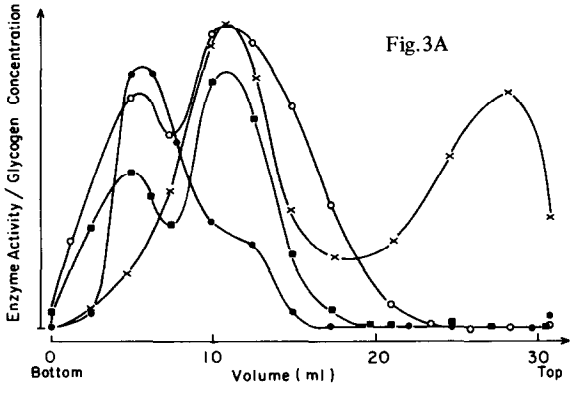
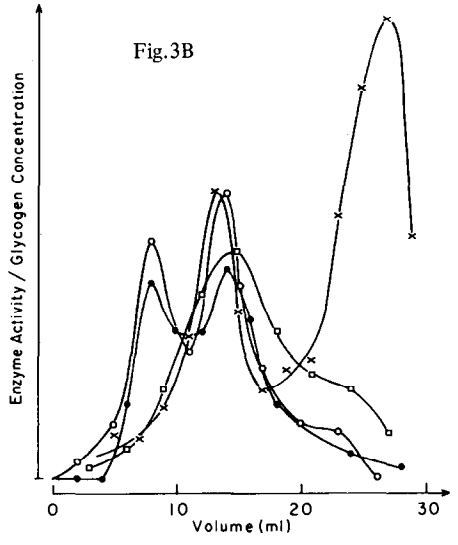


Fig.3A. Step glycerol gradient of crude glycogen particles. (○—○—○) Phosphorylase phosphatase activity. (■—■—■) Glycogen. (X—X—X) Phosphorylase kinase activity. (●—●—●) Glycogen synthetase activity. Fig.3B. Step glycerol gradient of crude glycogen particles. (X—X—X) Phosphorylase kinase activity. (□—□—□) Phosphorylase activity. (●—●—●) Debranching activity. (○—○—○) Glycogen.

In the middle of the gradient, two peaks of glycogen are clearly distinguished: one located at the lower part of the gradient is specifically associated with glycogen synthase, and partly debranching enzyme and phosphatase. The other one located in the upper part of the gradient contains the whole phosphorylase and phosphorylase kinase activities which are not free in solution, phosphorylase phosphatase and most of the phosvitin kinase and debranching enzyme activity. Part of UDPG pyrophosphorylase is also partially associated with this fraction, although most of it is in solution. The sedimentation coefficients of these two subfractions are respectively equal to 143 S and 113 S (fig.2). By electron microscopy these fractions are shown to be constituted of distinct particles devoid of any membranes (fig.1C). These particles have about the same diameter as the particles observed in the 'crude fraction'. The two subfractions observed in the glycerol gradient correspond therefore to two different types of glycogen particles, although up to now, we have not succeeded in distinguishing these two kinds of particles by electron microscopy.

The glycogen to protein ratio is equal to 15 in the 'heavy' particles, whereas it is only 2 in the 'light' particles: the two subfractions are therefore characterized by a very different glycogen composition.

The pattern of proteins observed on SDS gels show also a clear distinction in the protein composi-



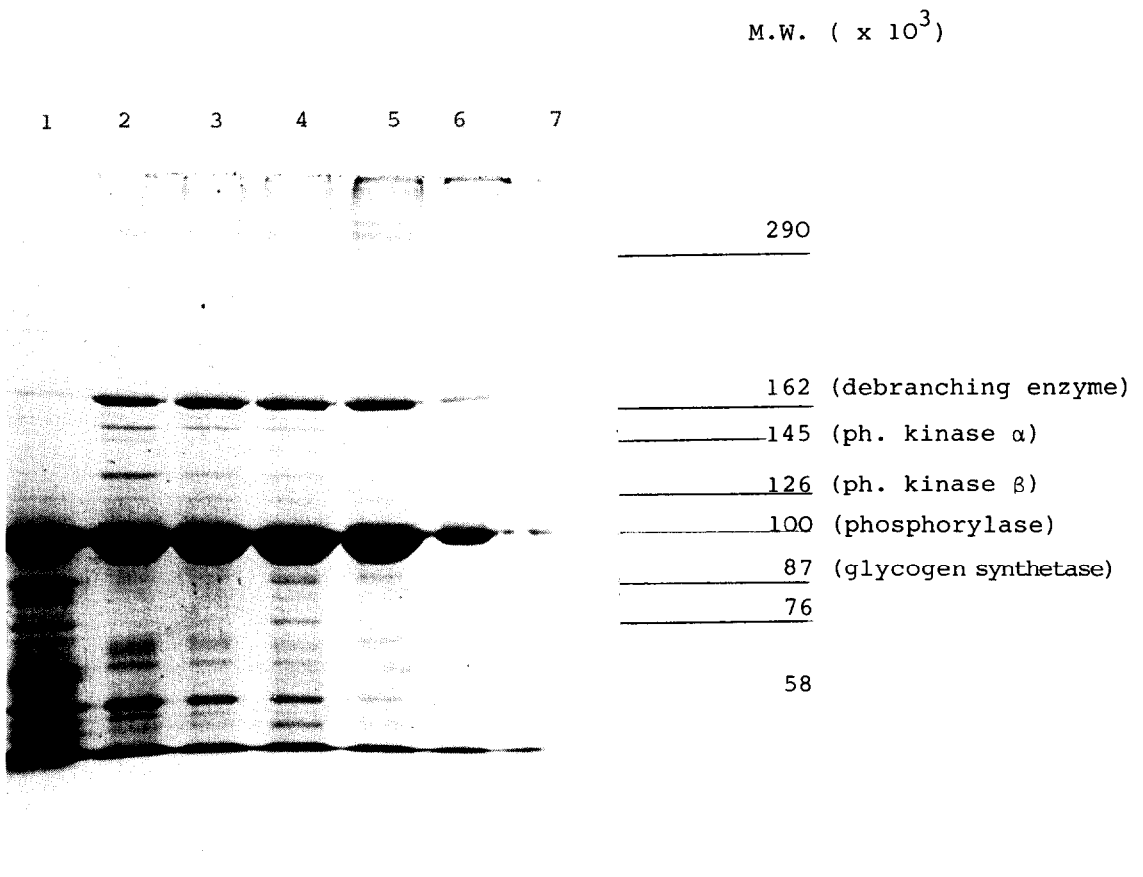


Fig.4. SDS–acrylamide gels of the different fractions of fig.2 glycerol gradient. 1 = Vol. 29 (free enzymes). 2 = Vol. 24 (light particles). 3 = Vol. 20 (light particles). 4 = Vol. 16 (heavy particles). 5 = Vol. 13 (heavy particles). 6 = Vol. 9 (heavy particles). 7 = Vol. 4 (heavy particles). The different molecular weights are determined from known values in the literature for phosphorylase glycogen synthetase, phosphorylase kinase and debranching enzyme.

tion of the different fractions separated on the glycerol gradient. The polypeptides present in the pellet (results not shown here) are clearly distinct from the ones present in the purified glycogen particle fractions. Moreover, the pattern of polypeptide bands observed on the SDS gel for the two subfractions of pure glycogen particles is also different: the intensities of the different bands are very different from one subfraction to the other one (fig.4).

Two remarks must be made: First, the number of proteins, clearly and specifically attached to the glycogen particles seems to be much higher than previously described by other authors [20]. Three bands of high molecular weight, of unknown function, can clearly be shown attached to the pure glycogen particle fractions.

Second, the differences between the two subtypes of particles are more quantitative than qualitative. In particular, while in the heavy subfraction little phosphorylase b activity can be detected, a relatively heavily stained band corresponding to phosphorylase b is present in the gel. This result suggests that the differences in the enzymatic activities observed for the two subfractions could be due to the presence or absence of specific inhibitors or activators in one or the other fraction.

4. Discussion

The data we presently report demonstrate the possibility of obtaining muscle glycogen particles in a more purified form than was previously possible. Under these conditions, it is possible to remove any sarcoplasmic reticulum component which precipitates in the pellet in a 5–30% glycerol gradient and the contaminant enzymes such as AMP deaminase and UDP glucose pyrophosphorylase. Both these enzymes represent contaminants of the glycogen particles even if they can eventually play a role in the overall muscle glycogen metabolism [21,22]. Our results only show that they do not participate in the supramolecular structure of the particle.

The particles which are obtained by the present procedure are highly purified but still very complex. They contain phosphorylase, phosphorylase kinase and phosphatase, glycogen synthetase, debranching enzyme and several other bands which further wait for identification in SDS–polyacrylamide electrophoresis. From the pattern of the glycogen gradient it is evident that at least two populations of particles exist. They differ by their sedimentation coefficients and the glycogen to protein ratio. Even their relative enzyme composition is quite different. The great majority of glycogen synthetase is found in the heavier component (approx. 143 S) while the lighter particles are enriched in the glycogenolytic enzymes. Beside this quantitative difference it is clear that several proteins are nearly exclusively found either in the lighter or in the heavier particles. The gel pattern of both types of particles reveals also the constant presence of 3 or 4 bands of app. mol. wt 300 000. Because of the denaturating procedure employed (TCA precipitation, boiling in SDS and β-mercaptoethanol) it is unlikely that these bands represent aggregated forms of particle-specific proteins. Because of their low R_F they are possibly glycoprotein in nature [23] and the eventuality that they are the analogue for muscle of the glycogen primer protein described by Krisman in the liver [24] is attractive.

We are presently attempting to purify these proteins to ascertain their chemical composition and their ability to accept glycosyl moieties in the glycogen synthetase reaction. The occurrence of two different types of glycogen particles raises several questions on their physiological significance, their function in muscle and the mechanism for switching from one to the other.

Obtaining pure glycogen particles will probably allow us to understand the structural basis of the differences in the properties of the enzymes when they are free in solution and associated in the glycogen particles.

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