APPLICATION OF A METRIZAMIDE DENSITY-GRADIENT TO THE PURIFICATION OF GLYCOGEN PARTICLES

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1. Introduction

Different enzymes, related to the glycogen metabolism have been reported to be associated in the rabbit skeletal muscle within a 'glycogen particle' [1–5]. In this purified particle one can detect the presence of glycogen phosphorylase [1,3,5], phosphorylase kinase [1,5], phosphorylase phosphatase [1], amylase [1], myokinase [1], adenylic deaminase [1], the enzymes of the glycolytic pathway [1], glycogen synthetase [3,5] and amylo 1-6-glucosidase [3].

These particles can be separated into two different components by sucrose-gradient centrifugation [1,5] or Sepharose 2B filtration [1]. The first fraction, called by the authors the glycogen fraction [1,5] has a sedimentation coefficient of approximately 120 S [1,5] and contains glycogen [1,3,5], glycogen phosphorylase [1,3,5], glycogen synthetase [3,5], phosphorylase kinase [1], phosphorylase phosphatase [1] and debranching enzyme [3]. The second fraction, which is a heavy one, has a sedimentation constant of approximatively 600 S [1] and contains no glycogen [1] (or only a very small fraction of it [5]), glycogen phosphorylase kinase (according to [5], but not to [1]), ATPase [1] and phosphatase [1]. This second fraction has been shown by electron microscopy to be formed by vesicles of sarcoplasmic reticulum [1,5] and is called sarcovesicle fraction.

Abbreviations: AMP, adenosine 5′ monophosphate; IMP, inosine 5′ monophosphate; AMP deaminase, adenosine 5′-monophosphate deaminase; ATPase, adenosine triphosphatase; glycogen phosphorylase *b* (EC 2.4.1.1)

In this sucrose-density gradient, Meyer et al. [1] observe a partial separation inside the light fraction between glycogen and the enzymes associated with it, glycogen phosphorylase, phosphorylase phosphatase and phosphorylase interaction between sucrose and glycogen particle enzymes.

In an attempt to improve the separation and purification of the different fractions of the glycogen particle, we have used a new method of fractionation by metrizamide density-gradient, which avoids the inconvenience of sucrose. Metrizamide is a heavily modified sugar which interacts only weakly with glycogen interacting enzymes (as we have verified for glycogen phosphorylase cf. below). Metrizamide density-gradients, which have been already successfully applied to many biological systems [6] proved to be very efficient in the separation of glycogen particles.

2. Materials and methods

2.1. *Materials*

AMP, ATP and glycogen are purchased from Sigma, metrizamide from Nyegaard and Co A/S, Oslo. Glycogen phosphorylase is prepared in the laboratory according to [7].

2.2. *Fractionation of glycogen particles*

Glycogen particles are isolated from rabbit skeletal muscle according to the procedure of Krebs et al. [7] and Meyer et al. [1]. After a first low-speed centrifugation of the muscle extract (4000 × *g*, for 40 min) eliminating most of the muscle and cell

debris, the particles are precipitated by lowering the pH of the crude extract to pH 6. After centrifugation at 4000 X *g* for 30 min, the sedimented material is taken up using an amount of 0.1 M glycerophosphate, 4 mM EDTA solution, pH 8.2, approximatively equal in volume to the protein pellet and further diluted with 0.05 M glycerophosphate, 2 mM EDTA, pH 7.0. The particles are further purified by centrifugation at 29 000 rev./min for 90 min using No. 30 rotor in a Beckmann L2 65 preparative centrifuge. The precipitate is taken up in an appropriate vol. 0.05 M glycerophosphate, 2 mM EDTA, pH 7.0.

Metrizamide dissolved in glycylglycine buffer 50 mM, KCl 50 mM, pH 6.9, corresponds to an average density of 1.25. The sample is layered on the top of the gradient which is run for 65 h at 40 000 rev./min in a 75 Ti rotor on a L3 Beckman ultracentrifuge. The final density for the different fractions of the gradient varies between 1.08 and 1.55 as deduced from the measurement of the refractive index. (Refractive indexes are measured with a Carl Zeiss refractometer.)

2.3. *Enzyme assays and glycogen estimation*

AMP deaminase activity is measured from the decrease of absorbance at 260 nm (corresponding to the disappearance of AMP) in presence of 0.1 mM AMP in glycyl–glycine buffer 50 mM, KCl 50 mM, pH 6.9. Before the assay, the different samples from the metrizamide-gradient are dialysed overnight against glycylglycine buffer to eliminate metrizamide which strongly absorbs at 260 nm.

Total phosphorylase activity is measured in presence of saturating amounts of AMP, according to Helmreich and Cori [8]. Phosphorylase *a* activity is measured in the absence of AMP.

Adenosine triphosphatase is assayed as follows: to a reaction mixture (0.33 ml total volume) containing 10 μl 20 mM $MgCl_2$, 50 μl 10 mM $CaCl_2$, 250 μl 0.01 M Tris–HCl buffer pH 6.8 and 20 μl 2 mM ATP, 20 μl the different fractions are added. The mixture is incubated 30 min at room temperature and the reaction stopped by addition of 0.4 ml 10% trichloracetic acid. After centrifugation, inorganic phosphate present in the supernatant is assayed by the procedure of Eibl and Lands [9]. A blank is made with 0.33 ml reaction mixture and 20 μl of water.

Glycogen precipitated three-times with ethanol is suspended in a phenol sulfuric acid medium according to [10]. Absorbance at 490 nm is proportional to glycogen concentration.

3. Results

In a preliminary experiment, we have compared the equilibrium densities of glycogen phosphorylase either alone or complexed with glycogen: one can see on fig.1 that phosphorylase alone interacts with metrizamide and that the peak corresponding to the free enzyme is divided into many components, corresponding to different states of association of the enzyme with metrizamide molecules (density comprised between 1.35 and 1.50). This result has already been observed with catalase [11] and is not due to a structural similarity between metrizamide and glycogen, since metrizamide does not compete with glycogen in kinetic experiments even at low glycogen concentration (13 mM in glucose residue) and high metrizamide

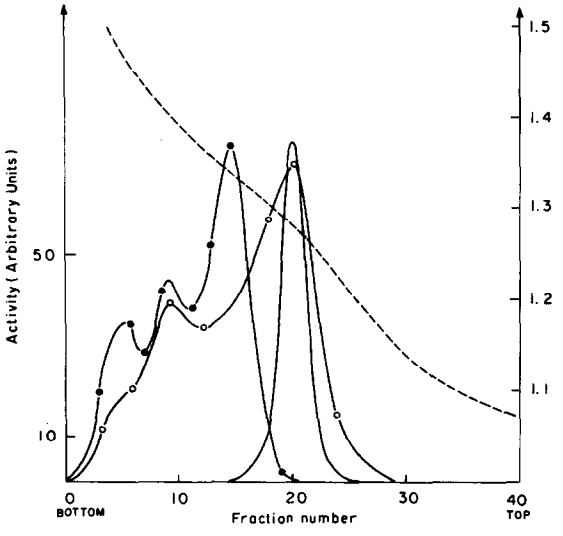

Fig.1. (a) Metrizamide density-gradient of phosphorylase alone. (——·——) Phosphorylase *b* activity. (b) Metrizamide density-gradient of glycogen phosphorylase *b* complex, (——) Glycogen, (–o–o–) phosphorylase *b* activity. The density is measured from the refractive index. A 6 ml gradient was charged with 0.1 ml glycogen phosphorylase *b* at about 10 mg/ml with or without 0.1 ml 5% solution of glycogen. The other experimental conditions are described in 'Materials and methods'.

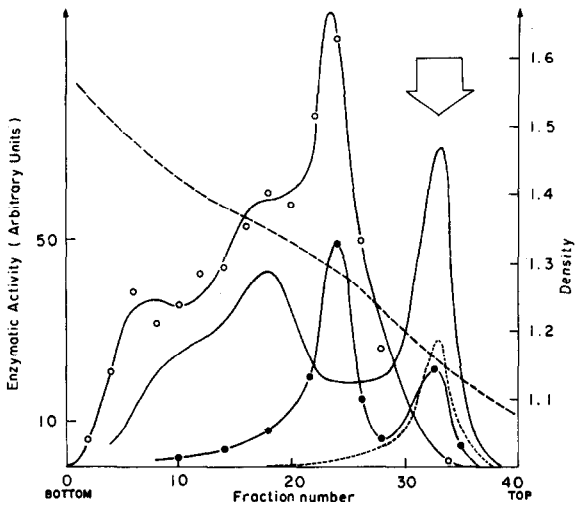

Fig.2. Metrizamide density-gradient of the glycogen particles. (- - -) Density, (—o—o—) phosphorylase *b* activity, (——) AMP deaminase activity, (. . .) ATPase activity and (—•—•—) glycogen present in the different fractions. The arrow indicates the presence of a milky-band in the gradient.

concentration (0.1 M). In the presence of glycogen, a new peak of phosphorylase *b* activity appears corresponding to the association of the enzyme with glycogen. It has about the same density as glycogen alone (d = 1.28).

When the experiment is performed with glycogen particles extracted from muscle, one sees on fig.2, that glycogen is present in two bands. One of these bands has the density corresponding to the phosphorylase glycogen complex as determined in the preliminary experiment (d = 1.28). This peak of glycogen is associated with very high phosphorylase activity and also with phosphorylase kinase activity (unpublished results). By comparison with the results of the other authors (d = 1.5), this fraction will be called 'glycogen fraction'.

The other minor glycogen peak corresponds to a lower density of the gradient (d = 1.16). This peak is associated with a strong ATPase activity, which is not sensitive to 1 mM ouabain. This fraction is probably the sarcovesicle fraction described by Fischer et al. [1] and Wanson and Drockmans [5]. It contains also a strong AMP deaminase activity, but no phosphorylase *b* activity.

Phosphorylase and AMP deaminase activities, but not ATPase activities, are also found at higher densities and correspond probably to free enzymes. The ratio of phosphorylase *a* to phosphorylase *b* is higher in the glycogen fraction than in this free enzyme fraction, probably because phosphorylase *a* binds more strongly to glycogen than phosphorylase *b* [12].

4. Discussion

With the help of metrizamide-gradients, we have shown that glycogen particles can be separated into two components: one fraction associated with phosphorylase and the other one associated with ATPase. Our results are in qualitative agreement with the ones of Meyer et al. [1] and Wanson and Drockmans [5], the first fraction corresponding to the 'glycogen fraction' and the second one to the 'sarcovesicle fraction'. Further characterization of the two fractions and of their protein content is now in progress. However, a quantitative estimate of the amount of free enzyme is difficult because of the non specific interactions between metrizamide and proteins, as exemplified in fig.1, in the case of glycogen phosphorylase *b*. This drawback can be overcome by using a gradient preformed in deuterium oxide [13]. However, it is already possible to notice some significant differences between previously published results and ours : Meyer et al. [1] and Wanson and Drockmans [5] found no significant amount of glycogen (less than 2%) in the sarcovesicle fraction. In our case, 20% of the total amount of glycogen is found in this fraction. This difference could be due to the competing effect of sucrose which is omitted in the present preparation. In agreement with Meyer et al. [1], but in disagreement with Wanson and Drockmans [5] no phosphorylase activity is found with the sarcovesicle fraction, which is shown here for the first time to contain AMP deaminase.

The clear distinction between the two subfractions forming the 'glycogen particle' and the repartition of the different enzymes in one or the other of these two subfractions show that these two subparticles are distinct 'in vivo' entities. However, this result does not exclude an association of them in the muscle cell [2,5].

Acknowledgements

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