SPIN-LABELLED PHOSPHORYLASE AS A PROBE IN A RABBIT MUSCLE GLYCOGEN PARTICLE FRACTION

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1. Introduction

Meyer et al. [1] have described the preparation of a particulate suspension from rabbit muscle containing glycogen and a number of enzymes. They suggest that the glycogen binding enzymes are attached to the large glycogen molecules in the suspension and they term these aggregates 'glycogen particles'. The enzymes present include phosphorylase *b*, phosphorylase *b* kinase, phosphorylase phosphatase and other enzymes of glycogen metabolism, trace glycolytic enzymes and some of the enzymes concerned with nucleotide interconversions.

Studies by the same group have shown that some of these enzymes have anomalous properties when bound to the glycogen particles and they postulate specific interactions either between the enzymes themselves or with the carbohydrate matrix to account for this. They also suggest that these protein–glycogen complexes are the glycogen granules seen in sections of muscle by electron microscopy and that the 'glycogen particle' is therefore a cellular oganelle [2–5].

Transient production of phosphorylase *a* could be generated in this glycogen particle rich fraction by the addition of ATP, calcium and magnesium ions. This 'flash activation', was completely calcium dependent because of the calcium requirement of phosphorylase *b* kinase, and was terminated by the action of phosphorylase phosphatase on the phosphorylase *a* after the ATP had been used up [2].

Using spin-labelled phosphorylase *b* it has been possible to monitor the conformational changes which occur both when ligands bind to the enzyme and when it is activated by conversion to phosphorylase *a* [6,7]. This clearly offers a way of studying the behaviour of phosphorylase attached to the glycogen particle and in this paper we describe the use of spin labelled phosphorylase *b* to monitor flash activation and the interactions of ligands with phosphorylase during this process.

2. Materials and methods

Phosphorylase *b* was prepared according to the method of Fischer and Krebs [8], substituting dithiothreitol for cysteine as a sulphydryl protecting reagent. Twice recrystallised phosphorylase *b* was spin labelled with *N*-(1-oxyl 2,2,6,6 tetramethyl-4-piperidinyl) iodoacetamide, purchased from Synvar Ltd., as described by Dwek et al. [7]. Spin-labelled phosphorylase *a* was prepared and purified according to the method of Dwek et al. [7]. Phosphorylase *b* was labelled with iodoacetamidosalicyclic acid (obtained from Koch-Light Ltd.) by a similar method.

Glycogen particles were prepared by the acid precipitation method [1] and were resuspended to give a fraction containing 80–90 μM phosphorylase monomers. Phosphorylase *a* assays were carried out as described before [10], on aliquots taken at various times during flash activation. ESR spectra of spin-labelled phosphorylase were recorded on a JEOLCO JESPEIX spectrometer and the spectra were characterised by the ratio of the low field peak height to the centre peak height in the differentiated form of the spectrum. It has been shown that the value of this ratio (R) for a spectrum is a monitor of the conformational state of the phosphorylase [6,7]. A decrease in this ratio implies that the spectrum is

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tending towards that produced by a less mobile nitroxide group.

3. Results and interpretation

3.1. *Binding of labelled phosphorylase b by glycogen particles*

In order to show that labelled phosphorylase was taken up by glycogen particles and that it could displace the native phosphorylase from the particles, the glycogen-rich fraction was centrifuged in the presence of labelled phosphorylase *b*. By comparing the label concentration with the phosphorylase activity in the supernatant it was possible to deduce the amount of labelled enzyme taken up and the amount of native phosphorylase displaced. Since the spin label moiety on phosphorylase is slowly reduced by glycogen particles, in these experiments phosphorylase labelled with a fluorescent probe (4-iodoacetamidosalicylic acid) was used. This label binds to the same sulphydryl group as the spin label and the acetamidosalicylate–phosphorylase *b* has identical enzymic properties to the spin-labelled enzyme. Different concentrations of fluorescent acetamidosalicylate–phosphorylase *b* were added to a glycogen particle suspension and centrifuged for 100 min at 80 000 *g*. The supernatant was removed from the glycogen particle pellet and the amount of acetamidosalicylate–phosphorylase *b* in the supernatant was deduced from fluorescence measurements. The overall phosphorylase concentrations in the supernatant and the pellet were derived from measurements of activities of diluted aliquots of each fraction. A plot of the amount of labelled phosphorylase *b* bound to glycogen particles vs. the concentration of added labelled enzyme is shown in fig. 1. When 50 μM labelled phosphorylase *b* is added, 95% of it is taken up into the glycogen particles. At high concentrations of labelled phosphorylase *b*, native enzyme was displaced from the particles, demonstrating that extrinsically added phosphorylase takes up the positions on the particle originally occupied by intrinsic phosphorylase *b*.

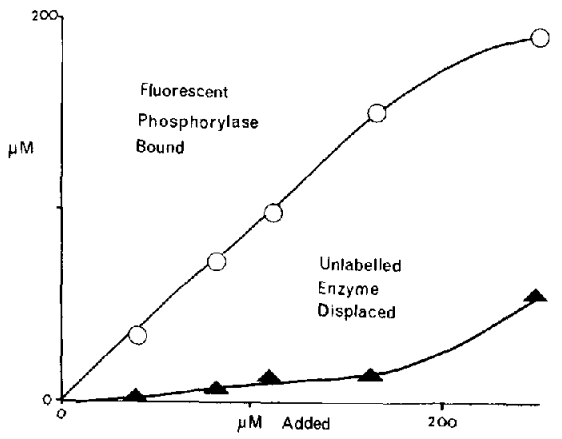

Fig. 1. (○) Amount of acetamidosalicylate phosphorylase *b* bound to aliquots of a glycogen particle suspension and (▲) unlabelled phosphorylase *b* displaced after pelletting glycogen particles by centrifugation in the presence of various concentrations of fluorescent labelled phosphorylase *b*.

3.2. *The behaviour of spin-labelled phosphorylase during flash activation*

When 50 μM spin-labelled phosphorylase *b* was added to a glycogen particle suspension the ESR ratio (the first open circle in fig. 2, R = 0.45) was found to be lower than that seen when the enzyme was dissolved in a 2% solution of purified glycogen made up in the same buffer (A in fig. 2, R = 0.59). This suggested that the conformation of the phosphorylase in the glycogen particle suspension was modified in some way, possibly by interaction with a ligand. On addition of spin-labelled phosphorylase to particles from which the glucose-6-phosphate had been removed by extensive dialysis into Tris buffer the ESR ratio became 0.60. In addition to glucose-6-phosphate the dialysate contained traces of IMP and glucose. Using glucose-6-phosphate dehydrogenase it was found that the particle suspension contained 300 μM glucose-6-phosphate, an inhibitor of phosphorylase *b* which binds tightly to and modifies the spectrum of the spin-labelled enzyme. When an appropriate concentration of glucose-6-phosphate was added to the spin-labelled phosphorylase in the purified glycogen medium the ESR ratio became the same as that seen with glycogen particles. The solution of 300 μM glucose-6-phosphate, 2% glycogen, 5 mM glycerophosphate pH 6.8 was then used as a simulated glycogen particle medium.

On addition of 2 mM calcium chloride, 10 mM

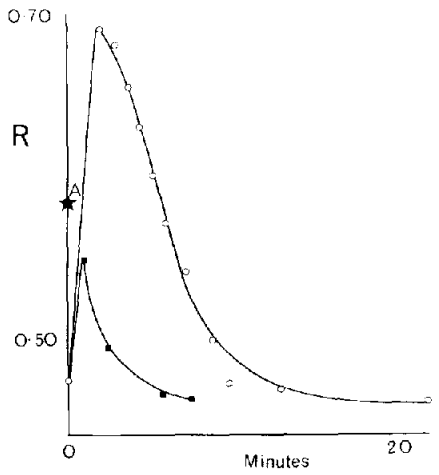

Fig. 2. Changes in the ESR ratio (R) of spin-labelled phosphorylase *b* in a glycogen particle suspension during flash activation. 50 μM spin-labelled phosphorylase *b*, 10 mM magnesium chloride, plus (○) or minus (■) 2 mM calcium chloride. Reaction started at zero time with 6 mM ATP. 'A' denotes the ratio exhibited by 50 μM spin-labelled phosphorylase *b* or *a* in 50 mM sodium glycerophosphate, 2 mM EDTA, 2% glycogen.

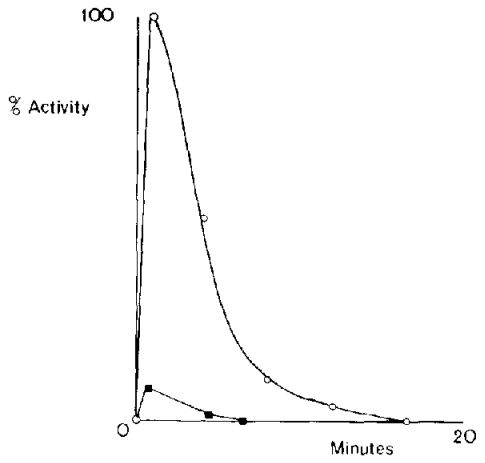

Fig. 3. Changes in the activity of phosphorylase during flash activation. Conditions as in fig. 2 plus (○) or minus (■) calcium. Aliquots were taken at various times, diluted 50 times as described by Heilmeyer [2] and assayed for phosphorylase *a* according to the method of Birkett et al. [10]. Activity is expressed as percentage of maximum phosphorylase *a* activity possible in the flash activation mixture.

magnesium chloride and 4 mM ATP to the spin-labelled phosphorylase *b* and glycogen particles a normal flash activation was observed (fig. 3) while the ESR ratio was seen to rise and fall synchronously with the change in the activity of the enzyme (fig. 2). Part of the increase in the ESR ratio can be explained by the observation that the very low ratio observed when glucose-6-phosphate binds to the *b* form will be lost when phosphorylase *a* is generated since glucose-6-phosphate binds much less strongly to the *a* form (K_d = 600 μM instead of 50 μM). Phosphorylase *a* gives an ESR ratio of 0.59 (A in fig. 2) in the simulated glycogen particle medium whereas phosphorylase *b* gives a ratio of 0.45 both in the simulated medium and in glycogen particles. The reason for the discrepancy between the ratio shown by phosphorylase *a* in a simulated glycogen medium (0.59) and the maximum ratio seen during flash activation (0.70) will be discussed later.

When the ATP added has been hydrolysed phosphorylase *a* is dephosphorylated by phosphorylase phosphatase present in the particles; as the phosphorylase activity falls the ESR ratio decreases due to the glucose-6-phosphate being taken up by the reformed phosphorylase *b*.

The flash activation of the spin-labelled phosphorylase on glycogen particles is absolutely dependent on calcium ions, but a small, transient change in the ESR ratio is seen when ATP is added in the absence of calcium or magnesium ions. The magnitude of the change depends on the concentration of nucleotide added and such changes can be produced by suitable concentrations of ADP, AMP or IMP. All these nucleotides inhibit the binding of glucose-6-phosphate to phosphorylase *b* and hence reverse the low ratio seen with phosphorylase *b* in glycogen particles. The effect is time dependent because the ATP added is hydrolysed to ADP and AMP and then deaminated to IMP by enzyme systems present in the particle suspension [3]. Since ADP and AMP, which are produced transitorily, bind very much more tightly to phosphorylase *b* than do ATP and IMP they will have a greater tendency to inhibit glucose-6-phosphate binding. Consequently they produce a transient rise in the ESR ratio (fig. 2).

All the effects seen in the ESR spectrum during

flash activation can thus be explained by analogy with experiments on phosphorylase in a simulated glycogen particle medium except for the anomalously high ESR ratio displayed by the phosphorylase *a* formed during flash activation. This very high ratio is never seen with phosphorylase *a* in free solution or in a simulated glycogen particle medium. Only one ligand causes the ESR ratio of phosphorylase *a* to rise in the simulated medium, that is IMP. The possibility that this anomalously high ratio was caused by IMP or another diffusible ligand other than those in the simulated glycogen particle medium binding to the added phosphorylase was tested in the following experiment.

Purified spin-labelled phosphorylase *a* was added to glycogen particles known to contain only 30 μM IMP. The ESR ratio which started above the value obtained for phosphorylase *a* in a simulated glycogen particle medium (containing 300 μM glucose-6-phosphate) fell exponentially as the enzyme was hydrolysed by phosphorylase phosphatase (fig. 4, open circles). Using a semi-log plot the ratio was extrapolated back to 0.69–0.70 at zero time. After dialysis of the particles for 6 hr into glycerophosphate buffer to remove small ligands the experiment was repeated. Under these conditions nucleotides, small sugars, etc., were removed whilst the glucose-6-phosphate level was maintained at that used in the simulated glycogen particle medium by the action of phosphorylase and phosphoglucomutase on the glycogen and free phosphate in the buffer. The ESR ratio fell at the same rate as before (fig. 4, closed circles) and the semi-log plot could be extrapolated back to 0.69–0.70. This is close to the anomalously high ratio seen during flash activation experiments. Clearly the high ESR ratio observed when spin-labelled phosphorylase *a* is added to glycogen particles cannot be due to diffusible ligands (including IMP). One must postulate that the high ratio seen in this experiment and during flash activation is due to interaction with a macromolecular constituent of the glycogen particle suspension.

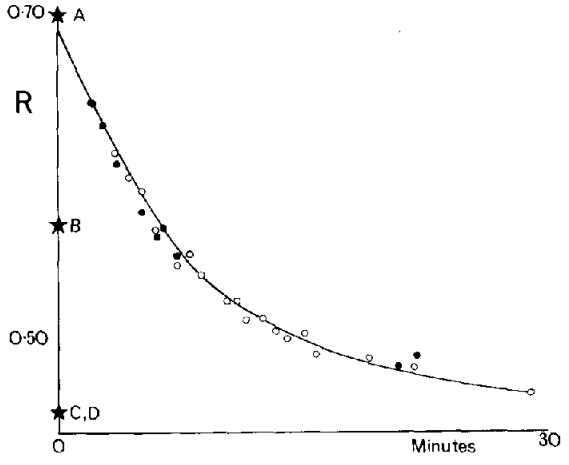

Fig. 4. Changes in the ESR ratio of spin-labelled phosphorylase *a* (50 μM) in the glycogen particle suspension. The curve was extrapolated back to zero time assuming an exponential decay to give the value at point 'A'. Points 'B' and 'C' are the ratios exhibited by 50 μM spin-labelled phosphorylase *a* in a 50 mM sodium glycerophosphate, 2 mM EDTA, 2% glycogen, 300 μM or 10 mM glucose-6-phosphate respectively. Point 'D' shows the ratio exhibited by spin-labelled phosphorylase *b* in the glycogen particle suspension.

4. Conclusions

Spin-labelled phosphorylase is a useful probe for following the flash activation of glycogen particles. The ESR spectrum of the label responds to the phosphorylation and dephosphorylation of the phosphorylase and to the binding of ligands to the two species of the enzyme.

The ESR ratio observed for spin-labelled phosphorylase *b* in the glycogen particle suspension is similar to those observed when the enzyme is dissolved in a simulated glycogen particle medium containing glycogen and small ligands but no other enzymes. In contrast the ESR ratio observed for phosphorylase *a* in the simulated system is not the same as that seen during flash activation or when phosphorylase *a* is added to glycogen particles.

The anomalously high ESR ratio seen in these systems which is not due to an interaction of the enzyme with a diffusible ligand in the suspension suggests that the phosphorylase *a* is in an unusual environment in the glycogen particle suspension. This could be due to an interaction with a macromolecular component of the glycogen particle aggregate.

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