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Electron microscopy of glycogen degrading enzymes

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Single molecules of glycogen phosphorylase *b* exhibit images in the electron microscope which are similar in shape and dimension to those derived from X-ray crystallography. Phosphorylase *a* exhibits tetramers but shows dimers in the presence of glucose. Glycogen debranching enzyme appears as a monomer with an unusual crescent or shrimp-like shape, with occasional isologous aggregation to circular dimers. The longest dimension of the monomer is very similar to that of the phosphorylase dimer, 11.5 nm. Strong binding of the debranching enzyme to glycogen is readily visualized in the electron microscope. It is suggested that the distinctive shape of the debranching enzyme may be related to its catalytic function.

Phosphorylase; Glycogen debranching enzyme; Electron microscopy; Glycogen binding

1. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) is the chief enzyme involved in glycogen degradation and has been the subject of several recent reviews [1,2]. There have been several previous electron microscopy studies of phosphorylase, the most recent being in 1972 [3], but instrumental improvements since then both facilitate observation and minimize specimen damage, thus improving the resolution of biological particulates in electron micrographs. The structures of the phosphorylated and non-phosphorylated forms of phosphorylase have been determined to high resolution by X-ray crystallography [4,5], so that electron micrographs of this enzyme can be compared to the known structure, and conditions for microscopy chosen which should preserve the overall structure of proteins which have not been examined previously.

Amylo-1,6-glucosidase/4-α-glucanotransferase (EC 3.2.1.33 + EC 2.4.1.25), commonly referred to as glycogen debranching enzyme, is a monomeric enzyme of approx. 165 kDa when isolated from rabbit muscle. It has the interesting property of possessing both glucosidase and transferase activities on a single polypeptide chain. In the degradation of muscle glycogen, phosphorylase can form glucose 1-P from the terminal α-1,4-linked glucose units until all chains have four glucose units left before each α-1,6-branch point. The debranching enzyme then transfers a unit of three glucose residues from the 'side-chain' to the 'main chain', leaving a single glucose unit attached by the α-1,6-link to the main chain. It then hydrolytically removes this single glucose residue. In the most recent comprehenisive review on the debranching enzyme, Nelson et al. [6] envision this enzyme as possessing a single overlapping or strongly interacting polymer-binding site(s), with the transferase catalytic site on one side and the glucosidase site on the other.

We have recently crystallized the debranching enzyme in a form suitable for X-ray diffraction studies when oligosaccharides were included in the medium [7]. Raising the maltotriose concentration to 60 mM improved the crystal properties with regard to the limits of diffraction and useful X-ray

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lifetime [8]. We have also determined the binding constants for glycogen, glucose and a series of oligosaccharides [9]. These studies, as well as earlier ones, suggested that conformational changes may occur on saccharide binding and may also be associated with the catalytic process. We reasoned that electron microscopy might provide information on this matter, and also be useful in the initial stages of structure determination.

2. MATERIALS AND METHODS

Phosphorylases *a* and *b* from rabbit muscle were prepared by previously described methods [10]. Glycogen debranching enzyme, also from rabbit muscle, was purified to homogeneity by the revised method described earlier [8].

For conventional negative staining of enzyme molecules, a 3 μl droplet of the sample (5–30 μg protein/ml) was placed onto a hydrophilic carbon film (prepared by glow discharge) which was supported by parlodion on a 300 mesh copper grid. After 1 min the droplet was removed and the carbon surface washed with 3 droplets of 10 mM ammonium acetate (pH 6.0). The adherent molecules were negatively stained by applying 3 droplets of an aqueous solution of sodium phosphotungstate (2%, w/v; pH 7.0) or uranyl acetate (2%; pH 4.4) or uranyl formate (1%; pH 4.0).

The Valentine method of negative staining [11,12] was also used, with some modifications. A carbon film, 5–7 nm thick, was evaporated by electron bombardment onto a square of freshly cleaved mica (5×5 mm) in a Balzers BA-511 M apparatus. The carbon film was partially detached from the mica by floating it onto a clean water surface, then blotted with filter paper. A 0.5 μl droplet of the enzyme solution (5–10 μg protein/ml) was touched onto the wetted edge of the carbon film, and allowed to spread between the carbon and the mica. After a min incubation to allow the enzyme molecules to attach to the carbon film, the carbon-mica sandwich was blotted onto filter paper. After washing with 10 mM ammonium acetate, the carbon film was floated free onto 2% aqueous uranyl acetate (pH 4.4), picked up from above with a 300 mesh copper grid, blotted with filter paper, and dried. For some experiments a modification of the Valentine method, called the 'pleated sheet' [13], was employed.

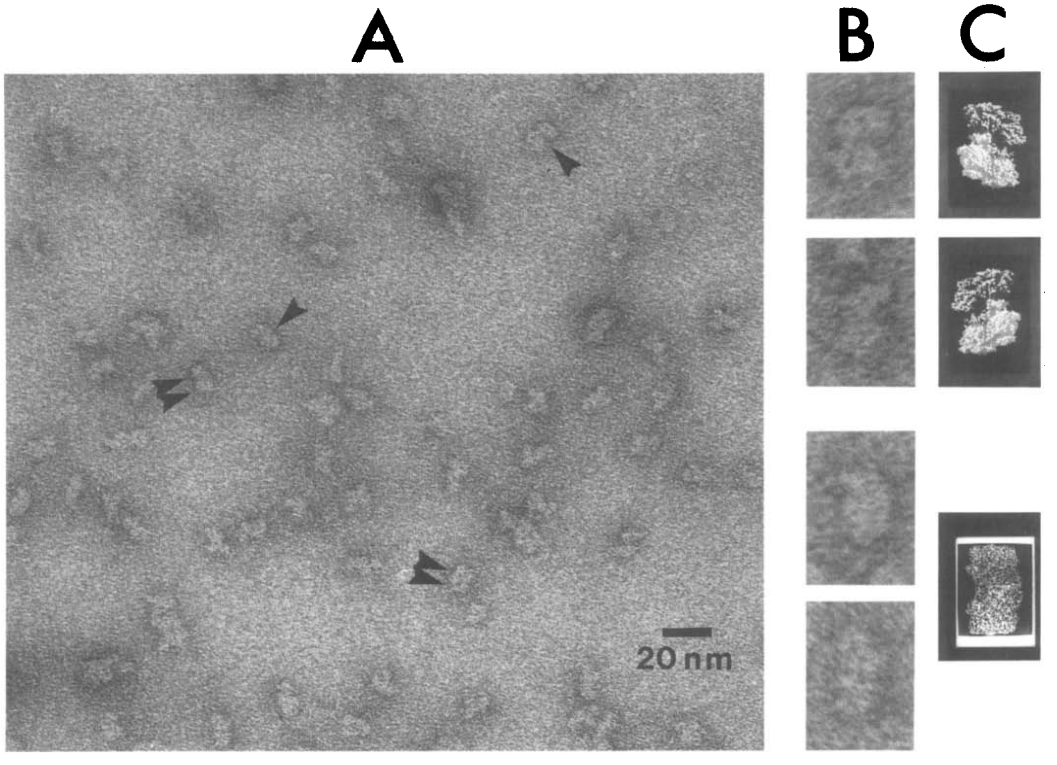

Fig.1. Glycogen phosphorylase *b* dimers: conventional negative staining with 1% uranyl acetate. (A) A representative field of molecules: the single arrowheads indicate the dimer in front view and the double arrowheads indicate the dimer in 'side' view. (B) Enlargements of two front views (upper two frames) and of two side views (lower two frames) are presented. (C) The X-ray crystallographic structure of the phosphorylase dimer presented as the van der Waals' contours of atoms on the surface [3]. The top frame is looking down the molecular two-fold axis at the catalytic of the dimer, the second frame shows the dimer rotated 180°, the bottom frame shows the dimer rotated 90° to either of the previous views.

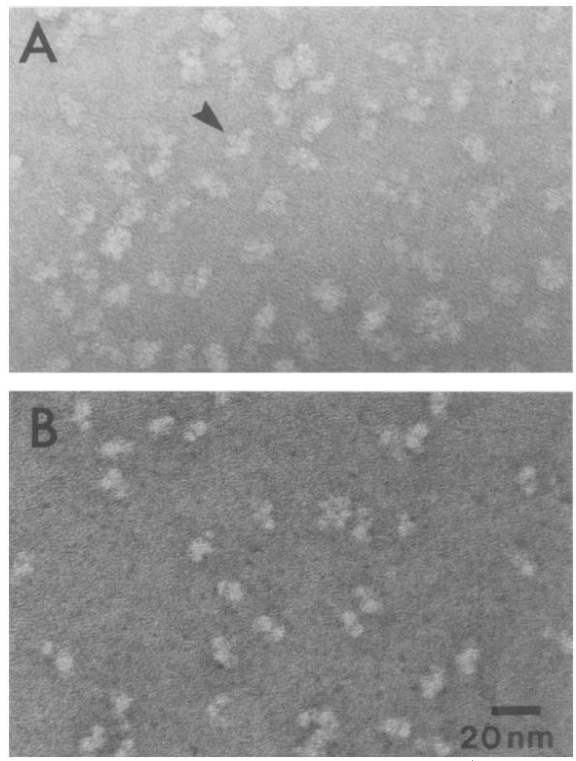

Fig.2. Glycogen phosphorylase *a* molecules. (A) Conventional negative staining with 2% uranyl acetate with an easily discernible tetramer indicated. (B) Negative staining with 2% uranyl acetate by the Valentine method shows that the enzyme becomes a dimer in the presence of 50 mM glucose.

Transmission electron micrographs were obtained with a Philips EM420 electron microscope operated at 100 kV. Off-axis focussing using the low-dose module was employed to minimize specimen damage. The microscope had been calibrated with catalase crystals as a magnification standard [14], but for these studies the molecular dimensions of phosphorylase *b* (length 11.5 nm [3]) were used as a measurement standard. Micrographs were taken at an instrumental magnification (calibrated) of 103500 and recorded on Kodak SO-163 sheet films (3¼ × 4 inch). Development was in D19 (1:2) for 4 min.

3. RESULTS AND DISCUSSION

Fig.1 shows that the phosphorylase *b* dimers exhibit the same shapes previously deduced from the X-ray crystallographic studies of the phosphorylase *a* dimers [4] (the two forms of phosphorylase have very similar molecular ar-

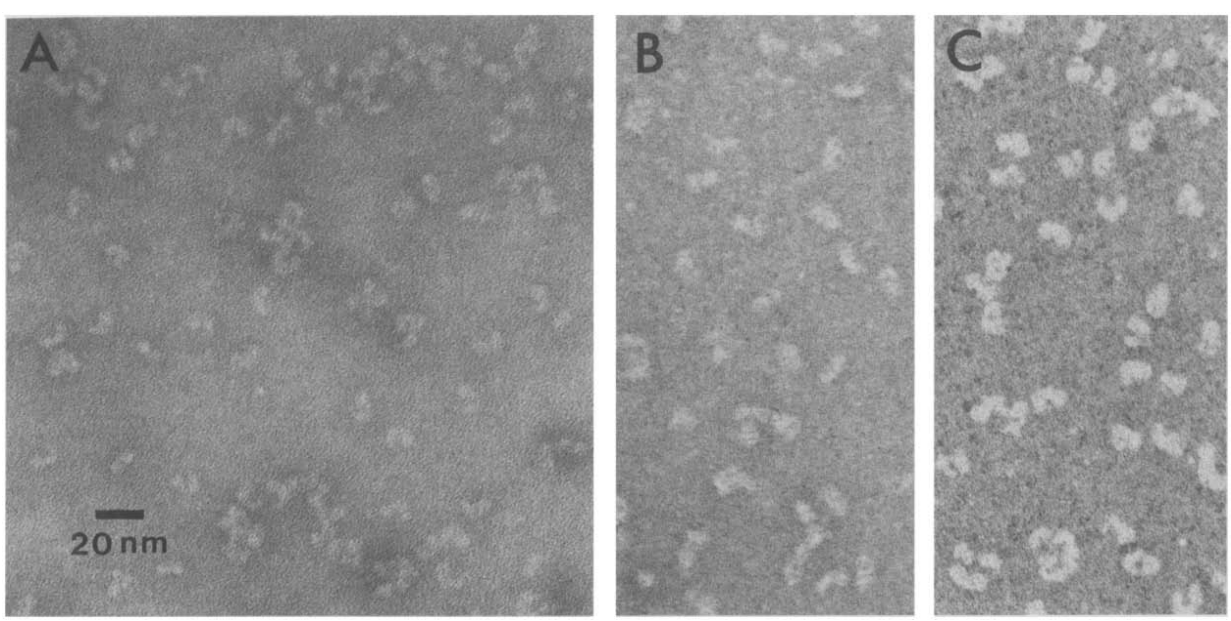

Fig.3 Glycogen debranching enzyme. (A) A field of molecules stained with 2% sodium phosphotungstate by the conventional method and (B) the result of staining with 2% uranyl acetate (Valentine method). (C) The pleated sheet method was employed and the negative stain was a combination of sodium phosphotungstate and uranyl acetate.

chitecture in their isomorphous tetragonal crystals [15,16]).

Fig.2A demonstrates that phosphorylase *a* behaves as a tetramer in solution, as has long been known from hydrodynamic studies [17]. Previous electron microscopy studies of phosphorylase *a* microcrystals alos indicated tetramers [3]. In the presence of 50 mM glucose the phosphorylase *a* dimer dissociates to a dimeric form [17], as is illustrated in fig.2B.

In fig.3 a comparison of the three negative staining techniques for the debranching enzyme is

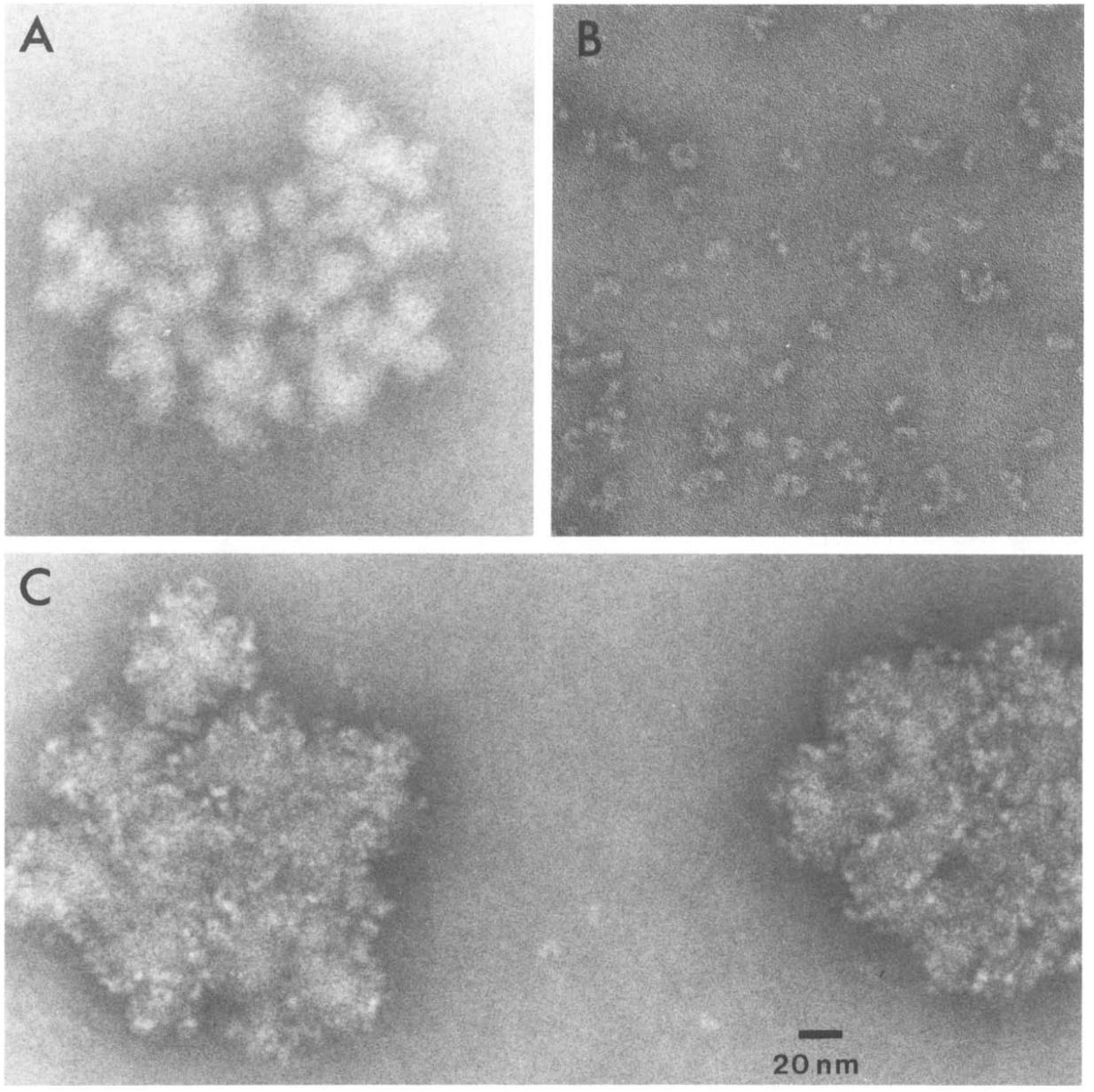

Fig.4. Association of debranching enzyme with glycogen. Conventional negative staining with 2% sodium phosphtungstate. (A) A particle of high molecular mass glycogen, in a control solution of 30 μg/ml; (B) the debranching enzyme molecules in a control solution of 30 μg/ml; (C) the result of mixing the two control solutions together (1:1) before application to a hydrophilic carbon support film. The buffer was 25 mM glycylglycine, 1 mM EDTA, 1 mM DTT (pH 7.3).

presented. Conventional staining with sodium phosphotungstate (fig.3A) gave the most reproducible results and revealed the characteristic crescent or shrimp-like shape of the enzyme. Although uranyl salts did not distort the shape of phosphorylase (fig.1), the debranching enzyme appears to be denatured somewhat by uranyl acetate or uranyl formate when prepared in the conventional manner or by the Valentine method, resulting in the rather larger and fuzzier images shown in fig.3B. The pleated sheet method produced excellent contrast, but the compression of the debranching enzyme molecules between the two carbon films is evident.

Monomers of the debranching enzyme occasionally undergo an isologous association to form a circular, doughnout-shaped dimer; these are especially noticeable in fig.4B. Hydrodynamic studies have indicated that the debranching enzyme exists as a monomer of 165 kDa at low concentrations, and that association to form dimers occurs at protein concentrations above 0.7 mg/ml [18,19].

Kinetic studies have shown that the enzyme binds to glycogen, which serves both as a competitive inhibitor of the reaction with the normal substrate, the phosphorylase limit dextrin, and as a poor substrate [6]. In addition, the debranching enzyme is a major component of the glycogen particle when the glycogen fraction of the cell is isolated under gentle conditions [18]. We have demonstrated binding of the enzyme to large molecular mass glycogen by direct methods, with a dissociation constant of 0.66 mM glucose end groups [9]. In fig.4 we show that when the debranching enzyme is mixed with glycogen, before being deposited on a carbon film and stained, the two molecules become associated. Few protein molecules left free remain in solution and the debranching enzyme in monomeric form is easily discernable on the surface of the glycogen particles. Although we did not try to saturate the glycogen particles with enzyme in these studies, our previous results indicated that the binding of these two macromolecules can be described by a saturation function [9]. From a consideration of surface areas, it can be calculated that approx. 2000 protein molecules would cover the surface of a glycogen molecule of 2×10^9 kDa (assumed).

The longest dimension of the debranching enzyme molecule is quite similar to that of the phosphorylase *b* dimer and, using the latter as a standard, we estimate this parameter to be approx. 11 nm. It is not immediately obvious how this dimension can be correlated with the dimensions of the unit cell for the $P2_12_12_1$ space group of the crystallized protein, which are 10.6, 19.6 and 9.3 nm [7], nor are the dimers seen in the current study easily reconciled with that space group. The unusual shape of the monomeric debranching enzyme may be related to its function. For example, the yeast hexokinase monomer has a somewhat similar shape and in this case the smaller lobe undergoes a major conformational change upon the binding of glucose, rotating as a unit to close down on the larger domain [20]. One might suggest that a similar conformational change in the debranching enzyme may serve to carry the maltotriose unit from the 'side-chain' of the limit dextrin to the 'main-chain' in the transferase activity of the enzyme [9]. Our attempts to demonstrate major conformational changes in the debranching enzyme by electron microscopy were not successful. For example, the enzyme in the presence of 60 mM maltotriose appears the same, and has the same tendency to form dimers, as in figs 3 and 4 (not shown).

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