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Glycogen contains phosphodiester groups that can be introduced by UDPglucose:glycogen glucose 1-phosphotransferase

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Rabbit-muscle glycogen contains covalently bound phosphorus, equivalent to 1 phosphate group per 208 glucose residues. This often disputed, minor component was previously thought to represent a phosphomonoester group at C-6 of a glucose residue. Here we show that more than half the phosphorus is present as a phosphodiester, the remainder being monoester. A novel enzyme activity has been found in muscle that can account for the presence of the phosphodiester in glycogen. This is a UDPglucose:glycogen glucose 1-phosphotransferase that positions glucose 1-phosphate on C-6 of glucose residues in glycogen, forming a diester. The phosphomonoester groups present may arise by removal of the glucose residue originally transferred as glucose 1-phosphate.

Glycogen; Glycogen phosphodiester; Glucose 1-phosphate transferase

1. INTRODUCTION

This is a report of a novel structural feature in the glycogen molecule and the enzyme responsible for its introduction. The structure is a phosphodiester group joining an α-glucosyl residue to the primary hydroxyl of a glucose residue in a glycogen chain.

While the presence of small amounts of esterified phosphate in starch has long been accepted, Fontana [1] noted that reports of covalently bound phosphate in glycogen have been disputed. He observed that covalently bound phosphate could be introduced into rat-liver glycogen by administration of $^{32}P_i$ to the whole animal. Where, in starch, it has been thought that the phosphate is present mostly on primary hydroxyl groups, as a monoester, i.e. glucose 6-phosphate [2], Fontana concluded that only part of the ^{32}P in glycogen was in this form. He also noted that the glycogen could be fractionated on DEAE-cellulose into components differing severalfold in their content of ^{32}P.

We have been studying minor components of the glycogen molecule. In particular, we discovered the presence in muscle glycogen of one molecular proportion of a covalently bound protein, glycogenin, which is the primer for glycogen synthesis [3]. It is an autocatalytic self-glucosylating protein [4,5]. Glycogenin contains a serine phosphate residue [6], accounting, at least in part, for the claimed presence of phosphate. We also detected glucosamine as a naturally occurring component of liver glycogen [7], but not muscle glycogen [8], and in the case of liver glycogen were able to repeat Fontana's [1] observation that it could be fractionated on DEAE-cellulose [9]. His belief that the fractionation was related to the presence of phosphate groups was confirmed in that our liver-glycogen fractions contained variable amounts of bound phosphate, with the phosphate content varying between extreme fractions by a factor of almost 100. Other differences in physical and chemical properties paralleled the changing phosphate contents. Liver glycogen was therefore revealed to be very heterogeneous in terms of this minor phosphate component.

We turned our attention to muscle glycogen which proved to contain more bound phosphate than did liver glycogen, and certainly more than could be accounted for by the serine phosphate residue in glycogenin [6]. Our intent has been to characterize the nature of the linkage of phosphate to glycogen. In doing so we have uncovered a novel structural feature involving phosphate and have detected an enzyme capable of introducing it.

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2. MATERIALS AND METHODS

2.1. *Materials*

[β-^{32}P]UDPglucose and [β-^{35}S]UDPglucose (β-phosphoro[^{35}S] thioate analog of UDPglucose) were synthesized as by Marchase et al. [10]. Their respective specific activities were 1400 Ci/mmol and 1100 Ci/mmol and were used without dilution of the isotope. QAE-Sephadex and molecular sieves were from Pharmacia. Glucose 6-phosphate dehydrogenase was from Boehringer. α-Amylase was the crystalline enzyme, prepared from saliva [11]. UDPpyridoxal was the gift of Professor T. Fukui, Osaka, Japan. Bovine intestinal alkaline phosphatase and all other reagents were from Sigma.

2.2. *Analytical methods*

Carbohydrate was measured by the phenol–sulphuric acid method [12] or, as glucose, with glucose oxidase [13]. Free and combined phosphate were assayed as by Hess and Derr [14]. When glycogen was treated with alkaline phosphatase, it was at pH 8.9 for 1.5 h at 37°C in 5 mM Tris-HCl with 6 mg of glycogen and 0.4 U of enzyme in a 0.2 ml digest.

2.3. *Purification of glycogen, digestion with α-amylase and separation of phospho-oligosaccharides*

Glycogen was isolated and purified from rabbit skeletal muscle as by Kennedy et al. [3]. Briefly, the method consists in blending the muscle in cold 10% trichloroacetic acid followed by ethanol precipitation and then shaking an aqueous solution with chloroform/octanol (3:1) to denature protein. The glycogen is recovered with ethanol and purified by chromatography on Sepharose CL-6B in 6 M guanidinium chloride. The glycogen that is eluted in the void volume is extensively dialyzed against distilled water and freeze-dried.

Glycogen (258 mg) was digested for 14 h at room temperature with α-amylase (5 U) in 1 mM Tris-HCl, pH 7.0 (10 ml.). The digest was brought to pH 8.0 with 1M Tris-HCl and the sample applied to a QAE-Sephadex column (5 ml) which was washed with 5 volumes of buffer and then with water to remove neutral carbohydrate. The adsorbed carbohydrate was eluted first with 20 mM NaCl in 1 mM Tris-HCl, pH 8.0 and then with 80 mM NaCl in the same buffer. The carbohydrate contents of the fractions are shown in Fig. 1. The total weights of carbohydrate eluted by 20 mM and 80 mM NaCl, were 2 9 mg and 2.1 mg respectively. Fractions 5–15, eluted by 20 mM NaCl were freeze-dried, dissolved and desalted on a small column of Sephadex G-25, again freeze-dried, dissolved in 0.02 N trifluoroacetic acid (0.5 ml.) and heated at 100°C for 20 min. After neutralization with ammonium bicarbonate, the solution was freeze-dried. Thereafter, water was twice added followed by freeze-drying. The product was dissolved in 1 mM Tris-HCl, pH 8.0 and refractionated on QAE-Sephadex in 20 mM and 80 mM NaCl (see Fig. 1).

2.4. *In vitro labelling of glycogen with ^{32}P*

The source of the glucose transferring enzyme used here was the ammonium sulfate precipitate obtained in the purification of the autocatalytic self-glucosylating protein from muscle [4]. It was solubilized in and dialyzed against 50 mM Tris-HCl, 2 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), pH 7.4. Purified glycogen (50 mg) was solubilized in 0 5 ml of 100 mM acetate buffer containing 10 mM dithiothreitol (pH 5.5). The muscle extract (200 μl, 5 mg protein) was added followed by ^{32}P-labeled UDPglucose (10–50 μCi). After overnight incubation at 37°C under toluene, the reaction was arrested by addition of an equal volume of 20% cold trichloroacetic acid. The pellet was discarded and glycogen was precipitated from the supernatant with two volumes of cold ethanol. It was redissolved in water, twice reprecipitated with ethanol and then solubilized in 6 M guanidinium chloride (0.5 ml) The solution was passed through a Sepharose CL-6B column (8 ml) in 6 M guanidinium chloride. The glycogen that eluted in the void volume was dialyzed extensively against water and freeze-dried. This last procedure served to remove the last traces of low molecular weight radioisotope. In six experiments the ^{32}P incorporated ranged from 7,800 to 12,200 counts/min. When treated with alkaline phosphatase, no radioactivity was released. All the label remained in the glycogen, after recovery.

The pH of 5 5 chosen to carry out this reaction was selected after an experiment in which we had tested the degree of ^{35}S labeling of glycogen at different pH values using [β-^{35}S]UDPglucose (Table I).

2.5. *Characterization of radiolabelled glycogen*

The recovered ^{32}P-labelled glycogen (100 mg) was digested with α-amylase (5 U) in 1 mM Tris-HCl buffer, pH 7.0 (2 ml). After 5 h the pH was brought to 8.0 with 1 M Tris-HCl and the sample applied to a QAE-Sephadex column (2 ml) equilibrated with 1 mM Tris-HCl buffer pH 8.0. The column was washed with 5 volumes of buffer and then with water and the washings discarded. The ^{32}P-labelled oligosaccharides retained by the column were eluted with 0.2 M trifluoroacetic acid, freeze-dried, hydrolyzed in 1 M trifluoroacetic acid for 3 h at 100°C and, after addition of ammonium bicarbonate to 100 mM final concentration, again freeze-dried. Dissolution in water and freeze-drying were repeated twice. The sample was dissolved in 22 μl of 400 mM Tris-HCl buffer pH 7.9 containing 10 mM oxidized NADP and divided into two equal portions. To one portion was added glucose 6-phosphate dehydrogenase (1 U). The second portion served as a control. After 1 h at room temperature, the samples were applied to Whatman 3 MM paper and subjected to high voltage electrophoresis at 1.5 kV for 1.5 h in 5% acetic acid/0.5% pyridine, pH 3.5 As a control, ^{14}C-labelled glucose 6-phosphate was subjected to all steps of the above procedure beginning with the hydrolysis in 1 M trifluoroacetic acid (see Fig. 2).

3. RESULTS

3.1. *Phosphate in muscle glycogen*

Muscle glycogen was purified by the procedure we use for the preparation of the covalently bound protein glycogenin, which involves treatments designed to remove all non-covalently bound proteins [3]. The resulting glycogen contained 0.064% by wt of phosphorus.

3.2. *Nature of linkage(s) of phosphate to glycogen*

In order to concentrate the phosphoester groups of glycogen, we hydrolysed it with α-amylase, an endohydrolase, and passed the products through QAE-Sephadex. The neutral oligosaccharides were not retained but the phosphorylated oligosaccharides were held back and were desorbed in two fractions by 20 mM and 80 mM NaCl. The respective weights from 258 mg of glycogen were 2.9 mg and 2.1 mg (Fig. 1). This experiment revealed that the phosphate was present in two forms, behaving as di- and mono-esters [15]. We tested this

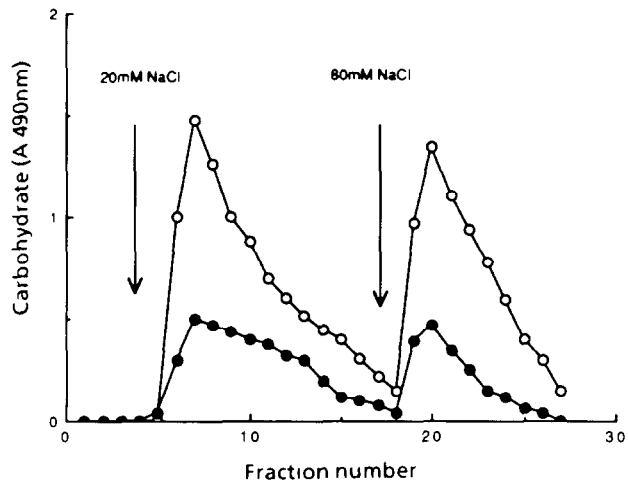

Fig. 1. Fractionation of glycogen-derived phosphorylated oligosaccharides. Glycogen (258 mg) was digested with α-amylase and the products fractionated on QAE-Sephadex (see section 3). Shown by open circles are the oligosaccharides eluted by 20 mM NaCl (diesters) and 80 mM NaCl (monoesters). Fractions 5–15 from the diesters were combined, hydrolysed in 0.02 N trifluoroacetic acid (20 min, 100°C) and again passed through the column, emerging as shown by the solid circles. For conditions, see section 2

deduction by hydrolyzing the supposed diester oligosaccharide fraction with mild acid under conditions that caused a 30% hydrolysis of glucose 1-phosphate while causing no significant hydrolysis of maltose (data not shown). Refractionation on QAE-Sephadex revealed that 36% of the fraction that had previously eluted as a diester now emerged as a monoester (Fig. 1). We conclude that the majority (60%) of the phosphate in the original α-amylase digest was present as a diester, of which one ester bond was acid-labile.

3.3. *Enzymic introduction of phosphate into glycogen*

The conclusion that an acid-labile phosphodiester is present in muscle glycogen led us to consider that it might be introduced after the manner in which phosphodiesters are synthesized on mannose residues in the carbohydrate chains of glycoproteins [15]. The *N*-acetylglucosamine 1-phosphate moiety of UDP-*N*-acetylglucosamine is transferred en bloc to the mannose, followed by the hydrolytic removal of the *N*-acetylglucosamine. In the present case, the donor to the primary hydroxyl group of a glucose 1-phosphate residue would be UDPglucose, accounting for the diester, followed by hydrolysis of the glucose to leave a phosphomonoester. A direct transfer of glucose 1-phosphate from UDPglucose to protein has already been noted [10].

A systematic search was made in a rabbit-muscle extract for an enzyme activity capable of carrying out such a glucose 1-phosphate transfer. We used [β-^{32}P]UDPGlc and, in some experiments, its β-phosphorothioate analogue ([β-^{35}S]UDPGlc). We obtained evidence of such transfer (Table I). The activity was not that of glycogen synthase since the transfer of ^{32}P occurred unimpeded in the presence of UDPpyridoxal which is a powerful glycogen synthase inhibitor [16] (result not shown).

Glycogen labelled with ^{32}P in this way was rigorously purified to remove non-covalently bound phosphorus. A residue of ^{32}P persisted. The glycogen was then hydrolysed with α-amylase. This resulted in the formation of ^{32}P-labelled oligosaccharides, indicating that the ^{32}P was indeed bound to glycogen. These were concentrated by ion-exchange, subjected to prolonged hydrolysis with acid to split the glycosidic bonds and diester bonds in order to release the phosphorylated monosaccharide(s), which were then subjected to electrophoresis before and after treatment with glucose 6-phosphate dehydrogenase. Fig. 2 shows that the ^{32}P-labelled product of acid hydrolysis of the labelled glycogen migrated during electrophoresis as glucose 6-phosphate, and was completely converted by glucose 6-phosphate dehydrogenase into a compound migrating during electrophoresis at the same rate as the product formed by dehydrogenase action on authentic ^{14}C-labelled glucose 6-phosphate, i.e. 6-phosphogluconate.

Table I

pH dependence of the incorporation of ^{35}S into glycogen, from [β-^{35}S]UDPglucose

pH	4.5	5.5	6.5	7.5	8.5	9.5
^{35}S incorporation (counts/min)	3,050	7,586	4,519	2,064	1,950	1,450

The buffers used were sodium acetate–acetic acid (pH 4.5, 5.5), imidazole-HCl (pH 6.5), Tris-HCl (pH 7.5, 8.5) and glycine-NaOH (pH 9.5). The digests were of the same composition as in the in vitro labelling of glycogen (section 2.4). The UDPglucose concentration was 63 nM and the specific activity was 1,100 Ci/mmol. The maximum incorporation, at pH 5.5, was equal to 3.13 fmol of glucose 1-phosphate transferred to the 50 mg of glycogen taken.

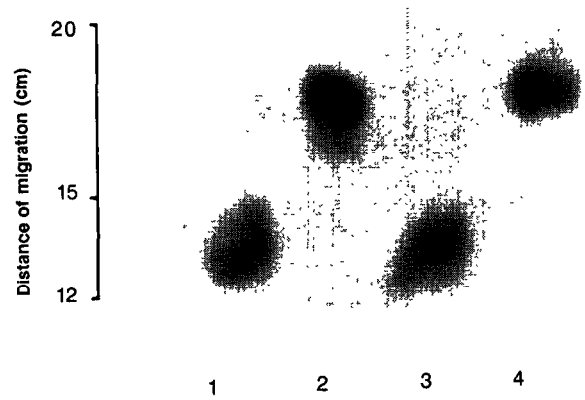

Fig. 2. Radioautograph showing the electrophoretic behavior of the ^{32}P-labelled product obtained by α-amylolysis, followed by acid hydrolysis, of ^{32}P-labelled glycogen (lane 3). Lane 4 depicts the electrophoretic behavior after the lane 3 product had been treated with glucose 6-phosphate dehydrogenase. Authentic ^{14}C-labelled glucose 6-phosphate is seen in lane 1, and in lane 2 after treatment with the dehydrogenase. For conditions, see section 2.

DISCUSSION

For proof of covalently bound phosphate in liver glycogen, Fontana [1] relied on the inability, by several methods, to remove ^{32}P from glycogen into which the isotope had been introduced by labelling in the whole animal. In our own case, we examined muscle glycogen that was carefully purified to remove non-covalently bound low and high molecular weight components and found that 0.064% P remained, by weight, with no detectable inorganic phosphate. This is within the range of phosphate contents reported for a variety of plant starches [17] and is 250 times the amount represented by the serine phosphate in glycogenin [6].

Although the phosphate ester in starch is thought to be present as a monoester [2,17], Fontana [1] had concluded from the chemical stability to acid and alkali of the ^{32}P in his liver glycogen that two types of phosphate were present. We were able to confirm this deduction and to identify the two types. The phosphate residues were concentrated by subjecting the glycogen to α-amy-

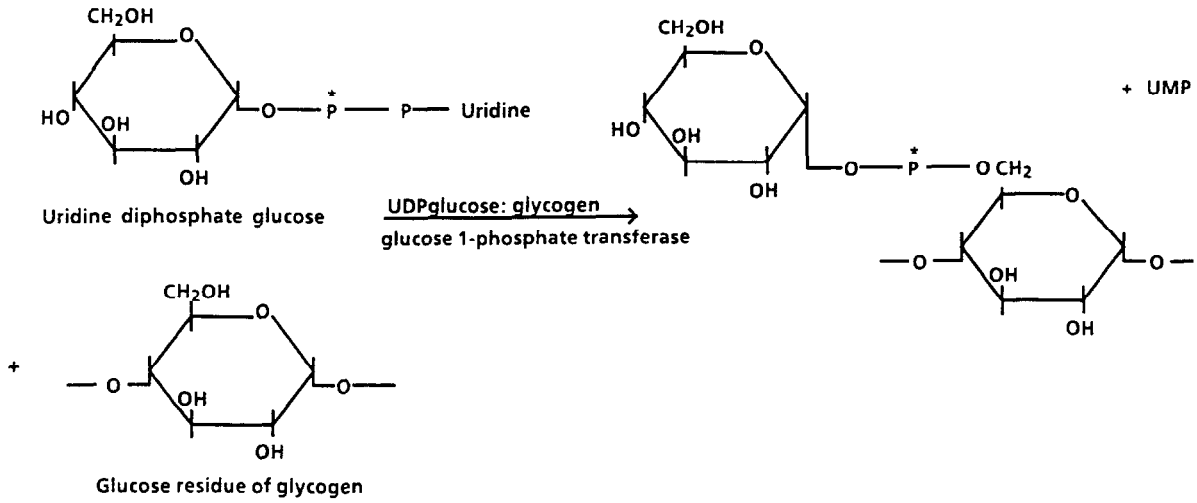

Fig. 3. Depicting the proposed method of introduction of phosphodiester groups into glycogen by UDPglucose:glycogen glucose 1-phosphate transferase. *P indicates the position of labelling by ^{32}P or ^{35}S in the UDPglucose

lolysis and ion-exchange chromatography. The charged oligosaccharides were separated into two fractions that behaved as mono- and di-esters (Fig. 1). The molar ratio of the two types of ester, based on the relative yields of phosphorylated oligosaccharides, appeared to be approx. 60 diester: 40 monoester.

Confirmation that an acid-labile linkage behaving as a diester was present, corresponding to phosphate joined to C-1 of a glucose residue, came from the fact that mild acid hydrolysis, sufficient to split glucose 1-phosphate by 30%, caused a 36% conversion of the oligosaccharide diester fraction into a monoester (Fig. 1).

We then considered how phosphate groups might be introduced into glycogen. One possibility was that of a hexokinase-like enzyme, able to phosphorylate C-6 of a glucose residue in glycogen from ATP. The alternative, as noted earlier, was based on an analogy with the phosphorylation of the carbohydrate chains of glycoproteins [15], involving the introduction of a glucose 1-phosphate residue from a nucleoside diphosphate glucose.

We were able to detect the latter type of activity by using β-^{32}P-labelled UDPglucose, demonstrating that ^{32}P could be introduced into glycogen from this substrate and that it was not removable in a denaturing environment.

Our evidence for the enzymic introduction of phosphodiester groups into glycogen is therefore that glycogen became phosphorylated when β-^{32}P-labelled UDPglucose was the donor, for which a logical explanation would be a transfer of glucose 1-phosphate by analogy with a similar glycosylphosphate transfer to glycoproteins [15]. The ^{32}P so incorporated could not be removed by alkaline phosphatase, consistent with its not being present as a phosphomonoester. We were able to concentrate the ^{32}P in charged oligosaccharides by the same procedure as for the phosphodiester groups of native glycogen (Fig. 1), from which it was released by acid hydrolysis as ^{32}P-labelled glucose 6-phosphate (Fig. 2). We therefore postulate that glucose 1-phosphate is transferred from UDPglucose to the primary (C-6) hydroxyl of a glucose residue in glycogen (Fig. 3).

The enzyme activity responsible for the glucose 1-phosphorylation appeared to have a pH optimum of 5.5 (Table I). It is possible that the actual pH optimum is at a more neutral pH and that pH 5.5 is an apparent optimum arising from competing reactions that also use UDPglucose and are catalyzed by other enzymes in the impure preparation of the glucose 1-phosphotransferase. The activity was not that of glycogen synthase. It appears to be a new enzymic activity for muscle glycogen metabolism, not hitherto described.

We cannot exclude the possibility that some phosphate groups of glycogen are introduced directly from ATP as phosphomonoesters by a hexokinase-like enzyme. On the other hand, a plausible scenario for the origin of the phosphomonoesters is that they are formed from the diesters. The 'capping' glucose could in time be removed by a glucose 1-phosphate phosphodiesterase, as detected in liver [18], or an α-glucosidase, of which there are several in muscle cytosol [19], leaving the phosphomonoester residue. If such a group could be phosphorolysed, it would provide the glucose 1,6-bisphosphate needed as a coenzyme in the onward conversion of glucose 1-phosphate into glucose 6-phosphate. We should also note that when glucose 6-phosphate residues have been detected in starches and glycogens, this has frequently been done by acid hydrolysis of the polysaccharide, thereby destroying any evidence of a phosphodiester [17].

The function of the phosphate groups remains ob-

scure, but the demonstration of a phosphodiester bond may explain long-standing observations of the behavior of glycogen when treated with dilute acid or alkali, as if there are present some particularly labile bond(s) [20]. We will later report evidence suggesting that the phosphodiester groups may function as chain initiators, such that a proportion of the branches in glycogen are not only the 1,6-glucosylglucose bonds but that some contain an intervening phosphodiester group as in Fig. 3. The partial depolymerization of glycogen seen after mild acid or alkali treatment [20] would be a consequence of the presence of such bonds.

The alkali lability of phosphoesters may in part explain why there has been a ready acceptance of the idea of phosphate in starch [2,17] but not in glycogen [1]. Starch is isolated by relatively mild treatment, at neutral pH without heating, making use of its water insolubility. Glycogen is most commonly isolated by the Pflüger method that takes advantage of the fact that glycogen is the only mammalian macromolecule that is largely stable to alkali. Accordingly, tissue is heated for several hours at 100°C in 30% KOH, conditions not conducive to retaining phosphoesters, di- or mono-. It was for the same reason of preparing glycogen in this way that the presence of protein, claimed as long ago as 1886 [21], remained controversial until 1985, when a covalently bound protein, glycogenin, was isolated from glycogen that had been purified without resort to alkali [3]. The use of drastic chemical procedures to isolate natural products is not without pitfalls.

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