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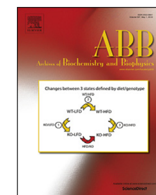

Incorporation of phosphate into glycogen by glycogen synthase

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ABSTRACT

The storage polymer glycogen normally contains small amounts of covalently attached phosphate as phosphomonoesters at C2, C3 and C6 atoms of glucose residues. In the absence of the laforin phosphatase, as in the rare childhood epilepsy Lafora disease, the phosphorylation level is elevated and is associated with abnormal glycogen structure that contributes to the pathology. Laforin therefore likely functions *in vivo* as a glycogen phosphatase. The mechanism of glycogen phosphorylation is less well-understood. We have reported that glycogen synthase incorporates phosphate into glycogen via a rare side reaction in which glucose-phosphate rather than glucose is transferred to a growing polyglucose chain (Tagliabracci et al. (2011) *Cell Metab* **13**, 274–282). We proposed a mechanism to account for phosphorylation at C2 and possibly at C3. Our results have since been challenged (Nitschke et al. (2013) *Cell Metab* **17**, 756–767). Here we extend the evidence supporting our conclusion, validating the assay used for the detection of glycogen phosphorylation, measurement of the transfer of ^{32}P from [β-^{32}P]UDP-glucose to glycogen by glycogen synthase. The ^{32}P associated with the glycogen fraction was stable to ethanol precipitation, SDS-PAGE and gel filtration on Sephadex G50. The ^{32}P-signal was not affected by inclusion of excess unlabeled UDP before analysis or by treatment with a UDPase, arguing against the signal being due to contaminating [β-^{32}P]UDP generated in the reaction. Furthermore, [^{32}P]UDP did not bind non-covalently to glycogen. The ^{32}P associated with glycogen was released by laforin treatment, suggesting that it was present as a phosphomonoester. The conclusion is that glycogen synthase can mediate the introduction of phosphate into glycogen, thereby providing a possible mechanism for C2, and perhaps C3, phosphorylation.

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1. Introduction

Glycogen is a branched polymeric storage form of glucose present in many cell types. The polymerizing glucose residues are linked by α-1,4-glycosidic bonds formed by the action of glycogen synthase ([1]; Fig. 1). The branches are introduced as α-1,6-glycosidic linkages by the branching enzyme. Glycogen contains other minor constituents besides glucose, the most important of which is covalently attached phosphate [2–5]. Measurements of the abundance of the phosphate have ranged from ~1:500 to ~1:5000 phosphates per glucose residue and depend on the source of the glycogen. Recent studies suggest that the phosphate exists as monoesters at C2, C3 and C6 carbons of glucose residues within the glycogen [6,7]. Plant amylopectin, which is a close relative of glycogen both chemically and functionally, also contains C3 and C6 phosphomonoesters of glucose [8–11]. No specific function for the phosphate in glycogen is known but its hyperaccumulation appears to disrupt normal glycogen structure [12]. The best evidence comes from studies of Lafora disease [13–17], a fatal teenage-onset myoclonic epilepsy in which an abnormal glycogen-like polymer forms insoluble deposits, Lafora bodies, in neurons, heart, skeletal muscle, skin and other tissues. Lafora disease has been linked, in about 90% of cases, to recessive mutations in one of two genes, *EPM2A* and *EPM2B*/*NHLRC1*, which encode respectively the proteins laforin [18,19] and malin [20]. Mice with either locus disrupted accumulate Lafora bodies and have a number of the neurological

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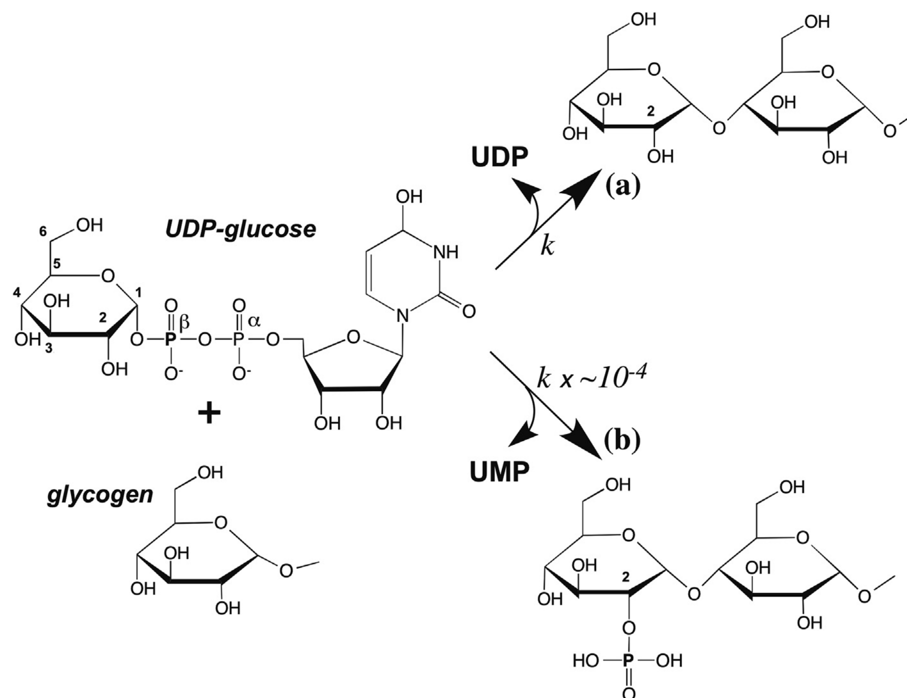

Fig. 1. Reactions of glycogen synthase. Shown are (a) the primary reaction catalyzed by glycogen synthase in which glucose is transferred from the substrate UDP-glucose to the non-reducing end of a polyglucose chain in glycogen and (b) the side reaction in which the β-phosphate of UDP-glucose is transferred to add a glucose-phosphate unit (see text). The rate constants are to indicate that the side reaction occurs at around one ten thousandth of the rate of the main reaction.

defects seen in the human disease [13–17,21]. Laforin, which by sequence can be placed in the sub-family of atypical dual specificity protein phosphatases [22], has been shown to dephosphorylate amylopectin [23,24], glycogen [24], and phospho-oligosaccharides *in vitro* [25]. Furthermore, glycogen isolated from laforin or malin knockout mice has an elevated phosphate content [6,24,26] and, with aging, the glycogen becomes less branched and less water soluble, consistent with the formation of Lafora bodies [12]. Therefore, laforin appears to act as a glycogen phosphatase *in vivo* and its absence results in abnormal glycogen structure which may underlie the pathology of Lafora disease.

The origin of the phosphate present in glycogen is less well understood [5]. Nonetheless, the mechanism of glycogen phosphorylation is an important issue, not only for our fundamental understanding of glycogen metabolism but also because it could guide efforts to suppress glycogen phosphate accumulation as a therapy for Lafora disease. The phosphate present in plant starch is known to be introduced by specific dikinase enzymes [27–29]. However, neither bioinformatic nor biochemical studies have so far revealed comparable enzymes in mammals. Another possibility, that we have proposed, is that glycogen synthase itself can introduce covalently-linked phosphate into glycogen. By using [β-^{32}P]UDP-glucose as a substrate, we had shown that glycogen synthase could incorporate ^{32}P into newly synthesized glycogen. We attributed this result to the transfer of the β-phosphate of UDP-glucose into glycogen as a rare side reaction, once every ~10,000 normal catalytic cycles ([25]; Fig. 1). We proposed that the mechanism might involve a cyclic phosphate diester intermediate that could explain phosphorylation at C2 and possibly at C3. It is difficult, however, to explain C6 phosphorylation by such a mechanism. Enzymological and structural studies did provide further support for the involvement of cyclic phosphate [30]. First, a crystal structure of glycogen synthase with glucose-1,2-cyclic phosphate bound indicated that the catalytic site could accommodate the cyclic ester in a manner consistent with the proposed mechanism. Secondly, incubation of glycogen synthase with UDP-glucose resulted in the generation of the cyclic phosphate. Others have challenged this conclusion, suggesting that the ^{32}P signal we detected in glycogen was due to the normal reaction product [β-^{32}P]UDP binding non-covalently to glycogen [7]. In the present study, we describe more extensive investigation of the phosphorylation of glycogen and provide further evidence that glycogen synthase is capable of transferring the β-phosphate of UDP-glucose to glycogen.

2. Materials and methods

2.1. Reagents

[γ-^{32}P]ATP (NEG002A001MC; specific activity 3000 Ci/mmol in 10 mM Tricine pH 7.6) and UDP-[U-^{14}C]glucose (NEC403V050UC; specific activity 250 mCi/mmol in ethanol:water 2:98) were from PerkinElmer. UDP for competition assays was from Sigma (#94330). High purity UDP (99.2%) used in laforin reactions was from Chem-Impex International, Inc. (#00310). [α-^{32}P]UDP (SCP230/37; specific activity 6000 Ci/mmol in 50 mM Tricine pH 7.4) was from Hartmann Analytic. Materials for gel filtration experiments were Sephadex G-50 (Sigma, G-50-150) and Spin Columns (Promega, C1281). Glycogen was purified from the skeletal muscle of New Zealand White rabbits as described previously [6].

2.2. Enzymes

Recombinant yeast Gsy2p was produced in *Escherichia coli* and purified as described by Baskaran et al. [31]. Recombinant human GYS1 glycogen synthase was produced in insect cells and purified as described by Khanna et al. [32]. Hexokinase (#1012765501) and pyrophosphatase (#91078329) were from Roche. Phosphoglucomutase (#46550003) was from Oriental Yeast Co., LTD.

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