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Substrate specificity of the autocatalytic protein that primes glycogen synthesis

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The autocatalytic protein that primes muscle-glycogen synthesis, and which glucosylates itself from UDPglucose, is inhibited by maltose. Investigation of the reason for the inhibition led to the finding that the protein will glucosylate substrates other than itself. *p*-Nitrophenyl α-glucoside, α-maltoside, α-maltotrioside and α-maltotetraoside each inhibit self-glucosylation of the protein by acting as alternative acceptor substrates. The α-maltoside is the best acceptor. The α-maltohexaoside did not act as an acceptor but was an effective inhibitor. These findings help to explain the self-limiting nature of the autocatalytic extension of the maltosaccharide chain of the protein and suggest that protein self-glucosylation may be an intermolecular event. They may also point to the mechanism by which the autocatalytic protein is initially glycosylated.

Glycogenin; Self-glucosylating protein; Glycogen biogenesis

1. INTRODUCTION

Glycogenin is the name we have given to covalently bound protein we originally found in muscle glycogen, in equimolar proportion [1]. The glycogen is linked via the hydroxyl group of tyrosine [2,3].

Subsequently we discovered in muscle and other tissues an protein that undergoes glucosylation when incubated with UDPglucose [4]. It has a similar M_r to glycogenin, cross-reacts with a polyclonal antibody to this protein [5,7], and therefore appears to be a glycogen-free form of glycogenin, although it already contains a bound maltosaccharide [5,7]. When purified to homogeneity, the protein proved to be autocatalytic [7]. Using UDPglucose, it extends the bound maltosaccharide to malto-octaose [8]. We believe that this protein is the primer for glycogen synthesis, accounting for its presence in glycogen [1,7]. Thus, rabbit muscle contains two parts of glycogenin bound to glycogen and one part in the glycogen-free form, with the potential to prime the synthesis of additional glycogen [7]. We refer to the latter as self-glycosylating protein (SGP) to distinguish it from glycogenin in glycogen. A similar protein, which primes glycogen synthase is, was found by Pitcher et al. to be present in purified muscle glycogen synthase [9] and to have extensive sequence homology to glycogenin, including the linkage of carbohydrate to tyrosine [3]. It also underwent self-glucosylation after inactivation of the synthase [10]. The complete amino acid sequence of muscle glycogenin has now been reported by Campbell and Cohen [11].

In an examination of endogenous activators and inhibitors of muscle SGP we were led to the discovery that maltosaccharide derivatives can compete with the self-glucosylation reaction by themselves acting as acceptors of glucose residues transferred from UDPglucose. This is a report of these findings and their implication for our knowledge of the structure, mechanism of action and biosynthesis of SGP.

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2. MATERIALS AND METHODS

2.1. *Materials*

SGP was purified to homogeneity from rabbit muscle as by Lomako et al. [7]. Maltosaccharides were purchased from Sigma and *p*-nitrophenyl saccharides from Boehringer. UDP[^{14}C]glucose was from ICN.

2.2. *Methods*

The [^{14}C]glucosylation of SGP was measured as by Lomako et al. [7]. In experiments where acceptors or inhibitors were added, the composition of the digests was otherwise identical to those used in measuring SGP activity [7]. In order to measure incorporation of [^{14}C]glucose into added acceptors, the digest (100 μl) was passed through a mixed-bed ion-exchange resin (50 × 8 mm, BioRex RG 501 × 8 mixed-bed resin, BioRad) to remove UDPglucose and glucosylated protein. The freeze-dried effluent was dissolved in water (150 μl) and internal standards of *p*-nitrophenyl α-glucoside and *p*-nitrophenyl α-maltosaccharides (6 μl, 50 mM) were added. The sample was loaded onto a C_{18} column (4.6 × 250 mm Vydac) and fractionated by reverse-phase HPLC using a gradient of acetonitrile-water (0–25% acetonitrile during 45 min). The separation was monitored by absorbance at 320 nm. Samples were collected by absorbance peaks and their radioactivity measured, as well as freeze-drying in order to confirm the identity of the product by thin-layer chromatography (Fig. 2).

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3. RESULTS AND DISCUSSION

3.1. *Effects of glucose and maltosaccharides on SGP activity*

We were led to the observations in this report by asking the question how SGP might function in vivo in a milieu in which its ability to glucosylate itself might be compromised by the presence of alternative acceptor substrates, for example, glycogen or maltosaccharides. This was in the context that we had evidence for the presence in muscle extracts of a heat-stable inhibitor which proved to be of low molecular weight.

A systematic testing of possible inhibitors led to the observation that in the maltosaccharide series from maltose to maltohexaose, maltose is uniquely inhibitory (Fig. 1). Nor do glucose or glycogen inhibit. Maltose, quantitatively the most important α-amylolytic product of glycogen breakdown [12], was therefore a candidate for the role of the endogenous muscle inhibitor we had detected.

3.2. *Mechanism of SGP inhibition by maltose*

We then tested whether the reason why maltose was inhibitory to SGP was that it is a competing substrate. We decided to not use maltose, because we would then be searching for the formation of ^{14}C-labelled maltosaccharides which could also be formed by hydrolysis of the maltosaccharide chains of SGP. Instead, we used *p*-nitrophenyl α-maltoside as a potential inhibitor/acceptor and UDP[^{14}C]glucose as donor, where evidence of acceptor ability would be seen by the formation of a ^{14}C-labelled *p*-nitrophenyl saccharide which could not be mistaken for the product of an endogenous acceptor.

When this was done, a dramatic difference was seen between *p*-nitrophenyl α-maltoside and maltose (Fig. 1), the former achieving 50% inhibition at 3 mM concentration vs 40 mM when maltose was used. It now became apparent also that where glucose and other maltosaccharides did not inhibit SGP (up to 50 mM concentration), the *p*-nitrophenyl α-anomeric forms did do so (Fig. 1). Accordingly, using UDP[^{14}C]glucose as donor, we examined such digests for the presence of ^{14}C-labelled *p*-nitrophenyl saccharides and were able to find them. Fig. 2 shows a thin-layer chromatogram and radioautograph of the products.

p-Nitrophenyl α-glucoside, added to a mixture of SGP and UDP[^{14}C]glucose, not only inhibited the ^{14}C-labelling of SGP, but a product was formed with the R_F value of *p*-nitrophenyl α-maltoside. Added maltoside caused the formation of what appeared to maltotrioside, maltorioside gave tetraoside, tetraoside gave what may be pantaoside. The hexaoside, although inhibiting self-glucosylation by SGP (Fig. 1), gave no labelled saccharide.

The products from the maltoside and maltotetraoside were accompanied by lesser amounts of substances migrating to about the same positions as the acceptor substrates (Fig. 2). Whether they resulted from hydrolysis of the larger product, we do not known. Certainly the acceptor substrates appeared to be chromatographically pure.

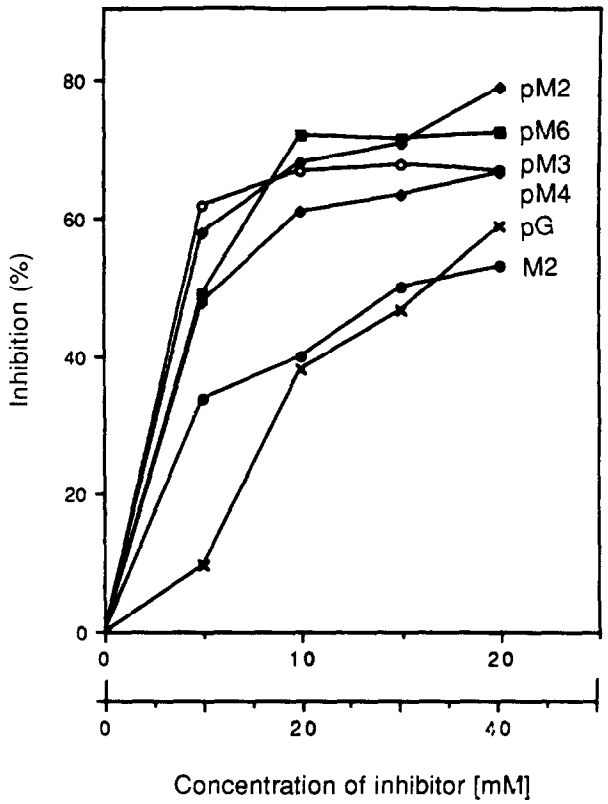

Fig. 1. Inhibition of protein self-glucosylation by maltose (M2), *p*-nitrophenyl αglucoside (pG) and *p*-nitrophenyl α-maltosaccharides (maltoside [pM2]-maltohexaoside [pM6]). Self-glucosylating protein was incubated with UDP[^{14}C]glucose in the absence and presence of the saccharide (see section 2). The degree of ^{14}C-glucosylation of the protein was determined by precipitating the protein with trichloroacetic acid and measuring the ^{14}C in the precipitate [7]. Glucose and maltotriosine-maltohexaose did not inhibit at 50 mM concentration (results not shown). The upper abcissa shows the concentration of the *p*-nitrophenyl saccharides, the lower of maltose.

It should be noted that during the HPLC fractionation of the products of the acceptor reaction, some radioactivity always appeared in the breakthrough volume. The amount varied with the amount of enzyme used but did not vary with the added acceptor or the amount of labelled *p*-nitrophenyl saccharide formed. The cpm associated with the breakthrough fraction was about 1000 cpm where the ^{14}C in the labelled *p*-nitrophenyl saccharide varied from 1100 to 13 600 cpm, depending on the acceptor substrate. Since the formation of this material appeared not to be associated with the acceptor, we did not examine it further.

That the glucosidic linkages being formed were α-1,4 was shown by the ability to degrade with β-amylase the presumed *p*-nitrophenyl α-maltotetraoside, formed from the trioside. The tetraoside disappeared and

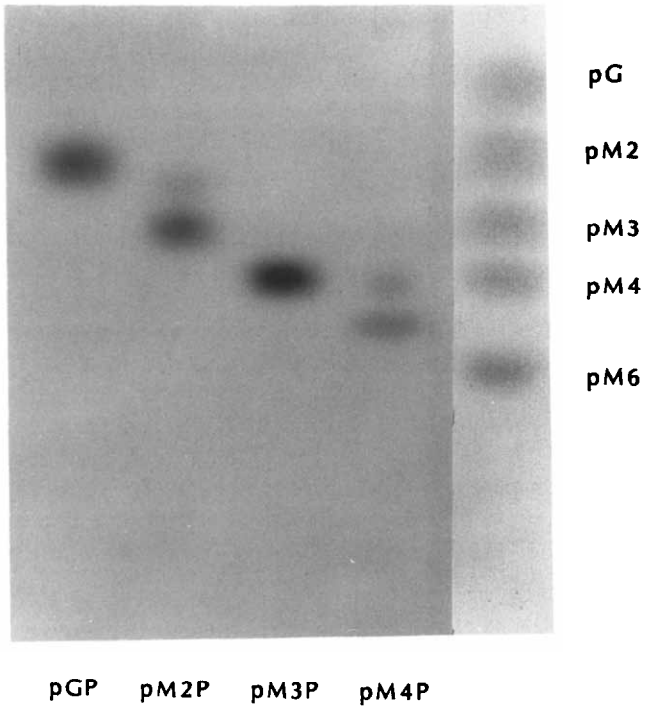

Fig. 2. Products of the reaction between self-glucosylating protein, UDP[^{14}C]glucose and *p*-nitrophenyl α-glucoside and α-maltosaccharides. The ^{14}C-glucosylated products were purified from the digest by being passed through ion-exchange and HPLC columns (see section 2). The products were applied to a thin-layer silica-gel chromatogram which was irrigated by ascent in butanol-ethanol-water (5:4:5, v/v). The left-hand panel is a radioautograph of the products. The right-hand panel is the chromatographic standards revealed with sulfuric acid. pG = *p*-nitrophenyl α-glucoside, pGP = product from *p*-nitrophenyl α-glucoside digest, similarly for pM2 (maltoside), pM2P (maltoside products) through pM4 (maltotetraoside).

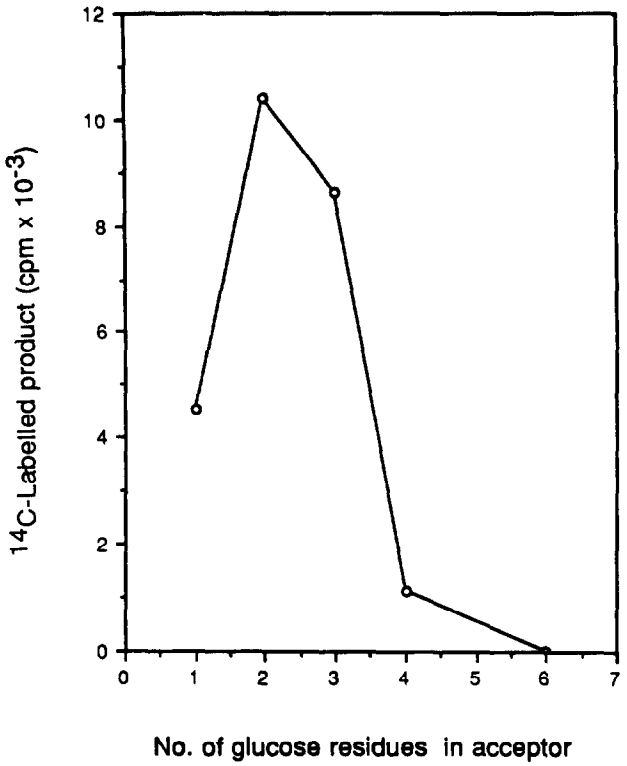

Fig. 3. Showing the relative rates of ^{14}C-glucosylation of *p*-nitrophenyl α-saccharides, α-glucoside-α-maltohexaoside) when incubated with self-glucosylating protein and UDP[^{14}C]glucose. The products were isolated by HPLC as in Fig. 2. The cpm recorded represents the products from a standard 100 μl digest containing 10 mM *p*-nitrophenyl saccharide [7].

[^{14}C]maltose (as judged by its R_F value in thin-layer chromatography), was the only labelled substance formed (result not shown).

Quantitative determination of the degree of labelling of the added acceptor showed that the maltoside is the most powerful acceptor, followed by the trioside, glucoside and tetraoside (Fig. 3). In these experiments, especially with the maltoside, the degree of labelling of the acceptor was much higher than of SGP, although it must be pointed out that the measurements were made with SGP at 0.2 μM concentration, UDPglucose at 2 μM and the acceptor at 10 mM. Thus, under conditions where self-labelling resulted in the incorporation of 2550 cpm of [^{14}C]glucose into protein, this dropped to 510 cpm in presence of maltoside, while the simultaneous degree of incorporation of [^{14}C]glucose into the maltoside was 17 708 cpm. Also, self-glucosylation is complete in 30 min, where labelling of the added acceptor continues for several hours.

The great superiority of *p*-nitrophenyl α-maltoside over maltose as an inhibitor of SGP (and as a competing acceptor) could be due to the fixed anomeric configuration of the former, or to the *p*-nitrophenyl residue mimicking the tyrosine to which the oligosaccharide in SGP is attached [2,3], or both. That the anomeric configuration is important was shown by the fact that the β-maltoside is non-inhibitory, even at 30 mM concentration. The absence of any inhibition by this anomer suggests that the nature of the aglycone is secondary to the anomeric configuration of the maltose. The result also suggests that the maltosaccharide chain attached to SGP is linked to tyrosine in the α-configuration, a structural feature that has not yet been established.

3.3. *Mechanism of glucosyl transfer by SGP*

We already reported that SGP as isolated contains a bound maltosaccharide, one per molecule [5,7]. We have recently reported that the maltosaccharide chains in SGP as isolated are of mixed length, with the shortest chain being maltose and the longest malto-octaose [8]. The self-glucosylating reaction observed *in vitro* is one in which all the saccharide chains are extended to 8 glucose residues [8].

The observations reported here bear an obvious relation to the foregoing. The maltoside is the best acceptor, corresponding to the shortest carbohydrate chain in SGP, as isolated. The act of self-glucosylation can be seen as one in which, as the carbohydrate moeity is

lenghtened, the rate of elongation decreases. Thus the maltotrioside is less effective as an acceptor than the maltoside, the tetraoside than the trioside, the hexaoside than the tetraoside (Fig. 3). The lengthening of the carbohydrate chain in SGP therefore becomes self-limiting because the longer chains are less and less efficient as acceptors.

That in our experiments the added acceptors were extended only by one glucose unit and not several (Fig. 2) is, we believe, explained by the 1000-fold excess of acceptor over the UDPglucose substrate, rendering it unlikely that any one acceptor molecule would participate more than once in glucose transfer.

That SGP will transfer glucose to an acceptor other than its own bound maltosaccharide allows one to speculate on the mechanism of self-glucosylation in vivo. What we have termed self-glucosylation is a reaction tht occurs when a single protein, homogeneous SGP, is incubated with UDPglucose [7]. The observation does not permit one to distinguish between intramolecular or intermolecular glucosylation, especially in light of the knowledge that under non-denaturing conditions SGP forms large aggregates [7]. The present observations do not exclude intramolecular glucosylation but the fact that a separate molecule, in this case a maltosaccharide, can accept glucose in a reaction catalysed by SGP, suggests that SGP in 'autocatalysis' may act by intermolecular glycosylation between aggregated protein molecules and that the aggregation may be purposeful.

We know that SGP binds UDPglucose strongly (unpublished observation). It is possible to think of the UDPglucose-binding site (the active site?) in one SGP molecule being in the proximity of the maltosaccharide chain of another molecule, even binding to that chain. The presence of a maltosaccharide-binding site in SGP is suggested from the behavior of the *p*-nitrophenyl saccharides as inhibitors of self-glucosylation (Fig. 1), as opposed to acceptors in transglucosylation (Fig. 3). Maltohexaose does not inhibit SGP. *p*-Nitrophenyl α-maltohexaoside does not participate in transglucosylation (Fig. 3). But the α-maltohexaoside inhibits self-glucosylation as strongly as any of the other maltosaccharides tested (Fig. 1). This, we consider, is evidence for the presence in SGP of a maltosaccharide-binding site. The large difference between maltohexaose as non-inhibitory to self-glucosylation and *p*-nitrophenyl α-maltohexaoside as a powerful inhibitor of this reaction, may implicate the aglycone, mimicking tyrosine, in the binding of the inhibitor by SGP.

A final consideration is that of how SGP acquires its initial substitution by carbohydrate. As mentioned, the shortest chain in SGP as we isolate it [7] is maltose [8]. Maltosylation of carbohydrate-free SGP may then be considered as leading to SGP. On the other hand, since SGP will glucosylate *p*-nitrophenyl α-glucoside, it could perhaps glucosylate itself even if only one glucose residue is attached to tyrosine. Therefore, an alternative to maltosylation of the protein, which would require an energized maltose derivative, is glucosylation by an enzyme capable of joining glucose to tyrosine followed by autocatalysis [10]. The fact that the smallest carbohydrate grouping found in isolated SGP is maltose does not preclude this second hypthesis. The intriguing question is, at what point does this protein become autocatalytic – as the glucosyl or as the maltosyl derivative?

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