Research Letters

The nature of the primer for glycogen synthesis in muscle

Joseph Lomako, Wieslawa M. Lomako and William J. Whelan

Department of Biochemistry and Molecular Biology, University of Miami, Miami, FL 33101, USA

Received 5 May 1990, revised version received 28 May 1990

We and others have reported that glycogenin, the covalently bound protein found in muscle glycogen, also exists in muscle in a glycogen-free form (M_r 38 000–39 000) that is autocatalytic, undergoes self-glucosylation and acts as a primer for glycogen synthesis. We now report that this entity is not present in a fresh muscle extract. Instead it exists within a pro form of much higher molecular mass which breaks down spontaneously to the M_r 38 000–39 000 form. Such breakdown is accelerated by the addition of α-amylase and is prevented by protease inhibitors. Multiple intermediates of the breakdown process have been detected, each capable of undergoing glucosylation.

Glycogenin; Glycogen biogenesis; Protein glucosylation

1. INTRODUCTION

Glycogenin is the name we have given to a protein that is covalently linked to rabbit-muscle glycogen via the novel glucose-tyrosine bond [1,2]. A protein of similar M_r (39 000) and free from glycogen is present in muscle and heart. It can be detected by its becoming glucosylated when tissue extracts are incubated with 2 μM UDP[^{14}C]glucose and 5 mM Mn^{2+} [3,4]. When purified to homogeneity the protein proved to contain a bound maltosaccharide residue and to be autocatalytic, catalysing the addition of glucose to its bound maltosaccharide [5] and lengthening it to malto-octaose [6]. This product primes glycogen synthesis [5]. The purified protein (self-glucosylating protein; SGP) gives a positive immunoblot with antibody to glycogenin from muscle glycogen [5]. Glycogenin and SGP appear to be similar, if not identical, but we will use the separate names here to distinguish the origin of the proteins.

Cohen and his collaborators found a glycogenin-like protein present in a muscle glycogen synthase preparation in 1:1 proportion with the synthase subunit [7]. They confirmed the linkage of glycogen to tyrosine in glycogenin and showed that the protein complexed with synthase also contained glucose bound to tyrosine [8], further that this protein was also autocatalytic [9]. Campbell and Cohen [10] have reported the complete amino acid sequence of glycogenin.

We now report that SGP, although isolatable from muscle in a form having approximately the same M_r as glycogenin from glycogen, does not seem to occur as such in vivo. Rather, muscle contains a pro form of SGP, of much higher molecular weight. This form breaks down to SGP via intermediate forms when a muscle extract is incubated.

Correspondence address: W.H. Whelan, Department of Biochemistry and Molecular Biology, University of Miami, PO Box 016129, Miami, FL 33101, USA

2. MATERIALS AND METHODS

2.1. *Materials*

All biochemicals were from Sigma unless otherwise stated. UDP[^{14}C]glucose was from ICN Biochemicals. Acarbose was a gift from Dr E. Truscheit (Bayer AG, Wuppertal, FRG). α-Amylase was prepared from human saliva as by Fischer and Stein [11] and was free from protease.

2.2. *Methods*

[^{14}C]glucosylation of proteins in muscle extracts was carried out as by Lomako et al. [5], using 2 μM UDP[^{14}C]glucose and 5 mM $MnCl_2$ in a 0.1 ml digest and incubating for 30 min at room temperature. A 50 μl portion was removed and added to 1 ml of 10% trichloroacetic acid (TCA) to precipitate protein which was filtered on a nitrocellulose HA filter, 0.45 μM, washed and the filter dried under an infrared lamp before the ^{14}C was counted in scintillation liquid for nonaqueous samples. The remainder of the digest was freeze-dried and used for SDS-PAGE followed by radioautography with Kodak X-Omat XAR-5 film at −70°C and developing in an automated Kodak X-Omat film processor. Western blotting, with an antibody to rabbit-muscle glycogenin, was carried out as in [5].

For the preparation of extracts of skeletal muscle, a rabbit was sacrificed by injection of nembutal through the ear vein. The animal was desanguinated and the freshly removed muscle was freeze-clamped in small portions in liquid nitrogen and then stored at −75°C. Portions (1–3 g) were blended in a Potter Teflon homogenizer in 3 vols of extracting buffer [5] and centrifuged at 16 000 × g for 15 min at 4°C. For the activation studies in Figs 1 and 2 the supernatant was immediately assayed for protein glucosylation and then incubated at 37°C in the absence or presence of α-glucosidase inhibitors (5 μg/ml). At intervals of 1 h a portion of each digest was incubated with UDP[^{14}C]glucose as above and used to determine the degree of labelling of protein as well as for SDS-PAGE of the labelled proteins.

For the study of protein glucosylation in gel-filtered extracts, a muscle extract prepared as above was precipitated by ammonium

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

sulfate at 50% saturation. After 1 h on ice the pellet was centrifuged as above, solubilized in 50 mM Tris-HCl buffer with 2 mM Chaps, pH 7.4, and desalted on a Sephadex G-25 column (8 ml) which was washed with the same buffer. The void volume was collected and used for the experiments shown in Fig. 3. The entire procedure from homogenizing the muscle to collecting the gel-filtered protein occupied no more than 1.5 h. In Fig. 3, when salivary α-amylase was used, it was in final concentrations of 0.95, 4.75 and 19 μg/ml, respectively, in the experiments of lanes 11–13. When protein glucosylation was subsequently studied in these digests, acarbose (30 μg/ml) was added to inhibit the α-amylase. When proteolytic inhibitors were added, these were as in the extracting buffer and at the same concentrations [5].

In order to test whether the [^{14}C]glucosylated proteins would act as primers for glycogen synthesis they were incubated with 2 μM UDP[^{14}C]glucose as above for 30 min and then freed from excess substrate by passing through Sephadex G-25. The void volume fraction was collected, unlabelled UDP-glucose to 5 mM and glucose 6-phosphate to 9 mM were added and the digests were incubated for 3 h at 37°C to allow endogeneous glycogen synthase and branching enzyme to utilize the [^{14}C]glucosylated proteins as primers. The digests were then freeze-dried and subjected to SDS-PAGE, radioautography and Western blotting.

The N-terminal sequencing of rabbit-muscle glycogen, prepared as by Kennedy et al. [1] and of SGP, prepared as by Lomako et al. [5], was attempted by Drs Keith Brew and Helmut E. Meyer, respectively. Both reported negative results that indicated a blocked N-terminus in each case.

3. RESULTS AND DISCUSSION

3.1. *Detection of glucosylated proteins of widely differing* M_r

We were led to the conclusions reported here by the observation that SGP activity in a fresh muscle extract is relatively low and increases when the extract is stored. An incubation period of several hours at 37°C was necessary to develop full activity (Fig. 1). We have observed that ATP is a powerful allosteric inhibitor of SGP, such that at the physiological concentration of ATP, SGP activity in muscle is largely inhibited [12]. However, ATP disappears within a few minutes when muscle is minced yet the activation of SGP on incubation of an extract was much slower (Fig. 1).

SGP 'activity' is measured by allowing the protein to glucosylate itself fully. This is not a kinetic measurement but measures the total amount of glucose that can be incorporated. We reasoned that the increase in extent of glucosylation seen on storing the extract might be the result of endogenous α-glycosidase(s) (α-amylase, α-glucosidase) shortening the pre-existing maltosaccharide chains such that more glucose was now required to lengthen them to malto-octaose [6], accounting for the increased 'activity'.

Accordingly, we tested the effect of adding α-glucosidase inhibitors to a muscle extract to learn whether activation was thereby prevented. Several inhibitors were tested. Only one of them, acarbose, had any effect (Fig. 1). (Acarbose is also an α-amylase inhibitor [13].) The effect of acarbose was, however, the opposite of that predicted. In its presence much more protein glucosylation occurred.

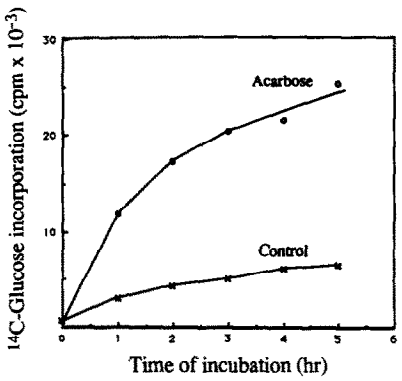

Fig. 1. Showing the changes in the extents of protein glucosylation when a fresh unfiltered muscle extract is incubated in the absence and presence of acarbose (5 μg/ml). A muscle extract (see section 2) was incubated at 37°C and assayed for protein glucosylation (see section 2) at zero time and at intervals of 1 h. The experiment was also performed with added castanospermin, deoxynojirimycin and turanose (5 μg/ml each) but in these cases no differences from the control were seen.

When we examined the [^{14}C]glucosylated proteins by SDS-PAGE we observed that in the presence of acarbose there was present a new series of labelled proteins with M_r values between 50 000 and 80 000. Protein occupying the position of SGP was not seen (Fig. 2, lanes 4,6). By contrast, these 'acarbose' proteins were absent from the acarbose-free extract. Instead, two major protein bands were seen (Fig. 2, lanes 3,5). That of lower M_r corresponded to the SGP that we have purified to homogeneity. The other, with a somewhat greater M_r, was more prominent. We will refer to it as p42, its approximate M_r.

All the labelled proteins gave positive immunoblots with antibody to glycogenin from rabbit-muscle glycogen [5]. All acted as primers for glycogen synthase, since when incubated with unlabelled 5 mM UDP-glucose, all the ^{14}C now moved to the top of the stacking gel, indicating its incorporation into glycogen-like material (results not shown). With the disappearance of the ^{14}C bands from their original positions on the gel there was a corresponding disappearance of the immunoblot. One can examine protein glucosylation in crude extracts without interference from the glycogen synthase present by using 2 μM UDP-[^{14}C]glucose since the K_m of SGP for UDP-glucose is 3 orders of magnitude lower than that of glycogen synthase [5]. Evidently this is also true for all the forms of labelled protein seen in Fig. 2.

The relation between the various forms of glucosylated protein became clear when we decided to remove low molecular weight substances from a muscle extract by salt precipitation followed by passage through a molecular sieve (Sephadex G-25). The activation of protein glucosylation previously seen only on incubating the muscle extracts (Fig. 1) now occurred immediately.

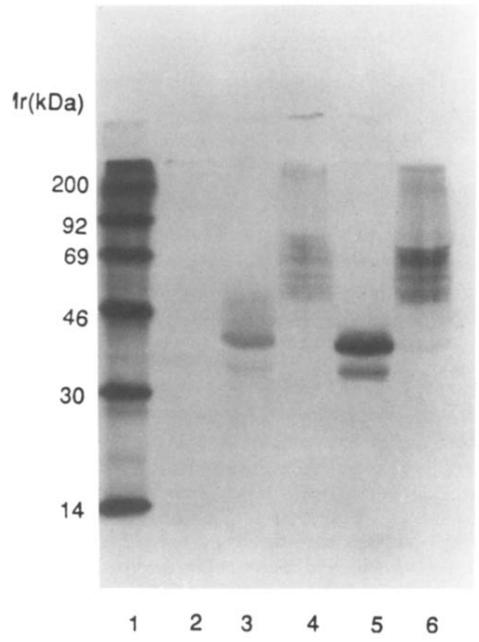

Fig. 2. Radioautograph of a fresh unfiltered muscle extract that had been incubated without and with acarbose (5 μg/ml) as in Fig. 1, then incubated with 2 μM UDP[^{14}C]glucose and 5 mM Mn^{2+}, freeze-dried, and subjected to SDS-PAGE. (Lane 1) ^{14}C-labelled mol. wt. standards; (lane 2), unincubated extract; (lane 3) extract after 3 h at 37°C; (lane 4) extract plus acarbose after 3 h at 37°C; (lanes 5 and 6) correspond respectively to lanes 3 and 4 for extracts that were stored for a further 4 days at 4°C. It is the lower of the two prominent bands in lanes 3 and 5 that corresponds to SGP [5] and the upper band that we refer to as p42.

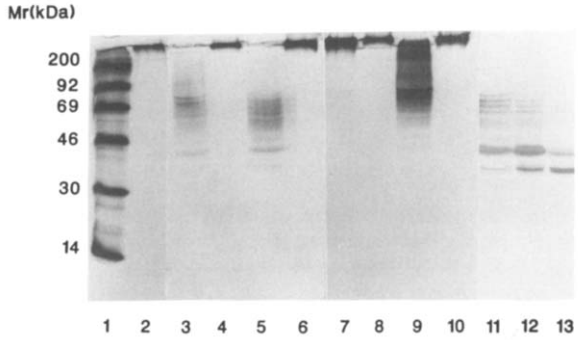

Fig. 3. A composite radioautograph showing the various forms of [^{14}C]glucosylated protein dectected in rabbit muscle. Lane 1 is ^{14}C-labelled protein standards. A fresh muscle extract was salt precipitated and gel filtered as in section 2. This fraction was incubated with UDP[^{14}C]glucose and the freeze-dried digest was subjected to SDS-PAGE (lane 2). The experiments in all other lanes derive from the same fraction. After the specified pretreatment, all digests were incubated with UDP[^{14}C]glucose and freeze-dried before SDS-PAGE. (Portions of all these digests were also assayed for ^{14}C-labelled, TCA-precipitable protein. Some of the results are quoted in the text.) Lanes 3 and 5, after prior incubation for 1 h and 3 h at 37°C, respectively. Lanes 4 and 6, as 3 and 5 but with added protease inhibitors (see section 2). Lanes 7–10 correspond to lanes 3–6 except that acarbose (5 μg/ml) was added to the incubated fraction. Lanes 11–13 represent the fraction of lane 2 incubated for 10 min at 37°C with increasing quantities of salivary α-amylase (see section 2). The control for lanes 11–13, i.e. absence of α-amylase, is not shown but was identical with lane 2. This figure is a composite photograph from four separate radioautographs (1–2, 3–6, 7–10, 11–13) lined up by means of the protein standards.

Measured as in Fig. 1, the activity was 12 000–14 000 cpm, a 20-fold increase. Evidently there is a low molecular weight inhibitor of, or competitor for, protein glucosylation in muscle and this is removed on G-25. The 'activation' phenomenon seen on incubation of an extract might instead represent the disappearance of the inhibitor/competitor.

When the protein was glucosylated immediately after gel filtration of a fresh extract, and then subjected to SDS-PAGE, yet another pattern of glucosylation was seen (Fig. 3, lane 2). The radiolabelled protein now heardly penetrated the running gel and, very approximately, had M_r 400 000 (p400). This occurred whether or not acarbose was present. It was after incubation of these filtered extracts that the patterns previously seen were restored (Fig. 2). If the extract was incubated without additives, breakdown with the formation of p42 and a barely visible amount of SGP occurred (lanes 3,5) and the activity fell to 7500 cpm after 3 h. In the presence of acarbose, breakdown took place, but more slowly. The products had a higher M_r than in the absence of acarbose and p42/SGP were absent (lanes 7,9). The activity increased to 29 000 cpm after 3 h.

It is instructive also to compare lanes 2 in Figs 2 and 3. Both refer to unincubated fresh muscle extracts. In Fig. 2, lane 2 no glucosylated protein is seen, corresponding to the very small degree of protein glucosylation measured by scintillation counting (Fig. 1, zero time). By contrast, Fig. 3, lane 2 corresponds to the same extract as Fig. 2, lane 2, but this had been gel-filtered before incubation with UDP[^{14}C]glucose. The p400 was now de-inhibited and could be glucosylated. A strong band of [^{14}C]glucosylated protein is seen at the top of the gel.

We deduce from the results seen in Figs 2 and 3 that the glucosylated species of M_r approx. 40 000 that we, Rodriguez and Fliesler [14] and Cohen et al. have previously reported as being involved in priming glycogen synthesis is not initially present in a muscle extract. This seems clear from Fig. 3 in which only glucosylated protein of high M_r is seen in a fresh, gel-filtered muscle extract (lane 2). The products of lower M_r are seen only when the extract is incubated under conditions that allow endogenous hydrolases to act on the high M_r form.

The high M_r glucosylated protein is already familiar to us. We have reported elsewhere that differentiated adipocytes and a rat mammary adenocarcinoma each contain a protein (or proteins) of M_r about 400 000, similar to that now seen in muscle [15]. Like muscle SGP, the M_r 400 000 protein already contains bound carbohydrate and undergoes glucosylation under the

same conditions as SGP, forming maltosaccharide chains up to DP 14 [15]. This was seen in unfiltered extracts. Evidently there is no (or less) low-molecular-weight inhibitor of protein glucosylation in the adipocyte or adenocarcinoma than in muscle, and less of the enzyme(s) that convert p400 into smaller products.

3.2. *The nature of the glycogen primer*

We are presented with an apparent paradox in that the protein in muscle glycogen is the M_r 37000 glycogenin [1]. A glycogen-free, self-glucosylating protein (SGP), seemingly identical, is present in muscle and acts as a primer for glycogen synthesis in vitro [5,9]. But now it appears that the latter is a breakdown product of a much higher molecular weight species (p400). What is the structural relation between p400, SGP and glycogenin from glycogen?

The answer to this question was partly revealed as the result of attempts to accelerate or arrest the breakdown of p400. We decided to fortify the gel-filtered muscle extract with α-amylase, or trypsin or proteolytic inhibitors and learn the results of incubation. The protease inhibitors were the same as we use when extracting muscle to prepare SGP [5] but which are removed in subsequent steps, as there were here, by salt precipitation and gel filtration. α-Amylase had a dramatic effect, rapidly causing the formation of p42 and SGP with the former predominating at the lower concentration of enzyme and the latter at the higher concentration (Fig. 3, lanes 11–13). Therefore, we might conclude that p400 is simply SGP protein (glycogenin) carrying a large amount of glycogen-like carbohydrate. The addition of trypsin (results not shown) did not materially change the pattern of conversion of p400 into p42/SGP, except that at a high trypsin concentration protein glycosylation was not seen.

The addition of protease inhibitors, however, essentially blocked the breakdown of p400 (Fig. 3, lanes 4,6,8,10), and the activity was practically unchanged at about 10000 cpm during 3 h of incubation whether acarbose was absent (lanes 4,6) or present (lanes 8,10). Therefore we must conclude that not only amylolysis but also proteolysis is necessary to cause the breakdown of p400, i.e. p400 contains more carbohydrate and more protein than does p42 or SGP. The latter conclusion depends for its validity on the specificity of the protease inhibitors only to inhibit proteases. The effect of acarbose in arresting the breakdown of p400 at an intermediate step (Fig. 2) suggests that at least two endogenous glycosidases are involved in the conversion seen in vitro and that acarbose inhibits one of them.

We also tested the effect of α-amylase on a filtered extract in the presence of protease inhibitors. The results were similar to those seen in the absence of the inhibitors (Fig. 3, lanes 11–13), except that at the lowest concentration of α-amylase (0.95 μg/ml) the glucosylating activity fell in 10–30 min to less than 10% of the original (it fell to 40% without protease inhibitors) and no [^{14}C]glucosylated protein could be seen by SDS-PAGE until after 2–3 h, by which time the activity had increased to 20% of the original and bands were seen in the region M_r 50000–80000. At the higher α-amylase concentrations, glucosylated proteins with M_r values near to SGP and p42 were seen at all times and the glucosylating activity fell to and remained at about 20% of the original.

We now intend to learn the structure of p400. In terms of protein content, a clue comes from the report by Campbell and Cohen [10] that glycogenin from muscle glycogen has a blocked N-terminus. We can now report the same observation and, in addition, that SGP also has a blocked N-terminus. Therefore, if p400 contains a larger protein moiety than SGP the extension is likely to be at the C-terminus.

Recognizing that p42 is a species distinct from SGP we have looked for both forms during the purification of SGP and find that both co-purify and are separated only in the final HPLC purification step [5]. By this means we obtained homogeneous p42 and, in an experiment not shown, could convert it into a species having the same M_r as SGP with the use of α-amylase, similar to the disappearance of p42 and the appearance of SGP seen in the gel-filtered muscle extract when α-amylase was added (Fig. 3, lanes 11–13). It seems that in the vicinity of the protein the last few residues of carbohydrate are less easily removed. (The homogeneous p42 underwent glucosylation when incubated with UDP-glucose and therefore can also be assumed to be autocatalytic.)

Our conclusions regarding the nature of the primer for glycogen synthesis need to be revised. What we have now detected in muscle in the form of p400 is a quite different glycoprotein than has previously been described. It appears to have a somewhat larger protein moeity and a much higher content of carbohydrate. SGP, in the form that we have previously isolated it, results from the breakdown of p400 during the purification process. We have still not detected a carbohydrate-free species of SGP, nor do we know how the initial glucosylation of the protein occurs, only that as isolated, in the form of SGP, the protein is autocatalytic and adds further glucose to itself. How such glucosylation participates in the formation of p400 remains to be elucidated.

Acknowledgements: This work was supported by grants from the National Institutes of Health (DK37500) and from the Florida Affiliate, American Heart Association.

REFERENCES

[1] Kennedy, L.D., Kirkman, B.R., Lomako, J., Rodriguez, I.R. and Whelan, W.J. (1985) in: Membranes and Muscle (Berman M.C., Gevers, W. and Opie, L.H. eds) pp. 65–84, IRL Press, Oxford.

[2] Rodriguez, I.R. and Whelan, W.J. (1985) Biochem. Biophys. Res. Commun. 132, 829–836.
[3] Rodriguez, I.R., Tandecarz, J.S., Kirkman, B.R. and Whelan, W.J. (1986) ICSU Short Rep. 4, 96–99.
[4] Kay, M.J., Kirkman, B.R., Lomako, J., Rodriguez, J.R., Tandecarz, J.S., Fliesler, S.J. and Whelan, W.J. (1987) Fed. Proc. 46, 2151.
[5] Lomako, J., Lomako, W.M. and Whelan, W.J. (1988) FASEB J. 2, 3097–3103.
[6] Lomako, J., Lomako, W.M. and Whelan, W.J. (1990) Biochem. Int. (in press).
[7] Pitcher, J., Smythe, C., Campbell, D.G. and Cohen, P. (1987) Eur. J. Biochem. 169, 497–502.
[8] Smythe, C., Caudwell, F.B., Ferguson, M. and Cohen, P. (1988) EMBO J. 7, 2681–2686.
[9] Pitcher, J., Smythe, C. and Cohen, P. (1988) Eur. J. Biochem. 176, 391–395.
[10] Campbell, D.G. and Cohen, P. (1989) Eur. J. Biochem. 185, 119–125.
[11] Fischer, E.H. and Stein, E.A. (1961) Biochem. Prep. 8, 27–33.
[12] Lomako, J., Lomako, W.M. and Whelan, W.J. (1990) BioFactors, (in press).
[13] Truscheit, E., Frommer, W., Junge, B., Muller, L., Schmidt, D.D. and Wingende, W. (1981) Angew. Chem. 20, 744–761.
[14] Rodriguez, I.R. and Fliesler, S.J. (1988) Arch. Biochem. Biophys. 260, 628–637.
[15] Lomako, J., Lomako, W.M. and Whelan, W.J. (1990) Biochem. Int. (in press).