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Proglycogen: a low-molecular-weight form of muscle glycogen

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

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

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We recently reported that muscle contains a trichloroacetic acid-precipitable component having M_r approx. 400 kDa that can be glucosylated by an endogenous enzyme acting on UDPglucose. This component contains within itself the autocatalytic, self-glucosylating protein glycogenin, the primer for glycogen synthesis. We now report that this substance, to which we give the name proglycogen, is a glycogen-like molecule constituting about 15% of total glycogen. It acts as a very efficient acceptor of glucose residues added from UDPglucose. Further, that the endogenous enzyme that adds the glucose to proglycogen is not the autocatalytic protein but a glycogen synthase-like enzyme. Proglycogen may be an intermediate in the synthesis and degradation of macromolecular glycogen and may exist and be metabolized as a separate entity. Consideration should now be given to the revival of the concept that tissue contains two forms of glycogen. One is proglycogen. The other is the 'classical', macromolecular glycogen. Additionally, proglycogen and glycogen may be glucosylated by different forms of synthase.

Glycogenin; Glycogen biogenesis; Protein glucosylation; Proglycogen

1. INTRODUCTION

Rabbit-muscle glycogen contains one molecular proportion of a covalently bound protein, which we have named glycogenin [1]. This has M_r 37 kDa and is linked to glycogen via the novel glucosyl-tyrosine bond [2,3]. Glycogenin appears to be the protein that Krisman and Barengo [4] suggested is the primer for glycogen synthesis. In addition to finding glycogenin as a covalently bound component of glycogen, we were able to identify, in muscle extracts, a glycogen-free form of glycogenin [5] which on purification to homogeneity proved to be an autocatalytic protein [6,7]. It already contains two covalently bound glucose residues, as maltose. Autocatalysis consists in lengthening the maltose residues to malto-octaose using UDPglucose as substrate [8,9]. At this point, glycogen synthase and branching enzyme can extend and branch the malto-octaose to form glycogen still containing bound glycogenin [6].

Despite our having isolated the glycogenin-like, self-glucosylating protein (SGP) from rabbit muscle, Smythe et al. [10] and we [11] recently reported that SGP as such does not exist in a fresh muscle extract. We had noted that if protein glucosylating activity was assayed immediately on preparation of a muscle extract, essentially none could be detected. This proved to be due to the presence of an endogenous, so-far-unidentified low-molecular-weight inhibitor which can be removed by gel filtration. The filtered extract readily undergoes protein glucosylation but SDS-PAGE and radioautography revealed that the [^{14}C]glucosylated protein (obtained by trichloroacetic acid (TCA) precipitation) is not SGP but an entity of much higher M_r very approximately 400 kDa (p400). When such an extract was stored before glucosylation was attempted, endogenous enzyme(s) brought about the breakdown of p400. Incubation of the extract with UDP[^{14}C]glucose revealed a series of glucoproteins with M_r values descending down to that of SGP, i.e. 37 kDa (p37). The addition of α-amylase accelerated the conversion of p400 into p37. The inclusion of acarbose, an α-glucosidase/α-amylase inhibitor [12], retarded the breakdown and largely prevented the formation of p37 and an associated, more prominent species, p42. Instead, there accumulated a group of sharply defined glucoproteins, with M_r values in the range 50–80 kDa (p50–p80). Protease inhibitors also retarded the breakdown of p400, suggesting that both amylolysis and proteolysis were involved in the breakdown of p400. Even before we had seen p400 in muscle extracts, we had seen it in extracts of adipocytes and a rat mammary tumor [13] but did not understand the connection to SGP (p37) because in these two sources the p400 was not inhibited and we did not encounter breakdown of p400 to p37.

This new report takes up from these observations and describes some properties of p400 and the manner of its glucosylation.

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

2. MATERIALS AND METHODS

2.1. *Materials*

All biochemicals were from Sigma or Fischer Scientific Co., unless otherwise stated. UDP[^{14}C]glucose was from ICN Biochemicals. Crystalline isoamylase and 'amylose EX-1' were gifts from Hayashibara Biochemical Laboratories, Hiroshima, Japan. UDP-pyridoxal was the gift of Dr T. Fukui, Osaka, Japan. Aluminum sheet Kieselgel 60 for thin-layer chromatography was from Merck. Reagents for SDS-PAGE were from BioRad. Extract-Gel D was from Pierce. Amplify was from Amersham.

2.2. *Methods*

Protein was measured as by Bradford [14]. Gel-filtered extracts of rabbit skeletal muscle were prepared as by Lomako et al. [11] and [^{14}C]glucosylation of proteins in the extracts carried out as by the same authors. SDS-PAGE analysis was performed with a Mini-Protan II electrophoresis system (BioRad) in 10% acrylamide according to Laemmli [15] followed by fluorography with Kodak X-Omat film at −70°C and development in a Kodak X-Omat film processor. For isoamylase digestion, [^{14}C]glucosylated p400 (80×10^3 counts/min) was precipitated with an equal volume of cold 20% trichloroacetic acid (TCA). The pellet was washed twice with cold 10% TCA and the excess TCA removed with ether. The protein was suspended in 100 mM ammonium bicarbonate containing 6% SDS and heated at 100°C for 3 min. After cooling to room temperature, the sample was applied to a column of Extracti-Gel D equilibrated with 100 mM ammonium bicarbonate. The column was eluted with the same solution. Fractions containing ^{14}C were collected and freeze-dried. The sample was solubilised in 100 μl of 100 mM sodium acetate buffer, pH 3.5, containing isoamylase (10 μg) and digested overnight at room temperature under a layer of toluene. It was deionised on a column of RG501-X8 mixed-bed ion-exchange resin and subjected to thin-layer chromatography in butanol/water/ethanol (5:4:5). The self-glucosylating protein referred to as p42 was isolated during purification of SGP (p37). It was obtained as an electrophoretically homogeneous product in the fractions emerging from the MonoQ column after the SGP had been eluted [6].

3. RESULTS AND DISCUSSION

3.1. *p400 is glucosylated by a synthase-like enzyme*

Using gel-filtered muscle extracts, assayed for protein glucosylating activity either immediately, or after incubation alone, or in presence of various activators and inhibitors, we have been able to distinguish between the types of glucosylated protein previously encountered in muscle. We treated muscle extracts in such a way as to display all forms of glucosylated protein between p37 and p400 [11]. These were incubated with UDP[^{14}C]glucose and various combinations of Mn^{2+}, glucose 6-phosphate and UDPpyridoxal. The amounts of TCA-precipitable ^{14}C-labelled protein were measured. Then the digests were subjected to SDS-PAGE and radioautography. Mn^{2+} was added as an activator of SGP [6], glucose 6-phosphate to activate synthase and UDPpyridoxal to inhibit it [16].

Table I shows that in a fresh gel-filtered muscle extract there is a powerful protein glucosylating activity that is not stimulated by Mn^{2+} but is markedly stimulated by glucose 6-phosphate and markedly inhibited by UDPpyridoxal. This activity is therefore like that of glycogen synthase and Fig. 1 shows that the substrate undergoing labelling in the fresh extract is p400. After such an extract has been preincubated for 120 min the protein glucosylation is of a quite different nature. p400 has disappeared. In its place are the species p37, p42 and p50-p80. The glucosylation of p37 and p42 is completely dependent on the presence of Mn^{2+}. Compare lane 6 (Mn^{2+} present) with lane 7 (Mn^{2+} absent). The glucosylation of p50-p80 is strongly activated by glucose 6-phosphate. Compare lane 7 (G6P present) with lane 6 (G6P absent). The glucosylation of p400 and p50-p80, but not of p37 and p42, is inhibited by UDP-pyridoxal. Compare lanes 5 and 8 (UDPpyridoxal present) with lanes 2–4 and 7 (UDP pyridoxal absent). In the presence of UDPpyridoxal and the absence of Mn^{2+}, little glucosylation occurs (lane 9).

What these results demonstrate is that at least two types of enzyme are at work in catalysing the glucosylation of the p37-p400 family of glucoproteins. One type carries out autocatalysis of p37 and p42 (see below). The other is a glycogen-synthase-like enzyme that glucosylates all larger species up to p400.

It is noteworthy, however, that the concentration of UDPglucose at which these reactions were carried out was 2.2 μM. The K_m of glycogen synthase for UDP-glucose using glycogen as a primer is around 5 mM. It has been by taking advantage of the fact that protein glucosylation occurs at a UDPglucose concentration three orders of magnitude lower than the synthase K_m for glycogen as the primer, that has made possible the selective detection of glucosylation of protein-bound carbohydrate in crude extracts. Macromolecular glycogen does not compete as a primer under these conditions (see below).

Table I

Protein glucosylation in muscle extracts[a]

	[^{14}C]glucose in TCA-insoluble precipitate (counts/min)			
Additions to digest:	None	Mn^{2+}	G6P	UDPpyridoxal
	19264	17854	36364	2798

[a]The fresh Sephadex G-25-filtered muscle extract was incubated for 5 min at room temperature with 2.2 μM UDP[^{14}C]glucose in the absence or presence of 5 mM $MnCl_2$, or 9 mM glucose-6-phosphate or 24 μM UDPpyridoxal, followed by addition of an equal vol of cold 20% TCA and scintillation counting of the precipitate. The precipitates were also subjected to SDS-PAGE and radioautography (Fig. 1). Similar experiments were carried out after a 2 h preincubation, followed by labelling with UDPglucose, when the digests were again subjected to SDS-PAGE (Fig. 1). In all, six types of experiments were performed (± Mn^{2+}, +G6P ± Mn^{2+}; +UDPpyridoxal ± Mn^{2+}). Protein glucosylation was measured under these conditions in a fresh extract and after the extract had been preincubated for 30 and 120 min. The incubations with UDP[^{14}C]glucose were carried out for 5 min (results shown above), 20 min and 60 min some 54 measurements in all. Each digest was subjected to SDS-PAGE and radioautography. It was by comparing all the numerical data and all the radioautographs that we were able to interpret the changes taking place. The results shown here and in Fig. 1 were chosen to illustrate these conclusions.

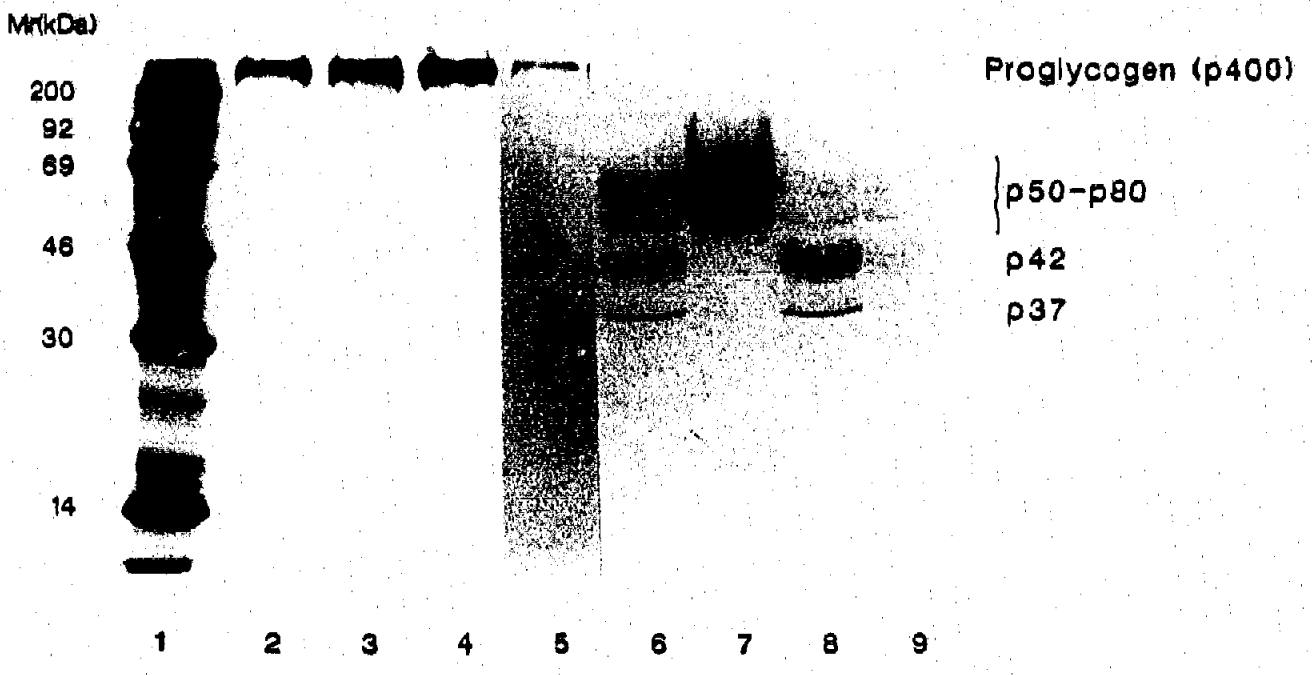

Fig. 1. A composite of radioautographs from muscle extracts incubated with UDP[^{14}C]glucose and subjected to SDS-PAGE. The comparison is between fresh, gel-filtered extracts (lanes 2–5) and extracts incubated for 2 h at room temperature (lanes 6–9) before incubating with substrate. Lane 1 is the radioactive M_r markers. Lane 2 is without any activator or inhibitor, lanes 3, 6, + Mn^{2+}; lanes 4, 7, + G6P; lanes 5, 9 + UDPpyridoxal; lane 8, + UDPpyridoxal, + Mn^{2+}. Conditions are as in Table 1 and lanes 2–5 correspond to the same experiments as in Table 1.

These analyses permit the following conclusions. First of all, although protein glucosylation occurs in all extracts and under all conditions, admittedly over a wide range of activity, it appears to be due not to a single entity but to at least two entities.

One of these, the activity in the fresh muscle extract, is a glycogen synthase-like activity characterized as such by its being stimulated by glucose 6-phosphate and inhibited by UDPpyridoxal.

The second glucosylating activity is that of the self-glucosylating protein, apparently in two forms, that which we have already isolated and which has M_r 37 kDa [6], the second form having M_r 42 kDa. Reference to Fig. 1 shows p37 as a very sharp band, indicating a species of uniform molecular weight. Since p37 is generated from p400 as a result of the action of endogenous hydrolyases [11], we may suppose the entity seen in Fig. 1 to represent the protein substituted by the minimum number of glucose residues required for it to be an autocatalytic entity, which we believe to be 2 or even 1 glucose residue [8,9].

It is interesting to compare p37 and p42. The latter, we have reported, can be converted into p37 by α-amylolysis [11] and therefore appears to differ in the amount of carbohydrate present. The fact that p42 is a diffuse band suggests that it is the same protein species was p37, but that the lengths of the carbohydrate chains as between different molecules of p42 are variable.

It is clear that p37 and p42 are totally dependent on manganese for their ability to become glucosylated. In the case of p37 this is self-catalysed. We have now isolated p42 as a homogeneous species. This too undergoes Mn^{2+}-dependent glucosylation when UDP-glucose is added and, therefore, may also be presumed to be autocatalytic.

The glucosylation of proteins p50-p80 is not Mn^{2+}-dependent; it is glucose 6-phosphate dependent. Therefore, we can tentatively conclude that the same type of glucose 6-phosphate-activated glycogen synthase as is responsible for the glucosylation of p400 (Table I) is also involved in glucosylating the p50-p80 species.

3.2. *The nature of p400*

We must now consider what is the nature of p400. Given that it is rapidly converted into p37 and p42 by added α-amylase [11], we can conclude that it differs from p37 mainly in containing more carbohydrate. That protease inhibitors retard the conversion of p400 into p37 [11] might also lead one to conclude that p400 contains a larger protein moiety than p37. We have pointed out [11] that this conclusion depends on the specificity or otherwise on the protease inhibitors only to inhibit proteases. For the purposes of this discussion we can make the assumption that the major if not the only difference between p37 and p400 is one of carbohydrate content.

p400 is glycogen-like but is precipitable by 10% TCA. This is the basis of the assay for protein glucosylation. This distinguishes p400 from glycogen,

most of which is soluble in TCA [1]. We have examined digests in which p400 was glucosylated in 2.2 μM UDP-glucose for the presence of TCA-soluble, ethanol-precipitable species labeled with [^{14}C]glucose, i.e. corresponding to macromolecular glycogen. We have added glycogen to such digests to 5% concentration. We find that relatively little label is incorporated either into endogenous or added glycogen at the 2.2 μM concentration of UDPglucose employed here. If, however, the UDPglucose concentration is raised to 5 mM, then p400 is converted into a labelled, TCA-soluble product conforming to the above definition of glycogen. We have developed the following hypothesis to explain the state of glycogen and its method of biogenesis in muscle.

In order to characterize p400 we arranged its [^{14}C]glucosylation in a muscle extract. Then we debranched it with isoamylase, which carries out the total debranching of glycogen [17]. The products were examined by thin-layer chromatography. The radioautograph seen in Fig. 2 displays a profile of unit chains in the range DP 4-22, similar to normal glycogen [17]. The ability of α-amylase and isoamylase to hydrolyze p400, coupled with the unit-chain profile, indicates that p400 is structurally very similar to glycogen.

It is clear from the present and previously published results [10,11] that SGP, which is the autocatalytic form of glycogenin, does not exist in the free state in a muscle extract. Instead it is present in p400 and in glycogen. That both entities appear to exist side-by-side in muscle can be shown by the simple device of adding TCA to 10% concentration in a fresh muscle extract. Some 15% of the carbohydrate is precipitated. We recall the concept of lyo- and desmo-glycogens introduced by Willstätter [18] whose hypothesis was that glycogen exists in protein-free (lyo) and protein-bound (desmo) forms, separable by solubility in TCA. This concept was extensively worked on in the 1950s [19], but, following the appearance of a report that the TCA fractionation procedure gave variable results [20], work on this topic largely ceased.

What the new results reported here appear to be pointing to, as a possibility, is that there are indeed two forms of glycogen, differing not in the presence or absence of protein, but in the proportion of protein relative to carbohydrate. One is the classical macromolecular glycogen, containing 0.35% protein (glycogenin [1]). The other is a glycogen in which the protein content (again, glycogenin) may be as high as 10%, and which in consequence is precipitable with TCA. Considering that p400 can be converted into glycogen in vitro, it merits the designation proglycogen. If its M_r is around 400 kDa then, at 15% of the total carbohydrate, there are about 5 mol of proglycogen per mol of glycogen (M_r 10^7 Da [1]).

Consideration should therefore be given as to whether there exist two forms of glycogen synthase that

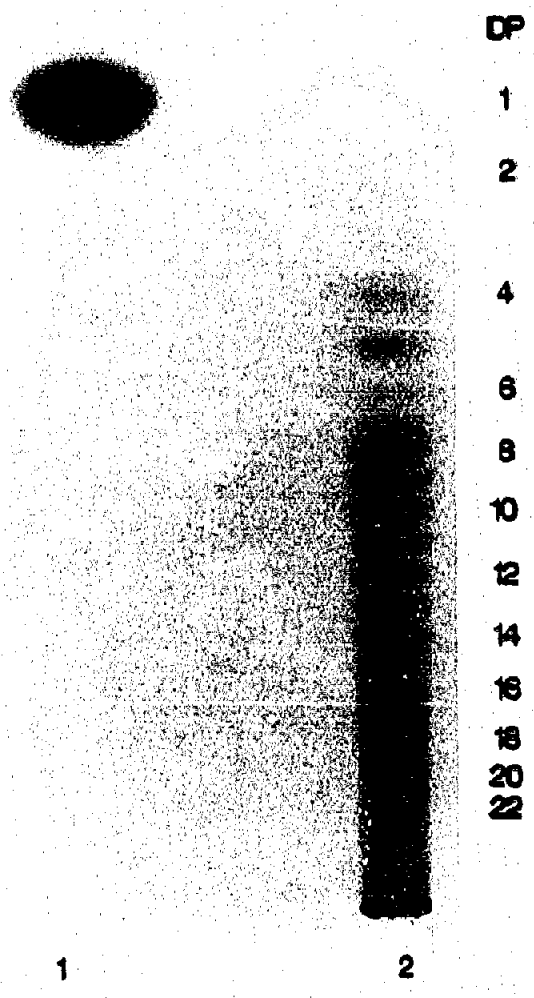

Fig. 2. Showing the [^{14}C]glucosylated maltosaccharide chains released from [^{14}C]glucosylated muscle proglycogen (p400) on debranching with isoamylase. The products were subjected to ascending thin-layer chromatography (lane 2). Lane 1 is [^{14}C]glucose. The degrees of polymerisation (DP) were assigned by comparative cochromatography of amylose EX-1, a mixture of glucose and maltosaccharides, detected by spraying with sulfuric acid.

give rise to proglycogen from SGP and to glycogen from proglycogen. Our structural analysis of proglycogen shows it to resemble glycogen, yet it is preferentially glucosylated in presence of glycogen. Perhaps synthases differing in the pattern of phosphorylation differ in their affinity for the two types of primer and this is one reason for the multi-site phosphorylation of synthase.

What also emerges from these observations is that if there are two forms of glycogen, whether interconvertible or not, measurements of tissue glycogen represent averages of the two entities. It is now necessary to devise separate assays for glycogen and proglycogen. When such separate assays are carried out it may prove that one form of glycogen will be seen to be selectively responsive to factors that cause glycogen stores to

change in amount, e.g. hormones. This, of course, in the case of lyo- and desmo-glycogens, is what had been recorded on numerous occasions [19] and some of those experiments should perhaps be revisited.

Recently we demonstrated a marked increase in the incorporation of glucose into TCA-precipitable material in extracts of 3T3 adipocytes which had been bathed in insulin [13]. We now realize that this was probably due to an increase in activity of the synthase which preferentially glucosylates p400. Therefore, if we no longer treat glycogen as if it is a single entity, or glycogen synthase as if it has only one substrate, we may clarify aspects of insulin's effects on glycogen synthesis which are still puzzling, such as fractional rather than integral changes in the stoichiometry of the phosphorylation of glycogen synthase [21]. Fractional changes may represent averages.

We should also comment on a distinctive property of the products of partial breakdown of p400. These are seen in Fig. 1 as [^{14}C]glucosylated TCA-precipitable entities with M_r values lying between that of p400 and the autocatalytic entities p37 and p42. The experimental evidence suggests that the intermediates represent products of the amylolysis of p400 such as might be formed by endogenous muscle α-amylase. Such degradation might be expected to yield a continuum of products in terms of M_r. Yet the bands are well-defined. Other examples are to be seen in Fig. 3 of [11]. This behavior suggests that the structure of p400 is such as to give rise to fragments of discrete size during amylolytic breakdown.

French [22] observed that starch molecules slowly break down into discrete entities when ungelatinized granules are immersed in cold, dilute acid. This led him to formulate the 'cluster' hypothesis whereby the amylopectin molecule is considered to grow as clusters of branched chains in which one chain is preferentially lengthened to become the basis for a succeeding cluster. Between clusters, the interlinking chain presents a point of vulnerability to enzyme attack. The cluster hypothesis is now well accepted as explaining amylopectin structure [23]. French [24] also studied α-amylase action on glycogen and concluded that 'there are at least several tens of densely branched regions per molecule which are connected by less densely branched, easily hydrolysed segments'. Our results are compatible with this conclusion and have, in addition, allowed the visualization of some of the discrete entities predicted from these conclusions. Support for glycogen structure, like that of amylopectin, also being based on clusters is thereby afforded.

Finally, we comment on recent reports by Miozzo et al. [25,26] who have studied a membrane-bound 'glycogen precursor' from retina which bears some resemblance to proglycogen since its claimed M_r is 4.7 $\times$ 10^5 and it contains an M_r 42 kDa protein, said to be similar to glycogenin. However, the protein content is only 3% which, if the protein is in covalent linkage, would suggest an M_r of 1.4 $\times$ 10^6. Additionally, although the precursor was initially TCA-insoluble, it became TCA-soluble after treatment with hot detergent. We tested the behavior of proglycogen under the same conditions and 85% remained TCA-insoluble. A further difference from our own results was that glucosylation of the retinal microsomal fractions, using UDPglucose, was accomplished by an endogenous enzyme without the apparent requirement for glucose 6-phosphate. Our endogenous, glycogen synthase-like activity is powerfully dependent on this cofactor (Table I, Fig. 1). Insufficient information therefore exists to permit a comparison of the retina 'glycogen precursor' with muscle proglycogen.

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