MULTIPLE PHOSPHORYLATION OF RABBIT MUSCLE GLYCOGEN SYNTHASE BY GLYCOGEN SYNTHASE KINASE-1

Relationship between phosphorylation state and kinetic properties

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1. Introduction

Glycogen synthase from skeletal muscle is regulated by phosphorylation and dephosphorylation [1]. It is generally accepted that the subunit of glycogen synthase can be multiply phosphorylated, as originally proposed [2,3]. The number of phosphorylated sites reported by different investigators ranges from 3–6/85 000 dalton subunit. Several protein kinases are able to phosphorylate this enzyme. Two phosphates can be introduced by the catalytic subunit of the cAMP-dependent protein kinase [4]. Values of 0.5–4 phosphates have been reported for different cAMP-independent protein kinases [5–9].

Conversion of glycogen synthase from the I into the D form has usually been determined by measuring changes in the

–glucose 6-phosphate/+ glucose 6-phosphate (-G6P/+G6P) activity ratio

(or %I activity when expressed as percentage)

By using this type of assay it has been concluded that some of the sites of phosphorylation determine predominantly the conversion into the D form (i.e., their phosphorylation drastically reduces the –G6P/+G6P activity ratio) while other sites are non-specific (i.e., their phosphorylation is not associated with changes in the –G6P/+G6P activity ratio) [4,10]. However, a continuous variation of the enzyme kinetic parameters with phosphorylation state was found [11] when analyzing the properties of a series of glycogen synthase samples of different phosphate content. Phosphorylation of these samples was achieved by unidentified endogenous protein kinases at an intermediate step of the purification process.

Here we have used purified glycogen synthase kinase-1 (GSK-1), a cyclic AMP-independent kinase known to be able to introduce $\leqslant 4$ phosphates/85 000 dalton subunit, which results in a total conversion into the G6P-dependent form [6,12]. Our results show that the kinetic properties of glycogen synthase, when phosphorylated with that single kinase, varied monotonically with the phosphate content. These results indicate that none of the phosphates introduced by GSK-1 is non-specific and that the introduction of the third and fourth phosphates, which could seem irrelevant as it produces only small changes in the –G6P/+G6P activity ratio, provokes, in fact, the highest changes in the properties of the enzyme.

2. Methods

2.1. *Purification and assay of glycogen synthase*

Glycogen synthase I was purified by the method in [13], as modified [14], using only fresh rabbit muscle. The method in [15] with 100–200 cpm/nmol UDP-[U-^{14}C]glucose was used as a standard assay for glycogen synthase activity. –G6P/+G6P activity ratio was calculated from the activities measured in the absence of glucose 6-phosphate and in the presence of 6.6 mM glucose 6-phosphate. For the study of the

dependence of reaction rate on UDP-glucose the following conditions were used. The reaction mixture (100 μl) contained 50 mM Tris–HCl (pH 7.8) 12.5 mM EDTA, 1 mM EGTA, 10 mM mercaptoethanol, 7 mg/ml rabbit liver glycogen, UDP-[U-^{14}C]glucose as indicated and 50–100 ng purified glycogen synthase. The specific activity of UDP-glucose was varied according to the reaction conditions and ≤50 000 cpm/nmol were used at low UDP-glucose concentrations. For study of the activation by glucose 6-phosphate, UDP-[U-^{14}C]glucose was present at 200 μM final conc. (spec. radioact. 7000 cpm/nmol). The reaction mixture also contained 50 mM Tris–HCl (pH 7.8), 12.5 mM EDTA, 1 mM EGTA, 10 mM mercaptoethanol, 7 mg/ml liver glycogen and 50–100 ng of purified glycogen synthase.

In all cases blanks were run in order to allow for the small ^{32}P radioactivity from the ^{32}P-labelled enzyme present in the filter paper which could interfere with the measurement of the [^{14}C]glycogen formed by the synthase.

Other details of the assay have been described in [16]. A unit of enzyme activity corresponds to the incorporation of 1 μmol glucose from UDP-glucose into glycogen/min in the standard assay.

2.2. *Purification of cyclic AMP-independent glycogen synthase kinase-1 (GSK-1)*

Cyclic AMP-independent glycogen synthase kinase-1 obtained as in [6] was further purified by the method combining chromatography on phosphocellulose and casein–Sepharose 4B described in [17] for the rat liver enzyme. The final enzyme preparation had spec. act 25 units/mg protein. Kinase activity was assayed at 30°C as in [6]. One unit of kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 nmol ^{32}P from [γ-^{32}P]ATP to casein per minute under the standard assay conditions.

2.3. *Glycogen synthase phosphorylation by cyclic AMP-independent glycogen synthase kinase-1 (GSK-1)*

Phosphorylation was carried out at 30°C. The reaction mixture (0.2 ml) contained 25 mM β-glycerolphosphate (pH 7.0), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 10 mM Mg^{2+}, 0.125 mM [γ-^{32}P]-ATP (300–400 cpm/pmol), 120–160 μg/ml glycogen synthase I and 0.4 units/ml kinase. At the indicated times, 20 μl aliquots were removed and assayed for ^{32}P incorporation or diluted with 50 mM Tris–HCl (pH 7.8) and 12 mM EDTA, and assayed for glycogen synthase activity. The amount of ^{32}P incorporated into protein was measured after separation from unreacted [γ-^{32}P]ATP by 'ITLC' chromatography according to [18].

2.4. *Other methods*

UDP-[U-^{14}C]glucose was prepared by the enzymic conversion of [U-^{14}C]glucose (Amersham) into UDP-glucose as in [15]. [γ-^{32}P]ATP was prepared as in [19]. Casein coupling to Sepharose 4B was carried out according to [20]. Protein was determined as in [21]. SDS–Polyacrylamide gel electrophoresis was as in [22].

3. Results

3.1. *Characterization of purified glycogen synthase I*

After SDS–polyacrylamide gel electrophoresis, one main band corresponding to the 85 000 dalton subunit was observed. A band corresponding to a proteolytic breakdown product [14] was only faintly visible.

The specific activity of the samples, as determined by the standard assay with saturating G6P, was 34 ± 3 U/mg protein. The –G6P/+G6P activity ratio was 0.82 ± 0.05. The $S_{0.5}$ value for UDP-glucose and the $M_{0.5}$ value for G6P calculated from Hill plots were 1.5 ± 0.5 mM and 12 ± 5 μM, respectively.

Glycogen synthase preparations were not contaminated with protein kinase activities as they did not incorporate any significant amount of ^{32}P when incubated under the conditions in section 2, in the absence of exogenous protein kinases, whether in the absence or in the presence (10^{-5} M) of cAMP.

3.2. *Phosphorylation and conversion of glycogen synthase with GSK-1*

Incubation of glycogen synthase with GSK-1 under standard phosphorylation conditions resulted in a progressive incorporation of ^{32}P into the synthase. Maximal phosphorylation averaged 4 mol phosphate/mol subunit. SDS–gel electrophoresis after incubation showed that all the ^{32}P incorporated was recovered in the position corresponding to the subunit of glycogen synthase. The rate of phosphorylation of glycogen

synthase by GSK-1 was more rapid for the introduction of the first two phosphates (~1 P/subunit per 15 min) than for the incorporation of the other two (~1 P/subunit per 30 min). The –G6P/+G6P activity ratio decreased throughout the incubation period whereas total glycogen synthase activity (+G6P) remained unchanged (fig.1A).

When the changes in the –G6P/+G6P activity ratio were plotted against the number of phosphates incorporated, it could be clearly observed that the introduction of the first two phosphates drastically decreased the –G6P/+G6P activity ratio (from 0.82–0.20) while the subsequent incorporation of 2 additional mol ^{32}P only slightly decreased this ratio (from 0.20–0.08) (fig.1B).

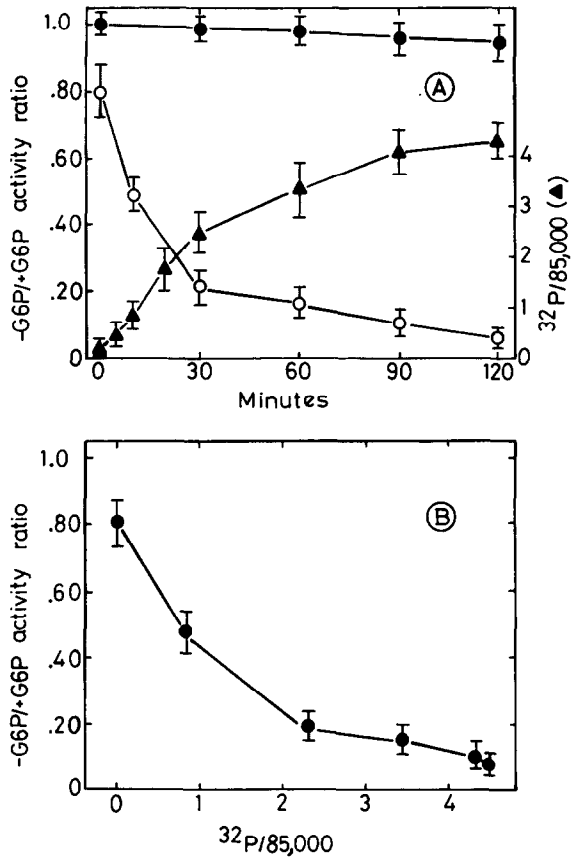

Fig.1.(A) Time-course of the phosphorylation of glycogen synthase by GSK-1. The reaction was carried out under standard assay conditions. Symbols are: (○) –G6P/+G6P activity ratio; (▲) ^{32}P incorporated; (●) activity +G6P/initial activity +G6P. (B) Data from fig.1A were replotted to show the relationship between the –G6P/+G6P activity ratio and the ^{32}P incorporated per subunit.

3.3. *Phosphorylation and kinetic behavior of glycogen synthase*

Kinetic properties ($S_{0.5}$ for UDP glucose and $M_{0.5}$ for G6P) were determined for glycogen synthase phosphorylated to different degrees by GSK-1. There was a slow phosphorylation-dependent increase in the $S_{0.5}$ for UDP-glucose for samples containing $\leq$3 phosphates/mol, $S_{0.5}$ increasing from the original 1.5 mM to 13 mM. Further phosphorylation produced a sharp increase in the $S_{0.5}$ reaching 250 mM for maximally phosphorylated samples (fig.2). When the $M_{0.5}$ for G6P was plotted versus the number of incorporated phosphates a similar pattern was observed. The $M_{0.5}$ value slowly increased with phosphorylation $\leq$2 phosphates/subunit. Rapid changes were observed when phosphorylation rose to >2 phosphates/subunit. The most dramatic increase was observed when the enzyme approached 4 phosphates/subunit (fig.3).

4. Discussion

In the present study it is shown that GSK-1 catalyzes a reaction in which $\leq$4 mol ^{32}P can be transferred from [γ-^{32}P]ATP to the glycogen synthase subunit, confirming studies with GSK-1 purified through a different procedure [6,12]. From these results it is clear that the properties of glycogen synthase with respect to both UDP-glucose and G6P concentrations varied extensively with the phosphorylation state of the enzyme. The $M_{0.5}$ value for G6P varied 100-fold over the phosphorylation range studied. The $S_{0.5}$ for UDP glucose showed a 250-fold variation as a function of the phosphate content.

Looking at the changes in the –G6P/+G6P activity ratio (fig.1B) as a function of the phosphate content, it could be concluded that glycogen synthase can be almost fully converted into the G6P-dependent form following phosphorylation to 2 mol phosphate/subunit, and that the third and fourth phosphates are non-specific as their introduction does not correlate to conspicuous changes in the –G6P/+G6P activity ratio.

In fact, it has been indicated that glycogen syn-

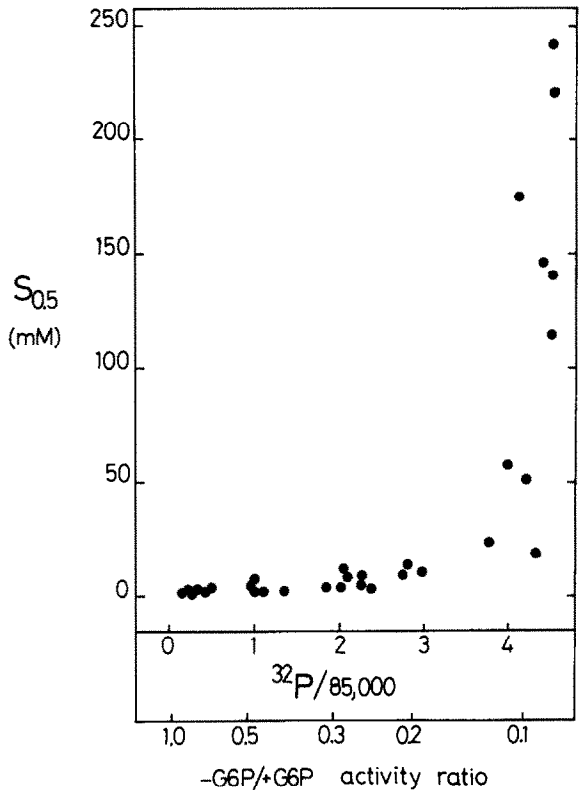

Fig.2. Relationship between $S_{0.5}$, the UDP-glucose (UDPG) concentration required for half-maximal rate, and ^{32}P incorporated into glycogen synthase. Samples were phosphorylated for different times. At the end of the incubation they were assayed for ^{32}P incorporation and their kinetic properties determined. $S_{0.5}$ was estimated from Hill plots of log $(V/V_{max}-V)$ versus log [UDPG]. V_{max} was calculated by extrapolation of Eadie-Hofstee plots of V/[UDPG] versus V. It was impossible to determine V_{max} with the more phosphorylated samples. The values of $S_{0.5}$ were then calculated from Hill plots on the assumption that the measured rate was equal to the rate measured in the standard assay in the presence of G6P. The lower abscissa shows the correspondence between ^{32}P incorporated and –G6P/+G6P activity ratio.

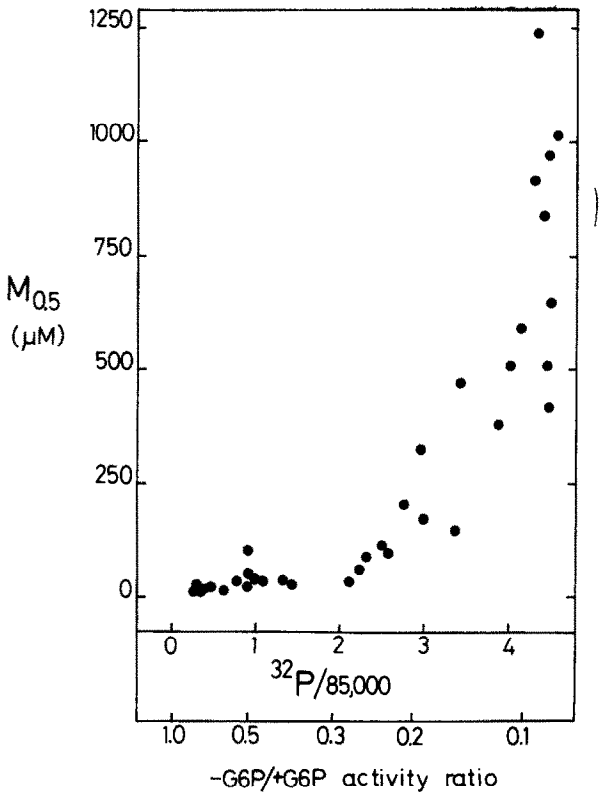

Fig.3. Relationship between $M_{0.5}$, the G6P concentration required for half-maximal activation at 0.2 mM UDPG, and ^{32}P incorporated into glycogen synthase subunit. $M_{0.5}$ was estimated from Hill plots of log $(V_a/V_a max-V_a)$ versus log [G6P]. In these cases the velocity increase, V_a, caused by glucose-6-P (activated rate minus rate in the absence of sugar phosphate) was used in place of velocity. $V_a max$ was calculated from Eadie-Hofstee plots of V_a/[G6P] versus V_a. Other details as in fig.2.

thase, when phosphorylated with GSK-1, is converted into the G6P-dependent form following phosphorylation to 2 mol phosphate/subunit [6]. Similarly, it has been suggested that only 2 of the sites phosphorylated by cyclic AMP-dependent protein kinases are involved in the I into D conversion and that phosphates >2 are not associated with changes in synthase activity [4].

However, these data demonstrate that none of the phosphates introduced by GSK-1 are non-specific, as all of them provoked changes in the kinetic properties of the enzyme. Furthermore, our results demonstrate that phosphorylation of synthase >2 mol phosphate/subunit produced very strong changes in enzymic properties that are not reflected in the –G6P/+G6P assay. In fact, those phosphates whose introduction appeared as almost non-specific in the –G6P/+G6P assay are precisely those which produced the greatest changes in the kinetic parameters of the enzyme.

The kinetic characteristics of a series of glycogen synthase samples of different phosphate content were studied [11,23] and it was also observed that the greatest changes occurred at >2 phosphates/subunit. This

coincidence is remarkable considering that their samples were phosphorylated by unidentified protein kinases present in the preparation and that phosphorylation was performed in the presence of high levels of ATP for long periods of time at 7°C. This raises the possibility of GSK-1s being involved in the phosphorylation of these samples.

The physiological meaning of the variations caused by the introduction of the third and fourth phosphates cannot be underestimated. If the assay is performed at concentrations of substrate and activator closer to their physiological values, the possible regulatory importance of these phosphorylations can be more clearly observed [24]. An additional regulatory possibility for these phosphates arises from the observation [25] that synthase containing 3 mol phosphate/subunit is a relatively poor substrate for glycogen synthase phosphatase.

An additional conclusion of this study is that the –G6P/+G6P activity ratio is not a sensitive enough indicator of regulation of the enzyme by phosphorylation. Thus, we have devised a new simple activity ratio assay which is much more sensitive than the standard –G6P/+G6P assay for the detection of changes in the enzymic properties caused by phosphorylation [24].

Acknowledgements

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