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INACTIVATION OF GLYCOGEN SYNTHASE *a* BY THE CATALYTIC SUBUNIT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE

KINETICS OF INACTIVATED FORMS

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Summary

Rabbit skeletal muscle glycogen synthase was phosphorylated to varying degrees with [γ-^{32}P]ATP and the catalytic subunit of the cyclic AMP-dependent protein kinase. Phosphorylation of glycogen synthase up to 1 mol phosphate per mol subunit had very little effect on the activity ratio (activity measured in the absence of glucose 6-phosphate divided by activity measured in the presence of glucose 6-phosphate), the $A_{0.5}$ for glucose 6-phosphate (concentration of glucose 6-phosphate which gives half maximal activation), or the K_m for UDPGlc measured in the absence of glucose 6-phosphate. Phosphorylation to the extent of 1.8 mol phosphate per mol subunit resulted in partial inactivation of glycogen synthase (activity ratio = 0.6) due primarily to an increase in the K_m for UDPGlc measured in the absence of glucose 6-phosphate. Phosphorylation to the extent of 2.6—2.8 mol phosphate per mol subunit resulted in further inactivation (activity ratio = 0.05—0.13) due primarily to a decrease in V (maximal velocity) measured in the absence of glucose 6-phosphate and partly to an additional increase in the K_m for UDPGlc measured in the absence of glucose 6-phosphate. The form of glycogen synthase containing 2.6—2.8 mol phosphate per mol subunit was unique in that activation by glucose 6-phosphate showed little or no positive cooperativity, $A_{0.5}$ for glucose 6-phosphate was relatively high, and V measured in the absence of glucose 6-phosphate was reduced. Phosphorylation of glycogen synthase had very little effect on either

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Abbreviations: activity ratio, activity measured in the absence of glucose-6-*P* divided by activity measured in the presence of glucose-6-*P*; $A_{0.5}$, concentration of glucose-6-*P* which gives half maximal activation; glycogen synthase *b*, glucose-6-*P*-dependent form(s) of glycogen synthase; glycogen synthase *a*, glucose-6-*P*-independent form of glycogen synthase; catalytic subunit, catalytic subunit of the cyclic AMP-dependent protein kinase; Mops, morpholinopropanesulfonic acid; SDS, sodium dodecyl sulfate.

the K_m for UDPGlc or the V measured in the presence of glucose 6-phosphate. We conclude from these studies that inactivation of glycogen synthase *a* by the catalytic subunit of cyclic AMP-dependent protein kinase in vitro is complex, and that no single kinetic parameter is the best index of inactivation.

Introduction

It is now well established that phosphorylation and inactivation of glycogen synthase can be catalyzed by the cyclic AMP-dependent protein kinase [1,2], by phosphorylase kinase [3—5] and by several other protein kinases [6,7], and that complete inactivation requires incorporation of up to 4 mol phosphate per mol subunit [8—10]. The significance of multiple phosphorylation and the several protein kinases in the regulation of glycogen synthase activity, however, is not clear. Changes in sensitivity to glucose-6-*P* with little or no change in the activity ratio [11—13] or in other kinetic parameters [14] have been observed in studies where more than one protein kinase was present. At least one of the sites phosphorylated by the cyclic AMP-dependent protein kinase appears not to be correlated with inactivation of glycogen synthase as measured by a decrease in the activity ratio [15,16], but has been shown in vitro to increase the rate of dephosphorylation of a second site which is involved with glycogen synthase inactivation [17]. In a recent abstract which reported inactivation of glycogen synthase by a cyclic AMP-independent protein kinase, changes in the K_m for UDPGlc and the $A_{0.5}$ for glucose-6-*P* were correlated with phosphorylation of different sites [18].

In order to better understand the role of multiple phosphorylation and the several protein kinases in the regulation of glycogen synthase activity, it will be important to characterize the forms of glycogen synthase produced by phosphorylation of glycogen synthase by a well defined preparation of each protein kinase. The object of the present study was to determine the kinetic properties of glycogen synthase phosphorylated by the cyclic AMP-dependent protein kinase to the extent of 1, 2, or 3 mol phosphate per mol subunit under conditions where incorporation of 3 mol phosphate per mol subunit is required for complete inactivation of glycogen synthase. The kinetic properties reported here differ significantly from those reported previously for glycogen synthase phosphorylated by the cyclic AMP-dependent protein kinase to the extent of 1 [19] or 2 [11] mol phosphate per mol subunit.

Materials and Methods

Materials

Mops, $pK_a = 7.2$ at 25°C, and oyster glycogen (type II) were obtained from Sigma Chemical Co. UDP[^{14}C]Glc was obtained from Amersham Corporation or ICN Corporation and evaporated to dryness at 30°C under vacuum to remove ethanol. Stock solutions of unlabelled UDPGlc (Sigma Chemical Co.) were added to achieve a final specific activity of either 2500—3000 cpm/nmol for kinetic experiments, or 75—260 cpm/nmol for assay of activity ratios.

Enzyme purification

Glycogen synthase *a* was purified to a specific activity of 39 units/mg by modification of the method of Soderling et al. [2]. One major protein band was detected on polyacrylamide gel electrophoresis in the presence of SDS. Details of this method will be published elsewhere. The catalytic subunit of the cyclic AMP-dependent protein kinase (specific activity = 1000 units/μg) was prepared from pig gastric mucosa as described previously [20,21]. One unit of catalytic subunit activity was defined as that amount of enzyme which catalyzes transfer of 1 pmol ^{32}P from [γ-^{32}P]ATP to histone in 1 min under the conditions described previously [22].

Phosphorylation

The reaction mixture for the phosphorylation of glycogen synthase *a* contained 20 mM phosphate, 7.7 mM glycerophosphate, 40 mM NaCl, 0.14 mM dithiothreitol, 0.4 mg glycogen/ml, 0.1 mg glycogen synthase *a*/ml, 0.2 mM [γ-^{32}P]ATP, 10 mM magnesium acetate, 0.7 mM EDTA, 1 mM EGTA, 2% glycerol and 0.6 mg gelatin/ml. The concentration of the catalytic subunit and incubation time were varied to achieve the desired level of phosphorylation (Table I). Control incubations were carried out under identical conditions except that either catalytic subunit or glycogen synthase was absent. Protein bound phosphate was determined on duplicate aliquots of all three samples as described previously [22]. Phosphate incorporation in the control which lacked catalytic subunit was $\leqslant$0.2 mol phosphate per mol of 85 000 dalton subunit of glycogen synthase in all experiments, and the kinetic properties of glycogen synthase *a* were unchanged as a result of this incubation. The amount of phosphate incorporation into glycogen synthase in the presence of catalytic subunit was corrected for the phosphate incorporation seen in the incubation which lacked glycogen synthase, but not for the low level of phosphorylation seen in the absence of catalytic subunit. After removal of aliquots for phosphate determination, the reaction was stopped by adding 20 volumes of cold buffer containing 50 mM Mops, 10 mM EDTA, 10 mg glycogen/ml and 25 mM KF at pH 7.0. Glycogen synthase was stable when frozen at $-70^{\circ}C$ at this dilution for several months

Glycogen synthase activity

The activity ratio was determined by assaying glycogen synthase in the absence and presence of 8.7 mM glucose-6-*P* as described previously [23] except that the buffer contained 50 mM Mops at pH 7.0 rather than 50 mM Tris at pH 7.8. Activity ratios were determined in the presence of 4 mM UDPGlc unless otherwise specified. Kinetic characterization of glycogen synthase was carried out under the same reaction conditions used to determine activity ratio. When glucose-6-*P* was varied, the UDPGlc concentration was 40 μM. When UDPGlc was varied, the glucose-6-*P* concentration was 0, 0.3 or 8 mM. The enzyme was diluted so that the reaction rates were linear for at least 4 min. In all cases product formation was measured at several time points to ensure that initial velocities were measured. A typical initial velocity plot is shown in Fig. 1. Kinetic constants were determined by fitting the data to either the Michaelis-Menten or the Hill equation by non-linear regression analysis [24].

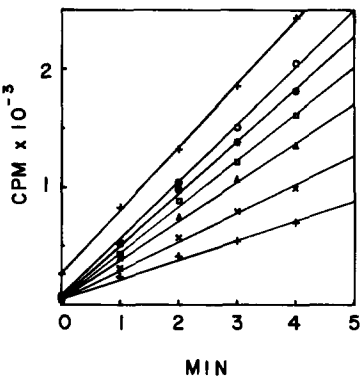

Fig. 1. Initial velocities for glycogen synthase phosphorylated to the extent of 1.8 mol phosphate per mol subunit were determined in the presence of 300 μM glucose-6-*P* with UDPGlc as the variable substrate. The concentrations of UDPGlc used in this experiment were 10 (+), 20 (X), 40 (△), 80 (□), 120 (*), 400 (○) and 4000 (+) μM. Blank values were obtained from a control assay which was identical to the glycogen synthase assay except that incubations were initiated with the control from the phosphorylation experiment which lacked glycogen synthase *a*.

Results

Inactivation of glycogen synthase a by catalytic subunit

Inactivation of glycogen synthase *a* to an activity ratio of 0.05—0.13 by phosphorylation with catalytic subunit required incorporation of 3 mol phosphate per mol subunit (Table I). Phosphorylation to the extent of 0.6 or 1.1 mol phosphate per mol subunit produced a negligible decrease in activity ratio (6%), whereas the second and third mol of phosphate each effected a substantial decrease in the activity ratio. Similar results were obtained when the activity ratios were measured at pH 7.8. The activity measured in the presence of 8 mM glucose-6-*P* and 4 mM UDPGlc was not affected by phosphorylation.

Effect of phosphorylation on the kinetics of glucose-6-P activation

Phosphorylation of glycogen synthase *a* to the extent of 1.1 or 1.8 mol phos-

TABLE I

INACTIVATION OF GLYCOGEN SYNTHASE *a* AS A RESULT OF PHOSPHORYLATION BY THE CATALYTIC SUBUNIT

Activity ratio of incubated and unincubated controls was 0.98.

Concentration of catalytic subunit (U/μl)	Incubation time (min)	mol ^{32}P/85 000 × *g*	Activity ratio
1	10	0.6	0.92
1	21	1.1	0.92
20	18	1.8	0.58
90	20	2.6	0.12
90	35	2.7	0.13
200	30	2.8	0.05

TABLE II

EFFECT OF PHOSPHORYLATION ON THE KINETICS OF GLUCOSE-6-*P* ACTIVATION

Numbers in parentheses represent the number of observations.

mol ^{32}P/85 000 × *g*	$A_{0.5}$ (μM)	Hill coefficient
(control) *	2.0 (7)	1.5 (7)
1.1	2.9 (2)	1.7 (2)
1.8	9.0	1.9
2.6	86	1.1
2.7	170 (2)	1.0 (2)
2.8	370	1.2

* Includes incubated and non-incubated glycogen synthase *a*.

phate per mol subunit resulted in little increase in the $A_{0.5}$ for glucose-6-*P* (Table II). The $A_{0.5}$ for glucose-6-*P* exceded 10 μM only after 2.6—2.8 mol phosphate per mol subunit were incorporated. The Hill coefficient for glucose-6-*P* activation was approx. 1 for glycogen synthase containing 2.6—2.8 mol phosphate per mol subunit, but showed positive cooperativity for all other forms. The difference in cooperativity for glucose-6-*P* activation is illustrated for glycogen synthase phosphorylated to the extent of 1.8 and 2.6 mol phosphate per mol subunit in Fig. 2.

Effect of phosphorylation on the kinetics for UDPGlc

The K_m for UDPGlc of glycogen synthase *a* measured in the presence of saturating concentrations of glucose-6-*P* was about 16 μM. Phosphorylation to the extent of 2.8 mol phosphate per mol subunit increased the K_m to only 35 μM. Higher values for the K_m were observed for highly phosphorylated glycogen synthase when less than saturating concentrations of glucose-6-*P* were present (Table III). The K_m for UDPGlc measured in the absence of glucose-6-*P* was about 110 μM for glycogen synthase *a* and increased little as a result of phosphorylation to the extent of 0.6 or 1.1 mol phosphate per mol subunit.

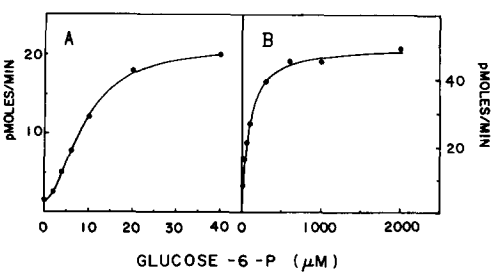

Fig. 2. Activation by glucose-6-*P* of glycogen synthase phosphorylated to the extent of 1.8 (panel A) and 2.6 (panel B) mol phosphate per mol subunit. Values for the kinetic constants in these experiments are: $A_{0.5}$ = 9 μM, Hill coefficient = 1.9 for panel A, and $A_{0.5}$ = 86 μM, Hill coefficient = 1.1 for panel B. Lines represent the best fit to the Hill equation. The glycogen synthase concentration for panel A was approximately half that for panel B.

TABLE III

EFFECT OF PHOSPHORYLATION ON THE K_m FOR UDPGlc, V AND ACTIVITY RATIO MEASURED WITH 40 mM UDPGlc

Numbers in parenthesis indicate the number of observations.

mol ^{32}P/85 000 × g	K_m for UDPGlc		Activity ratio measured with 40 mM UDPGlc	$\frac{V(-\text{glucose-6-}P)}{V(+\text{glucose-6-}P)}$
	no glucose-6-*P*	300 μM glucose-6-*P*		
(control) *	110 (7)	16 (3)	0.98	—
0.6	110	14	—	—
1.1	230	25	0.92	—
1.8	1100 (2)	24	0.82	0.9 (2)
2.6	1400	66 **	0.29	0.34
2.7	3000 (2)	61 **	—	0.35 (2)
2.8	—	35 ***	0.09	—

* Includes both incubated and non-incubated glycogen synthase *a*.

** 300 μM glucose-6-*P* is less than saturating.

*** Measured in the presence of 8 mM glucose-6-*P*.

At higher levels of phosphorylation the K_m for UDPGlc measured in absence of glucose-6-*P* increased progressively to a maximum value of 3 mM. There was no evidence for either positive or negative cooperativity in any of the above K_m determinations. Glycogen synthase with 2.6—2.8 mol phosphate incorporated per mol subunit was unique in that V was stimulated by glucose-6-*P* (Table III). This observation is also reflected in the fact that the activity ratio for this form measured in the presence of saturating UDPGlc (40 mM) was less than 1 (Table III).

Discussion

Inactivation of glycogen synthase (activity ratio <0.2) by the cyclic AMP-dependent protein kinase has been reported previously to require phosphorylation to the extent of 1 [19], or 2 [11,15] mol phosphate per mol subunit. In another study incorporation of 2 mol phosphate per mol subunit by the cyclic AMP-dependent protein kinase reduced the activity ratio only to about 0.4 [9]. In the present study, incorporation of 2 mol phosphate per mol subunit decreased the activity ratio to 0.6 and incorporation of 3 mol phosphate per mol subunit was required for complete inactivation of glycogen synthase (activity ratio 0.05—0.13). The first mol phosphate per mol subunit incorporated by the catalytic subunit was kinetically silent. Addition of the second and third mol phosphate per mol subunit differentially affected the K_m for UDPGlc and the V determined in the absence of glucose-6-*P* as well as the $A_{0.5}$ for glucose-6-*P*. This suggests that there are at least two different phosphorylation sites involved with inactivation. If a minimum of one kinetically silent site and two sites involved with glycogen synthase inactivation can be phosphorylated by the catalytic subunit, then it is not surprising that 3 mol phosphate per mol subunit are required for maximal inactivation. Variations among laboratories in the number of phosphates per subunit required to

inactivate glycogen synthase by the cyclic AMP-dependent protein kinase may result in part from the properties of the glycogen synthase *a* used. It is important, therefore, to note that the glycogen synthase *a* used in the present study has an activity ratio of approx. 0.98, whereas, in other studies, glycogen synthase *a* had some dependence on glucose-6-*P*, i.e. an activity ratio of 0.6—0.8 [9,11,15,19]. Furthermore, the $A_{0.5}$ for glucose-6-*P* for our glycogen synthase phosphorylated to the extent of 2 mol phosphate per mol subunit is lower than that observed for the glycogen synthase *a* used in the studies of Nimmo et al. [19] and Brown et al. [11]. Although the differences in the number of phosphates per subunit required to inactivate glycogen synthase using the cyclic AMP-dependent protein kinase in vitro cannot be explained at present, the fact that multiple phosphorylation of glycogen synthase in the present study was associated with significant kinetic changes argues against the idea that multiple phosphorylation under these conditions is nonspecific, and suggests instead the possibility that multiple phosphorylation of glycogen synthase by the catalytic subunit may have physiological significance.

Two previous studies [15,16] have identified a site on glycogen synthase which appears to correspond to the kinetically silent phosphorylation described in the present study. In those studies 40—75% of the first mol phosphate per mol subunit introduced by the cyclic AMP-dependent protein kinase was incorporated at a site which was not associated with a decrease in the activity ratio, and which became soluble in trichloroacetic acid after mild trypsin treatment. In our study, the first mol phosphate per mol subunit introduced by the catalytic subunit is kinetically silent. Although we have not established that the first mol phosphate per mol subunit is introduced exclusively at one site, it is introduced exclusively into peptides which are rendered soluble in trichloroactetic acid after mild trypsin digestion [10], and would thus appear to be incorporated at the silent site described previously. In the previous studies [15,16], incorporation of 1 mol phosphate per mol subunit resulted in a decrease in the activity ratio from about 0.8 to about 0.2. Our results provide a direct demonstration of a 'silent phosphate' since this level of phosphorylation had no appreciable effect on either the activity ratio or the kinetic properties of glycogen synthase.

Some kinetic properties for glycogen synthase inactivated by phosphorylation with the cyclic AMP-dependent protein kinase have been reported previously [11,19]. Incorporation of 1 mol phosphate per mol subunit was found by Nimmo et al. [19] to decrease the activity ratio of glycogen synthase *a* from 0.8 to 0.18, and to increase the $A_{0.5}$ for glucose-6-*P* from 50 to 600 μM. Incorporation of 2 mol phosphate per mol subunit was found by Brown et al. [11] to decrease the activity ratio of glycogen synthase *a* from 0.64 to 0.17, to increase the $A_{0.5}$ for glucose-6-*P* from approx. 15—295 μM, but not to significantly increase the K_m for UDPGlc measured in the presence of saturating concentrations of glucose-6-*P* (46—61 μM). The present study agrees with those studies in that phosphorylation with the catalytic subunit can reduce the activity ratio to 0.18—0.05 and markedly increase the $A_{0.5}$ for glucose-6-*P* without greatly increasing the K_m for UDPGlc measured in the presence of saturating concentrations of glucose-6-*P*. In contrast to the above reports, we found that the $A_{0.5}$ for glucose-6-*P* was less than 10 μM except

when 2.6—2.8 mol phosphate per mol subunit were incorporated into glycogen synthase. The present study is the only study which documents significant changes in the K_m for UDPGlc and the V as a result of phosphorylation by the cyclic AMP-dependent protein kinase.

There has been disagreement in the literature regarding the role of glucose-6-*P* in increasing the activity of skeletal muscle glycogen synthase *b* [25]. Glucose-6-*P* has been reported to either increase the V of glycogen synthase *b*, to decrease the K_m for UDPGlc or to do both. In our studies, the role of glucose-6-*P* in increasing the activity of glycogen synthase *b* depended on the level of phosphorylation. With low levels of phosphorylation (less than 2 mol phosphate per mol subunit), glucose-6-*P* increased the activity of glycogen synthase by decreasing the K_m for UDPGlc; with higher levels of phosphorylation, glucose-6-*P* increased the activity of glycogen synthase both by decreasing the K_m for UDPGlc and by increasing the V.

In the present study, the relationship between phosphorylation of glycogen synthase and activity ratio was found to involve (1) a kinetically 'silent' phosphorylation, i.e., phosphorylation which is ineffective in producing inactivation, (2) an increase in the K_m for UDPGlc measured in the absence of glucose-6-*P*, and (3) a decrease in the V measured in the absence of glucose-6-*P*. We conclude from the data presented here that inactivation of glycogen synthase *a* by cyclic AMP-dependent protein kinase in vitro is complex, and that no single kinetic parameter is the best index of enzyme inactivation under all conditions. Although the in vivo significance of multiple phosphorylation by a single kinase remains to be established, the data presented here would suggest that even a single protein kinase can produce forms of glycogen synthase with distinctly different kinetic properties.

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