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Kinectics of the D and I Glycogen Synthetase Forms and Their Interconversion, in the Sea Scallop *Pecten maximus* *

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In extracts from the adductor muscle of the shell-fish, *Pecten maximus*, glycogen synthetase (EC.2.4.1.11) was found. The enzyme occurs predominantly as D form (glucose-6-P dependent for activity). An I form (G-6-P independent) was also present. Kinetics of glycogen synthetase showed that the Ka for G-6-P in the D form was 10 fold higher than in the I form.

Both forms of glycogen synthetase were interconverted through reactions catalyzed by phosphatase and kinase enzymes respectively. Glucose-6-P and Mg^{+2} must be present to stabilize glycogen synthetase and to activate the synthetase D phosphatase, found in the 90,000 × *g* protein-glycogen complex.

The conversion of synthetase D to I was inhibited by F^-, glycogen, ATP and UTP. When F^- was present the effect of G-6-P on synthetase and phosphatase suggested that conversion involved the existence of more than a single glycogen synthetase phosphatase enzyme.

ATP and Mg^{+2} were necessary for the conversion of synthetase I to D, and the conversion was stimulated by cAMP.

In skeletal muscle the glycogen synthetase enzyme (UDPG-glucose: glycogen α-4-glucosyltransferase EC.2.4.1.11), exists in two interconvertible forms (16, 17, 20), a phosphorylated form (synthetase D) which is dependent on glucose-6-P for activity, and a dephosphorylated form (synthetase I) that is active in the absence of glucose-6-P (4). Synthetase I is considered the physiological active form (15) and is converted to synthetase D by a cyclic AMP-dependent protein kinase (12).

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Abbreviations: Glucose-6-P, glucose-6-phosphate; cAMP, adenosine 3':5'-monophosphate; UDPG, uridinediphosphate glucose.

Intensive accumulation of glycogen in tissues is, as a rule, accompanied by the conversion of glycogen synthetase into the I form, catalyzed by synthetase D phosphatase. This conversion is inhibited by physiological concentrations of ATP and the effect of this nucleotide is enhanced by glycogen (5, 21). However when synthetase phosphatase is highly purified, ATP has no effect (10)..

Glycogen synthetase and the interconversion of the D and I forms have been studied in crude extracts and in the protein-glycogen complex (8) obtained from the central adductor muscle of *Pecten maximus*, a shell-fish with a high glycogen content (28).

Materials and Methods

Condition of organisms. The *Pecten maximus* used in this study came from a culture park set up in «Ría de Arosa» (Galicia, Spain), periodically sent by air under optimal conditions to insure survival (i.e. in poliexpan boxes wherein an elevated humidity and a temperature of less than 10° C was maintained). The bivalves were utilized either at the moment of arrival in the laboratory, 10-20 hours after being taken from the sea, or after having spent a period of adaptation of 24, 48, 72 or 88 hours after arrival in an environment similar to their natural habitat (i.e. tanks of aerated sea water containing diatom and fagellate algae, in order to provide a convenient food supply), in the «Instituto de Investigaciones Pesqueras» in Barcelona. Organisms which have spent longer periods of time were considered anoxic. The central adductor muscles used in the experiments were isolated from an average of 4-6 organisms for each experiment. This tissue is characterized by its white color, vitreous appearance, striated fiber and rapid contraction (6). It was used either immediately after dissection or after being frozen at —20° C for no longer than a month after dissection.

Enzyme preparations. The adductor muscle suspensions (1:5 w/v) in 50 mM Tris-HCl, 5 mM EDTA buffer (pH 7.8) were homogenized in a motor driven Potter-Elvejhem homogenizer with teflon pestle. This and subsequent steps were carried out at 4° C. The homogenate was centrifuged at 6,500 × *g* in a Sorvall RC2 for 20 minutes and then filtered through glass wool. The resulting supernatant was referred to as the «crude extract» and it was used to assays of glycogen synthetase as well as the interconverting enzymes. In some experiments this crude extract was further centrifuged at 90,000 × *g* for 60 minutes in a Beckman L2 ultracentrifuge. The pellets thus obtained were resuspended in 50 mM Tris-HCl (pH 7.8) without EDTA for use in glycogen synthetase kinetic studies, or in the same buffer with 5 mM EDTA for studies of phosphatase and kinase activities.

To obtain a high percentage of I form, pellets of 90,000 × *g* resuspended in 50 mM Tris-HCl, 5 mM EDTA (pH 7.8) were incubated with 1 mM G-6-P and 5 mM Mg^{+2} for 90-120 minutes at 25° C. This preparation was centrifuged at 90,000 × *g* for 60 minutes and, 85-95 % of glycogen synthetase I appeared in the resulting pellets.

Analytical assays. Glycogen synthetase activity was determined by the method of THOMAS *et al.* (26). The reaction mixture, incubated at 25° C for 15 minutes, contained: 1 % glycogen, 6.6 mM UDP-(^{14}C) glucose (uniformly labelled and with specific activity 20,000-28,000 c.p.m./ μmol), 100 mM KF, 50 mM Tris-HCl (pH 7.8) and 30 μl of the enzyme source. Synthetase I activity and total activity (I + D) were determined in reaction mixtures which also contained either 10 mM Na_2SO_4 (21) or 6.6 mM glucose-6-P.

Glycogen synthetase D phosphatase activity was assayed in «crude extracts» and in 90,000 × *g* pellets before and after they were incubated at 25° C in the presence or absence of metabolites. The phosphatase activity was determined by the synthetase D to I conversion. The glycogen synthetase present in the crude extracts or in the glycogen-complex was used as substrate for phosphatase.

The synthetase I kinase was determined by conversion from the I to the D form. For these studies the 90,000 × *g* pellets were incubated with 1 mM glucose-6-P and 5 mM Mg^{+2} for 60 minutes at 25° C. At this point, 2.5 mM ATP and either 5 or 10 mM Mg^{+2} were added to aliquots and the incubation was continued. At various times 30 μl aliquots were removed and added to 60 μl of the reaction mixture to assay glycogen synthetase I and (I + D) respectively.

Glycogen was determined by the Anthrone method (2). Assays to determine protein were done by LOWRY *et al.* (14) method following the modifications of JI (9).

Chemicals. Glycogen (rabbit liver), glucose-1-P, glucose-6-P, ATP, UTP, UDPG and 3′:5′ cyclic AMP were obtained from Sigma Chemical Company. The UDP-(^{14}C)glucose from New England Nuclear Co. or Radiochemical Center (Amersham). KF, NaF, EDTA, $CaCl_2$, $MgCl_2$, and $MnCl_2$ were obtained from Merck.

Results

Subcellular localization and kinetic properties of glycogen synthetase. In central adductor muscle of *Pecten maximus* the enzyme glycogen synthetase was found to be present and its activity was highly dependent on G-6-P (27, 28). The specific activity was between 8-13 nmol/mg prot./min, being the I form 11 % (±5) of the total glycogen synthetase activity. 70 % of the activity present in the homogenates was recovered in the supernatant fraction following centrifugation at 2,500 × *g* for 20 minutes, and

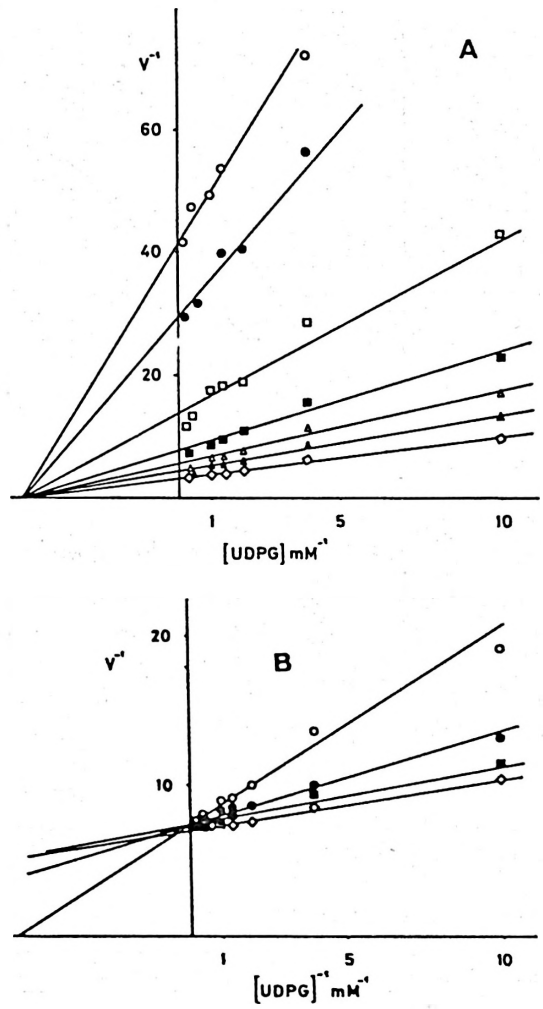

Fig. 1. *Double reciprocal plot of velocity of synthetase D and I vs.* UDPG concentration was between 0-5 mM (350,000 c.p.m./μmol). The reaction mixtures do not contain glucose-6-P (○) or they have: (●) 0.05 mM; (□) 0.2 mM; (■) 0.5 mM; (△) 1 mM; (▲) 1.5 mg and (◇) 5 mM glucose-6-P. Assays were carried out for 5 min, at 25° C, pH 7.8 with glycogen particulate pellet of 90,000 × *g* containing the synthetase as D form (A) or I form (B).

subsequently 58 % was found in the protein-glycogen complex.

Kinetic studies of the effect of increasing UDPG concentration on D form were carried out. Little activity was observed

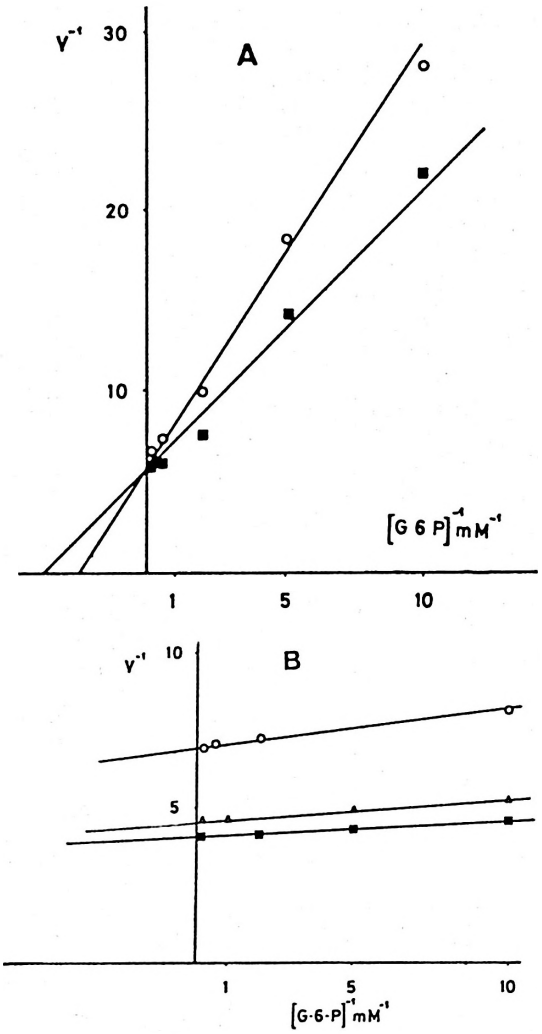

Fig. 2. *Kinetic plot activation of glycogen synthetase D and I form by glucose-6-P.* The assay conditions were similar to those indicated in fig. 1 UDPG concentration was 0.4 mM (400,000 c.p.m./μmol). Mg^{-2} was absent (O) or it was present in the amount of: (Δ) 2 mM or (■) 5 mM in the reaction mixtures. The enzyme source was as D form (A) or I form (B).

in the absence of G-6-P. The K_m, was around 0.2-0.3 mM and it was not altered by G-6-P as has been observed with enzyme from other organisms (3, 7, 20) (fig. 1A). With the I form (fig. 1B), the V_{max} reaction remains virtually unchanged in the presence of different concentrations of G-6-P. However, the K_m decreased from 0.15 mM, when G-6-P was absent from the reaction, to 0.084 and 0.05 mM when it was at 0.05 mM and 0.5 mM, respectively. The G-6-P concentration necessary to activate glycogen synthetase D to 1/2 V_{max} was between 0.3-0.5 mM (K_a). 5 mM Mg^{+2} decreased the K_a value from 0.5 mM to 0.27 mM with little modification of V_{max} (fig. 2A). In I form, the concentration of G-6-P giving half-maximal activation was 0.02-0.025 mM and 0.012 if 5 mM Mg^{+2} was present in the reaction (fig. 2B). In this case, Mg^{+2} also modified the V_{max} value, suggesting an increase of the enzyme's affinity for G-6-P. Although the two enzyme forms were activated by G-6-P, the K_a of I form was approximately ten fold less than that of D form.

ATP had an inhibitory effect on glycogen synthetase D from *Pecten maximus*, which can be reversed by an increase in G-6-P. The inhibition caused by ATP on the activation of the D form by G-6-P causes the K_a value to increase from 0.3 ($\pm$0.2) mM to 0.46, 0.62, 0.87, 1.11 and 2.12 mM when the assays were conducted wi.h ATP at 0, 0.1, 0.5, 1, 2 and 5 mM respectively. However the V_{max} did not change. The K_a of synthetase I increased from 0.025 ($\pm$0.005) mM to 0.15 mM if 5 mM ATP was in the reaction mixture (graph not shown).

$SO_4^{=}$ and $PO_4^{\equiv}$ inhibited glycogen synthetase D assayed in the presence of G-6-P (27). The inhibition was competitive as previously found by ROSELL *et al.* (19).

In vitro *interconversion of the D to the I glycogen synthetase.* Conversion

of glycogen synthetase D to I was observed when crude extracts from *Pecten maximus* were incubated at 25° C. However this conversion was not followed with 90,000 × *g* pellets; likewise, the total synthetase activity decreased during the preincubation, while I form activity decreased slightly. 1 mM G-6-P and 5 mM Mg^{+2} were effective in inhibiting total glycogen synthetase inactivation while D to I conversion was activated by these compounds. The effect was observed

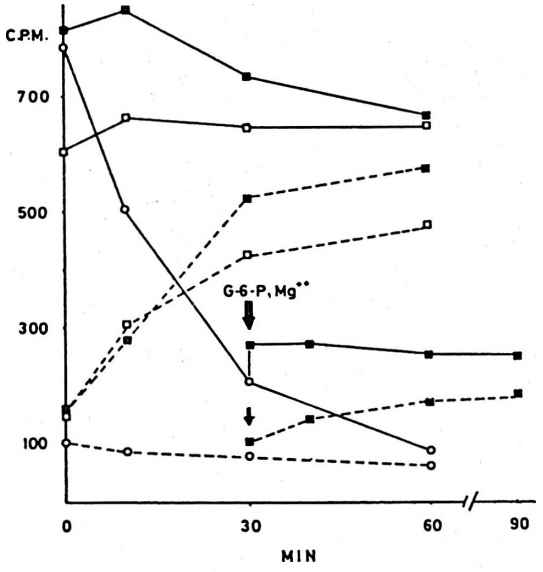

Fig. 3. *Dependence of glycogen synthetase D phosphatase from Pecten maximus upon glucose-6-P and* Mg^{+2}.

The enzyme source was a glycogen particulate fraction of 90,000 × *g* from a crude extract not preincubated. This fraction was resuspended in 50 mM TRIS-HCl, 5 mM EDTA (pH 7.8), and 10 mM mercaptoethanol and then incubated, at 25° C. Synthetase activity was determined at the intervals indicated in the enzyme source previously incubated without additions (○) or with 1 mM glucose-6-P and 5 mM Mg^{+2} (■). The open squares (□) represent the activity in crude extracts incubated without additions, from which pellets of 90,000 × *g* were obtained. The broken lines and solid lines represent the glycogen synthetase I and the total activity respectively.

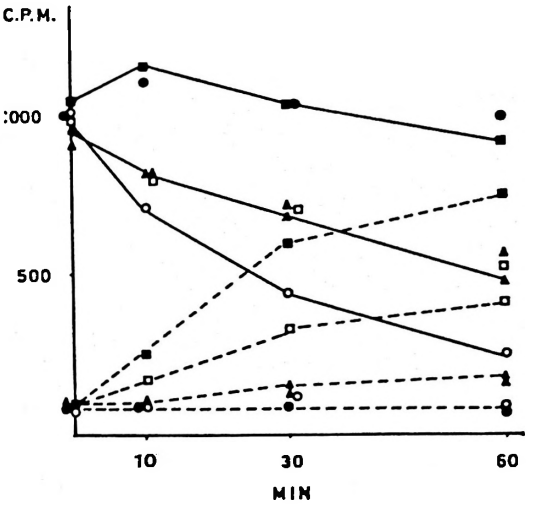

Fig. 4. *The effect of NaF and EDTA on synthetase D phosphatase.*

The enzyme sources were prepared as described in fig. 3. Incubations were run: (○) without additions or either (□) 1 mM glucose-6-P; (■) 1 mM glucose-6-P and 5 mM Mg^{+2}; (●) 1 mM glucose-6-P and 100 mM NaF; (△) 1 mM glucose-6-P, 5 mM Mg^{+2} and 5 mM or (▲) 10 mM EDTA. The broken and solid lines represent the form I and total activity respectively.

either at the beginning of the preincubation or after some loss of total glycogen synthetase had occurred (fig. 3).

Both G-6-P and Mg^{+2} are necessary to stabilize glycogen synthetase activity and to stimulate the phosphatase. If 90,000 × *g* pellets were preincubated with 1 mM G-6-P alone the level of total synthetase appears similar to the activity observed when G-6-P was absent; however the sugar phosphate stimulated the phosphatase activity (fig. 4). On the other hand, Mg^{+2} alone was insufficient to stabilize total synthetase or to promote the D to I conversion (unpublished results). 1 mM G-6-P and 5 mM Mg^{+2} bring the I form up to 85-95 % of the total activity in 90,000 × *g* pellets after 60 minutes of preincubation. The requirement of Mg^{+2} to the former conditions was followed

through experiments were EDTA was also included (fig. 4). 5 mM Mg^{+2} was found to be the optimal concentration to activate the glycogen synthetase D phosphatase (table I). At higher concentrations the conversion was somewhat slower. The conversion of the D to the I form activated by G-6-P was completely inhibited by 100 mM NaF, however, under this condition the total glycogen synthetase remain as constant as when Mg^{+2} and G-6-P were present (fig. 4). Ca^{+2} has an effect similar to that of Mg^{+2}; however, Mn^{+2}, which has been shown to be a good activator of phosphatase (25), did not activate the glycogen synthetase from *Pecten maximus* (27).

The inhibition of the conversion to synthethase I caused when ATP was added to crude extracts, was increased by

Table I. *Effect of different concentrations of Mg^{+2} on the D to I form conversion.*
The D and (D + I) activity assayed in 90,000 × *g* pellets (previously resuspended in TRIS-HCl 50 mM pH 7.8) added with G-6-P and Mg^{+2}.

Concentration of addition		Time of preincubation	
G-6-P mM	Mg^{+2} mM	30 min % I	60 min % I
1	5	100	100
1	10	85	95
1	15	66	92
1	—	39	43

Table II. *Influence of glycogen, ATP and UTP on D to I form conversion.*
The crude extracts utilized were prepared as described in Materials and Methods. G-6-P, ATP, UTP and glycogen were added before the incubation was done at 25° C for 30 min. The % of I form observed at 30 min of incubation was considered as 0 % of inhibition (100 % of activation) when only G-6-P was present.

Compounds			
G-6-P mM	ATP mM	Glycogen mg/ml	% Inhibition of I form
1	—	—	—
1	0.5	—	23
1	1.5	—	48
1	3	—	73
1	0.5	1.6	54
1	0.5	4.8	64
1	0.5	8.0	65
G-6-P mM	UTP mM	Glycogen mg/ml	% Inhibition of I form
1	—	—	—
1	0.5	—	33
1	1	—	49
1	3	—	81
1	0.5	8.0	81

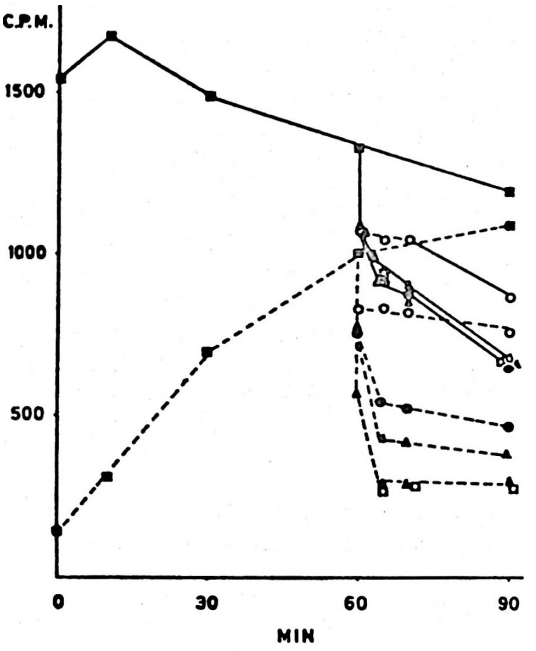

Fig. 5. *Synthetase I kinase activity.*
Conditions of fig. 3 were followed to obtain the enzyme source. C.p.m. represent the total (solid lines) and the glycogen synthetase I (broken lines) activities. 1 mM glucose-6-P, 5 mM Mg^{+2} (■) were added to the enzyme source before incubation and then incubated for 60 minutes at 25° C. Next 2.5 mM ATP and Mg^{+2} 5 mM (●), 10 mM (△), 10 mM and 50 μM cAMP (▲) or 10 mM and 100 μM cAMP (□) were added to different aliquots and time course of synthetase I kinase was done.

glycogen. The behavior of UTP was similar to that of ATP (table II).

Synthetase I kinase. Glycogen synthetase I kinase activity was assayed in the 90,000 × *g* pellets. The enzyme sources were incubated with 1 mM G-6-P and 5 mM Mg^{+2} for 60 minutes. After the incubation and by addition of ATP and Mg^{+2} the conversion from the I to the D form took place as fig. 5 shows, and cAMP increased that conversion. UTP-Mg^{+2} was found to be inadequate to make this conversion.

Discussion

The results of this work show that glycogen synthetase activity, is present in the adductor muscle of *Pecten maximus*. Two forms, glycogen synthetase D and I were found. Although synthetase of crude extracts was found principally as D form, the D and I form were interconvertible through phosphorylation and dephosphorylation reactions (1, 4, 5, 10, 18), catalyzed by kinase and phosphatase enzymes respectively. The D to I form conversion was observed when crude extracts were incubated at 25° C. However the extent of the conversion did not reach more than 70 % of I form.

Experiments carried out with protein-glycogen complex as enzyme source showed that glycogen synthetase lost activity during the preincubation (fig. 3). During the time course, D to I form conversion was not observed. However, glycogen synthetase inactivation was prevented at the moment G-6-P and Mg^{+2} were added to the protein-glycogen complex. At the same time the phosphatase started to act and the D to I conversion began. It might be considered which metabolites in the 90,000 × *g* supernatant are responsible for holding the glycogen synthetase activity and promoting D to I form conversion. From our results it seems reasonable to conclude that G-6-P and Mg^{+2} are these metabolites (11, 21, 25). The addition of G-6-P alone starts the D to the I form conversion. The conversion to the I form becomes complete after 60-90 min of incubation in the presence of 5 mM Mg^{+2} and 1 mM sugar phosphate. Under the latter circumstances, no glycogen synthetase inactivation was observed. It seems probable that the effect of these metabolites was to effect a change in reconformation of glycogen synthetase in order to allow the phosphatase enzyme to act on D form.

The trapping of Mg^{+2} by EDTA leaves the G-6-P to act alone on activation of phosphatase and stabilization of glycogen synthetase.

Studies previously done by LELOIR and GOLDEMBERG (13) and by STEINER (23) using partially purified fractions of rat liver glycogen synthetase also showed a loss of activity when they were incubated at 37° C at least in the presence of G-6-P.

The D to I form conversion was inhibited by: ATP, UTP, glycogen and F^-. The effect when NaF was added to the protein-glycogen complex should be underscore. The phosphatase activity was completely inhibited by F^-, even when G-6-P was present. However, NaF, stabilized the synthetase D activity preventing the loss of activity as completely as G-6-P and Mg^{+2} did. The results probably indicate that more than one phosphatase enzyme takes part in glycogen synthetase dephosphorylation. Perhaps one phosphatase is more dependent on Mg^{+2} for its activity. The idea of different glycogen synthetase forms with two or more phosphates in their structure (22, 24) support this explanation. Accordingly the phosphatase removes one of the phosphates responsible for the D form activity, but not enough phosphates to complete the D to I conversion. The I to D conversion was observed when ATP and Mg^{+2} were added to the protein-glycogen complex and it increased when cAMP was also present.

The D and I glycogen synthetase forms of the shell-fish *Pecten maximus* have a different affinity for the substrate UDPG. Both increased activity by G-6-P, being the D form ten fold more dependent on G-6-P than the I form. The K_a values of these forms increased if ATP was present in the reaction. It is probably that the inhibitory effect of the ATP on the synthetase D hinders the phosphatase activity, however the inhibition of the D to I form conversion may be a direct effect of ATP on the phosphatase.

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Resumen

El enzima glucógeno sintetasa (EC.2.4.1.11) se encuentra presente en extractos de músculo aductor del molusco *Pecten maximus*. Se presenta predominantemente en forma D (dependiente de G-6-P para mostrar actividad). También se confirma la presencia de actividad independiente de G-6-P forma I. Los estudios cinéticos de la glucógeno sintetasa indican que los valores de Ka para la G-6-P de la forma D son 10 veces superiores a los de la forma I.

Ambas formas del enzima resultan interconvertibles por enzimas de tipo fosfatasa y quinasa, respectivamente. Se requiere la presencia de glucosa-6-P y Mg^{+2} para la estabilización de la glucógeno sintetasa y activación de la sintetasa D fosfatasa, presentes en el 90,000×*g* complejo glucógeno-proteína.

La conversión de sintetasa D en I es inhibida por F^-, glucógeno, ATP y UTP. El efecto del fluoruro, en presencia de G-6-P, sugiere la posible presencia de más de una glucógeno sintetasa fosfatasa implicada en dicha conversión.

La conversión de sintesa I en D requiere de la presencia de ATP y Mg^{+2} y resulta estimulada por cAMP.

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