IDENTIFICATION OF A THIRD FORM OF GLYCOGEN SYNTHETASE IN RAT CHLOROMA TUMORS

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1. Introduction

The glycogen synthetase (uridine diphosphate glucan-glycogen glucosyltransferase, EC 2.4.1.11) and phosphorylase (α-1,4 glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) of all animal tissues examined thus far exist in a phosphorylated and an unphosphorylated form (except mouse strain I_{FnLn} which apparently lacks an active phosphorylase kinase system [1]). In the case of glycogen phosphorylase, the phosphorylated form (phosphorylase *a*) is active independent of its activator 5′-AMP; while in that of glycogen synthetase, it is the unphosphorylated form which is active independent of its activator G-6-P. This active form of synthetase has been called independent or I-form as opposed to the phosphorylated dependent or D-form which is inactive in absence of the activator G-6-P.

Rat chloroma, a tumor composed of immature granulocytes and having many similarities to human myelocytic leukemia [2] was found to have a fairly efficient dephosphorylating system for glycogen phosphorylase [3, 4]. In sharp contrast, however, the present study shows that the dephosphorylation process in chloroma glycogen synthetase under similar conditions was negligible except when Mg^{2+} is added. Besides the unphosphorylated I-form, chloroma glycogen synthetase appears to exist in two G-6-P dependent forms which differ in their maximal velocity and their G-6-P affinity. While one form is maximally active at an activator (G-6-P) concentration of 6×10^3 M similar to the D-form in other tissues [5–9], the other form called D′ was found to be activated only when the activator concentration is increased by 10 fold.

2. Materials and methods

2.1. *Materials*

Glucose-6-P, UDPG, shellfish glycogen and fibrous DEAE-cellulose were obtained from Sigma Chemical Co., St. Louis, Mo., USA and ^{14}C-UDPG was obtained from New England Nuclear. The glycogen was further purified as previously described [10].

2.2. *Methods*

2.2.1. Preparation of chloroma tumors and enzyme

Two chloroma-bearing Sprague Dawley rats were obtained from Dr. W.C. Moloney, Peter Bent Brigham Hospital, Boston. Repeated transfer of the tumors was done by subcutaneous injection of whole chloroma cell suspension into 3 to 7 day old rats [3]. The tumors were homogenized in 3 volumes of 0.3 M 2-mercaptoethanol, 0.005 M EDTA, pH 7.0, in a Virtis 45 homogenizer for 90 sec. Glycogen synthetase activity was measured by a radioactive assay method [11] according to Chandler and Moore [12]. Enzyme purification procedure was the same as that employed by Yunis and Arimura [4] for purification of chloroma glycogen phosphorylase. Only one synthetase activity peak was obtained following the DEAE-cellulose column chromatography as shown in fig. 1. The enzyme remained mainly in the G-6-P

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Table 1
Glycogen synthetase activity at 0.0, 0.006, 0.06 and 0.12 M G-6-P (cpm/ml).

Incubation medium	No G-6-P	% Total Activity	C.006 M G-6-P	% Total Activity	0.06 M G-6-P	% Total Activity	0.12 M G-6-P	% Total Activity
0.0004 M ATP 0.002 M magnesium acetate 0.04 M mercaptoethanol 5% sucrose 0.025 M glycerophosphate pH 7.0.	1,500	4%	15,300	43%	27,900	80%	25,900	73%
0.01 M magnesium acetate 0.04 M mercaptoethanol 0.002 M EDTA 5% sucrose 0.05 M Tris pH 7.5.	24,700	70%	35,500	100%	38,800	109%	36,000	101%

Total enzyme activity is determined in presence of 0.006 M G-6-P after enzyme incubation and conversion to independent form with EDTA and Mg^{2+}. The activity is expressed as cpm per ml of enzyme solution. The incubation medium shown in the upper part of the table was made for the purpose of converting any independent synthetase present to the D-form. The activity in absence of G-6-P was only 10–15% of the total for enzyme assayed in homogenates (not incubated with Mg-ATP) or following purification. Freezing of chloroma tissue did not prevent the EDTA-magnesium stimulated conversion (in crude homogenates) to the G-6-P independent form as shown in the lower part of the table. The response to sodium sulfate of the D and I forms thus prepared is similar to that reported for liver [24].

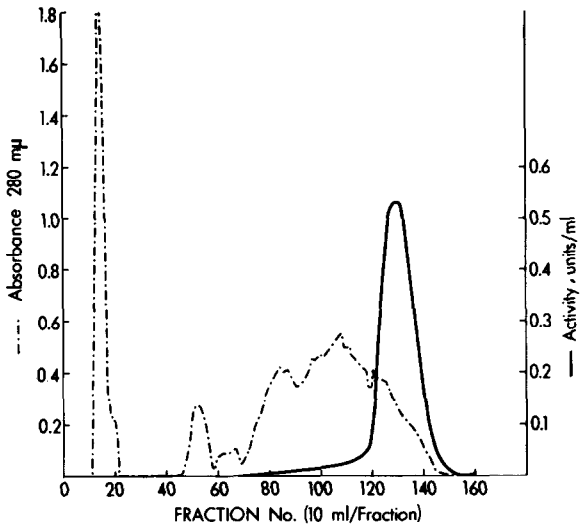


Fig. 1. Elution pattern of chloroma glycogen synthetase D-form from DEAE-cellulose column. Enzyme preparation and chromatography is similar to that published previously [4].

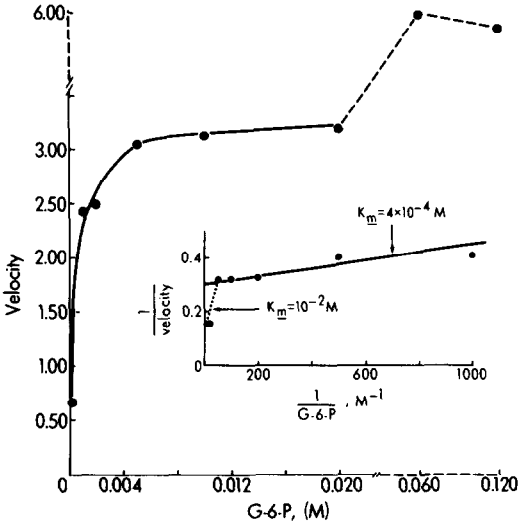


Fig. 2. Double reciprocal plot of purified chloroma glycogen synthetase with respect to its activator, G-6-P. The enzyme initial velocity was determined at pH 8.5 and 37° and expressed in units as described under Methods. The concentration of UDPG was fixed at 6.6×10^{-4} M final concentration.

dependent form throughout the purification. Specific activity increased from 0.008 units/mg* for the homogenate to 0.83 units/mg following purification with a 30% yield.

3. Results and discussion

In their polymorphonuclear leukocytes synthetase D ⇌ I interconversion studies, Rosell-Perez and coworkers [13, 14] noted a marked increase in the total enzymatic activity upon conversion to the I-form. They attributed this increase in total activity to the possible existence of an inactive synthetase form. Similar observations have been made previously on other tissues by other workers [9, 15].

The data shown in table 1 for chloroma glycogen synthetase also confirms the finding of other workers of an increase in the total activity (assayed in 0.006 M G-6-P) upon conversion of D → I. In addition this table illustrates that the activity of phosphorylated chloroma synthetase approaches that of the dephosphorylated form only when the phosphorylated enzyme is assayed at a G-6-P concentration of 0.06 M, which is ten times in excess of that known to be saturating for synthetase from other tissues [5–9]. These results were essentially the same (∓ 8%) in six separate rat chloroma extract preparations. This prompted further investigation of the purified enzyme. The data illustrated in fig. 2 for the purified phosphorylated enzyme showing non-linearity in the activator saturation curve and the double reciprocal plot suggest the presence of two inactive phosphorylated forms differing in their affinity for activator. One form denoted herein as D′ has a poor affinity for G-6-P (its K_m for G-6-P is approximately 50 times greater than that of the D-form and is maximally active at 0.06 M G-6-P). This situation is analogous to that found in glycogen phosphorylase which was also found to contain two activator dependent forms, denoted phosphorylase *b* and *c* by Cowgill and Cori [16] and Cowgill [17] or b_I and b_{II} by Assaf and Graves [18] and Assaf [19]. The two phosphorylase forms found in lobster muscle differed in their maximal velocities and their Michaelis constants for the activator 5′-AMP [17] and were separable by ion exchange chromatography [19]. As found in this work for chloroma synthetase, the two activator-dependent phosphorylase forms also differed in their optimal requirements for activator by ten fold. In studying the sedimentation velocity patterns of rabbit muscle glycogen phosphorylase *b* in presence of its activator 5′-AMP, Assaf and Yunis [20] demonstrated recently that the effect of this activator on enzyme structure extends beyond the kinetically determined saturating level of the nucleotide.

An alternative explanation to the non linearity observed here is that a phsophorylated form exists with two sites for activator. Negative cooperativity [21] between the two binding sites can result in two different affinities and two maximal velocities as was also observed by Yunis and Assaf [22] in bovine

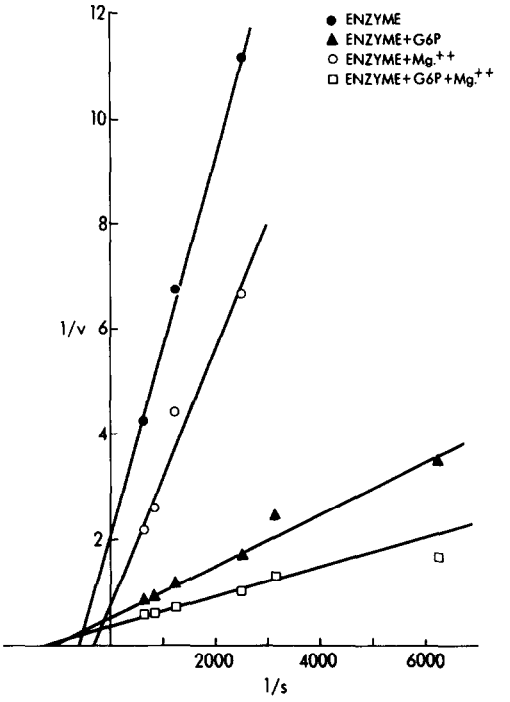


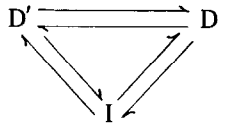
Fig. 3. Double reciprocal plot of purified chloroma glycogen synthetase with respect to its substrate, UDPG. Enzyme activity was determined at pH 8.5 and 37° and expressed in units as described under Methods. The concentration of G-6-P and magnesium chloride when present were fixed at 6.6×10^{-3} M and 6.6×10^{-2} M, respectively.

* Units are expressed as micromoles of ^{14}C-UDPG incorporated per 10 min per ml of enzyme solution.

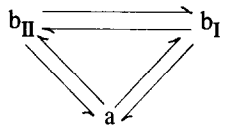
corpus luteum phosphorylase. In the case of corpus luteum phosphorylase, however, negative cooperativity was observed between the substrate (G-1-P) sites and not those of the activator. Kinetic studies on purified synthetase with respect to its substrate UDPG with and without the activator G-6-P gave a linear double reciprocal plot (see fig. 3) which shows that while G-6-P affects V_{max} it has little or no effect on the K_m for UDPG (K_m UDPG = 1 to 1.6 mM) suggesting little or no cooperativity between substrate binding sites under these conditions. Thus with respect to substrate, chloroma glycogen synthetase is not different from that of other synthetases [5, 9, 23].

Also of interest in this study was the dephosphorylation of phosphorylated chloroma synthetase. This enzyme could not be dephosphorylated by simply incubating in EDTA and mercaptoethanol as performed by Larner and co-workers for this enzyme from muscle of various animals [6] and as was successfully employed in dephosphorylation of chloroma glycogen phosphorylase [3]. On the other hand magnesium, which is known to stimulate the conversion of synthetase D → I in liver [9, 24, 25] and polymorphonuclear leukocytes [14, 26] was successful in the conversion of D → I in chloroma (see table 1), illustrating a significant difference in the dephosphorylation system of chloroma synthetase and phosphorylase.

The presence of more than one dependent form of glycogen phosphorylase and synthetase suggests that the regulation of glycogen synthesis and degradation must encompass a mechanism of control which involves a system other than simply the change from one dependent form to the independent form. Control of glycogen synthetase and phosphorylase activity by hormones and metabolites has been demonstrated by various workers [6, 27] and the presence of partially phosphorylated enzyme forms, demonstrated in phosphorylase, probably serves as another means for regulating enzymatic activity [27]. Irrespective of how control is exerted, the sequence of events for the turning "on and off" [8] of glycogen synthesis (in tissues containing the two G-6-P dependent forms) may involve three transformation steps depicted as follows:



This scheme is analogous to what presumably occurs in the interconversion of glycogen phosphorylase where:



Acknowledgement

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