Studies on UDPG-Glycogen Transglucosylase. II. Species Variation of Glucose-6-Phosphate Sensitivity of UDPG-Glycogen Transglucosylase*

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Received January 12, 1962

Muscle UDPG-glycogen transglucosylase activity in two species, *Opsanus tau* (toadfish) and *Rana pipiens* (frog), was studied. In both species the activities recovered in high yield in the 100,000 × *g* particulate fraction were glucose-6-P dependent, and in the case of the frog there was an absolute requirement for this co-factor. These activities were not stimulated in the absence of glucose-6-P by Mg^{++}. The kinetic constants for UDPG were different in the different species. These findings demonstrate a marked species variation in glycogen-synthesizing enzymes in nature.

In the preceding paper (Rosell-Perez *et al.*, 1962) the preparation and characteristics of two different activities of UDPG-glycogen transglucosylase[1] from rat muscle were reported. Studies were done with the toadfish (*Opsanus tau*) because initial experiments showed that the enzyme had a marked dependence on glucose-6-P for its action. A casual experiment with muscle from frog (*Rana pipiens*) revealed that this enzyme also was markedly dependent on glucose-6-P and led us to undertake further studies. After purification of the enzyme by ultracentrifugation, an absolute requirement for glucose-6-P by the enzyme from frog muscle was demonstrated. To compare these enzyme activities with the two activities from rat muscle, studies were done of the UDPG concentration dependence with and without glucose-6-P and Mg^{++} and of the behavior with SH reagents. These experiments are reported in this paper.

MATERIALS

Chemicals and enzyme assays were described in the preceding paper.

EXPERIMENTAL

Enzyme Preparations (Toadfish).—Two fish were received in the frozen state (dry ice) from the Marine Biological Laboratory, Woods Hole, Mass. After defrosting, the abdominal muscles were dissected and refrozen in liquid N_2-isopentane.[2] Aliquots of this frozen muscle were used for enzyme preparations. The frozen muscle was reduced to a powder in a stainless steel homogenizer cooled in liquid N_2. The powder was suspended (1:10 w/v) in 0.05 M Tris–0.005 M Versene buffer (*p*H 8.2) and was homogenized in a Potter-Elvejhem homogenizer which was chilled in ice. Samples of this homogenate were assayed (Step I). The homogenate was then centrifuged at 3,000 rpm in the International refrigerated centrifuge for 20 minutes. The supernatant fluid had the activity (Step II). An equal volume of 0.05 M Tris, 0.005 M Versene, and 1 M NaCl was added. The extract was recentrifuged for 2 hours in the Spinco centrifuge (Model L Preparative) at 100,000 × *g*. The small pellet that was obtained had the activity (Step III). In Table I the purification and recovery at each step are shown.

Enzyme Preparations (Frog).—The procedure used for preparation of the enzyme was similar to that used for the toadfish enzyme. Fresh muscle was used for preparing homogenates. Frogs were pithed, and the hind leg muscles were removed and homogenized (1:10 w/v) in 0.05 M Tris–0.005 M Versene buffer, *p*H 8.2, in a Potter-Elvejhem homogenizer as described above (Step I). The other two steps were the same as with toadfish and, as in that case, the supernatant fluids from the International centrifuge (Step II) were tested for transglucosylase activity before addition of the Tris-Versene-NaCl buffer. In Table II the purification and recovery at each step are shown.

Incubation with Mercaptoethanol. — Enzyme preparations (Step II) were incubated in 0.05 M mercaptoethanol for comparison with the enzyme from rat muscle. The activity of toadfish preparations increased during the incubation. It followed a time course somewhat similar to that observed with the enzyme from rat muscle. However, in the case of the frog preparation, the activity with added glucose-6-P increased markedly (more than 2-fold) during the incubation period. In neither case was there a marked change in the activity without added glucose-6-P, indicating no transformation to a glucose-6-P independent activity.[3] These experiments are presented in Figure 1.

* This work was supported in part by the Cleveland Fund, by the Fund for the Study of Diabetes and Related Metabolic Disorders, and by a grant from the National Institute of Arthritis and Metabolic Diseases of the U. S. Public Health Service (A-2366). A partial report of this work has been presented (Rosell-Perez and Larner, 1961).

[1] Abbreviations used are the same as those used in the preceding paper.

[2] We wish to express our gratitude to Drs. Paul Nace and S. J. Cooperstein for supplying the toadfish and for their help with the dissections.

TABLE I
PURIFICATION OF THE TOADFISH ENZYME[a]

Fraction	Volume (ml)	Activity (μmole/ml/hr)		Specific Activity (μmole/mg Prot/hr)		Recovery (%)	
		−G-6-P[b]	+G-6-P[c]	−G-6-P	+G-6-P	−G-6-P	+G-6-P
I. Homogenate (1:10 w/v)	11	4.3	19.5	0.03	0.14	100	100
II. Supernatant fluid (International)	10	3.1	16.1	0.13	0.7	65	75
III. 100,000 × g particulate fraction resuspended	8.9	0.96	17	0.33	6.0	18	70

[a] Purification steps as described in the text. [b] −G-6-P = activity measured in the absence of glucose-6-P. [c] +G-6-P = activity measured with added glucose-6-P.

TABLE II
PURIFICATION OF THE FROG ENZYME[a]

Fraction	Volume (ml)	Activity (μmole/ml/hr)		Specific Activity (μmole/mg Prot/hr)		Recovery (%)	
		−G-6-P[b]	+G-6-P[c]	−G-6-P	+G-6-P	−G-6-P	+G-6-P
I. Homogenate (1:10 w/v)	12	9.0	62.1	0.03	0.23	100	100
II. Supernatant fluid (International)	11	0.95	60.7	0.01	0.7	9.6	89
III. 100,000 × g particulate fraction resuspended	10.5	0	55.5	0	9.94	0	78

[a] Purification steps as described in the text. [b] −G-6-P = activity measured in the absence of glucose-6-P. [c] +G-6-P = activity measured with added glucose-6-P.

Kinetic Studies (Frog Enzyme).—The 100,000 × *g* particulate fraction (Table II, Step III) was resuspended in 0.05 M Tris–0.005 M Versene–0.05 M mercaptoethanol buffer. No activity was detected when the enzyme was tested in the absence of glucose-6-P with or without Mg^{++}. The enzyme was active, however, if glucose-6-P was added. If Mg^{++} was added in the presence of glucose-6-P, an additional stimulation was obtained.

In Figure 2 are shown the normal and reciprocal plots for the UDPG concentration dependence in the presence of glucose-6-P and glucose-6-P together with Mg^{++}.

Mg^{++} added to glucose-6-P increased V without a significant change in K_m. In Table III these constants are summarized.

Kinetic Studies (Toadfish Enzyme).—The effect of increasing UDPG concentration on enzyme activity was studied with the 100,000 × *g* particulate fraction (Table I, Step III). Little activity was detected in the absence of glucose-6-P. Mg^{++} alone did not stimulate this activity. Because the activity without glucose-6-P was so low, kinetic constants were not calculated. Activity was markedly stimulated by added glucose-6-P. Additional stimulation (about 30%) was noted when Mg^{++} was tested in the presence of glucose-6-P.

Figure 3 shows the normal and reciprocal plots for the UDPG concentration dependence of the enzyme from toadfish. The activity in the presence of glucose-6-P always had several points at low concentrations of UDPG that appeared to be out of line. Thus, the reciprocal plots were also non-linear. In the presence of Mg^{++} together with glucose-6-P the reciprocal plots were linear. The possible interpretation of these kinetics will be discussed later.

Table III presents the kinetic constants determined for the enzyme from toadfish (average of three experiments) and for the enzyme from frog (two experiments).

Inhibition by p-Hydroxymercuribenzoate.—Since the enzyme from frog was completely dependent on added glucose-6-P, the inhibition with *p*-hydroxymercuribenzoate was tested. It appeared that the enzyme from frog was less sensitive to this inhibitor than the enzyme from rat; *i.e.*, 1×10^{-5} M *p*-hydroxymercuribenzoate did not inhibit, and 7.5×10^{-5} M inhibited only 30% (in rat 4.5×10^{-5} M inhibited 100%). Activity was completely inhibited at 4×10^{-4} M *p*-hydroxymercuribenzoate. In the presence of 0.05 M mercaptoethanol, this concentration of *p*-hydroxymercuribenzoate did not inhibit.

[3] When the 100,000 × *g* particulate fraction was incubated in mercaptoethanol under similar conditions, no time-dependent activation was observed. (See footnote 5 in the previous paper.)

Discussion

In a preliminary experiment transglucosylase activity was studied in homogenates of various tissues from the toadfish. The activities with and without glucose-6-P at a single low concentration of UDPG indicated a marked dependence of the enzyme on this sugar phosphate.[4] After it was shown that a glucose-6-P dependent enzyme activity could be prepared from stored frozen rat muscle, we decided to investigate systematically the UDPG concentration dependence of the enzyme from toadfish muscle in the presence and absence of glucose-6-P. As we had observed high ratios of transglucosylase activity with and without glucose-6-P in frog muscle, this enzyme was also included in the present studies.

The enzyme from toadfish muscle that was associated with the 100,000 × *g* particulate fraction was stimulated about 18-fold when glucose-6-P was added. The activity without glucose-6-P was very low. The enzyme from frog muscle associated with the 100,000 × *g* particulate fraction was active only when glucose-6-P was added. In neither the toadfish nor the frog was the enzyme that was associated with the 100,000 × *g* particulate fraction stimulated by Mg^{++} in the absence of glucose-6-P. This lack of stimulation by Mg^{++} was similar to that observed with the glucose-6-P dependent activity from rat muscle (prepared after aging).

Although the enzyme activity in the 100,000 × *g* particulate fraction in the toadfish and frog, in its glucose-6-P dependence, was similar to the D activity prepared from rat skeletal muscle, some facts indicate that the enzymic proteins may be different. The apparent K_m for UDPG for the enzyme from frog muscle was 1.1–1.8 × 10^{-3} M, and for the enzyme from toadfish muscle 1.5–2.5 × 10^{-4} M. For the enzyme from rat muscle, the apparent K_m for UDPG of the D activity was 4 × 10^{-4} M, which is closer to that of the toadfish. Mg^{++} in the presence of glucose-6-P produced a greater increase in activity in the frog than in the toadfish (100% as compared to 30%). In the rat no such effect was observed. The sensitivity of the enzyme from frog muscle to *p*-hydroxymercuribenzoate was less than either the I or D activities from rat, but was closer to the D activity. When the enzyme was incubated with mercaptoethanol, the activation observed, especially in the case of the frog was considerably different from the rat. If with the enzyme from rat muscle the activation observed suggested a conversion of the activities, then in the frog the explanation would appear to be different. It might possibly be explained in the frog by an activation of an inactive enzyme or inactive precursor(s).

Analysis of glucose-6-P in supernatant fluids gave a value of 3 to 5 × 10^{-4} M per gram of muscle (wet weight). In spite of a similar glucose-6-P content to that observed in rat hemidiaphragms (Larner *et al.*, 1959), the enzyme activity differs markedly from that of the rat and in a

[4] This experiment was done by C. Villar-Palasi in collaboration with S. J. Cooperstein and A. Lazarow at the Marine Biological Laboratory, Woods Hole, Mass.

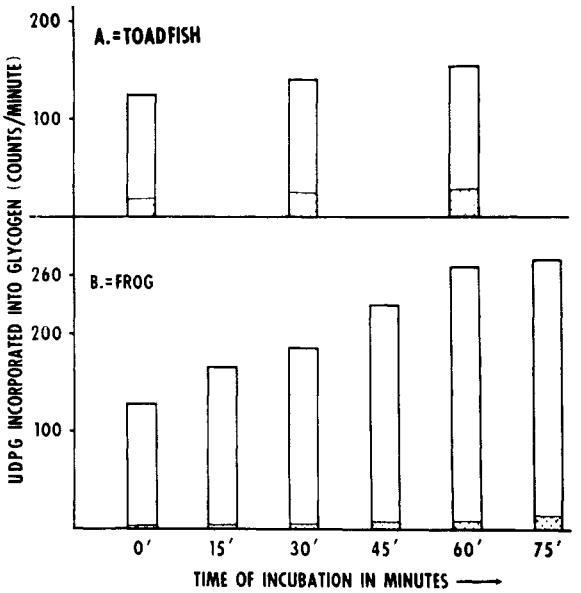

FIG. 1.—Time course of incubation of transglucosylase from frog and toadfish in the presence of 0.05 M mercaptoethanol. Total bars represent activity in the presence of added glucose-6-P. Cross-hatched bars represent activity without glucose-6-P.

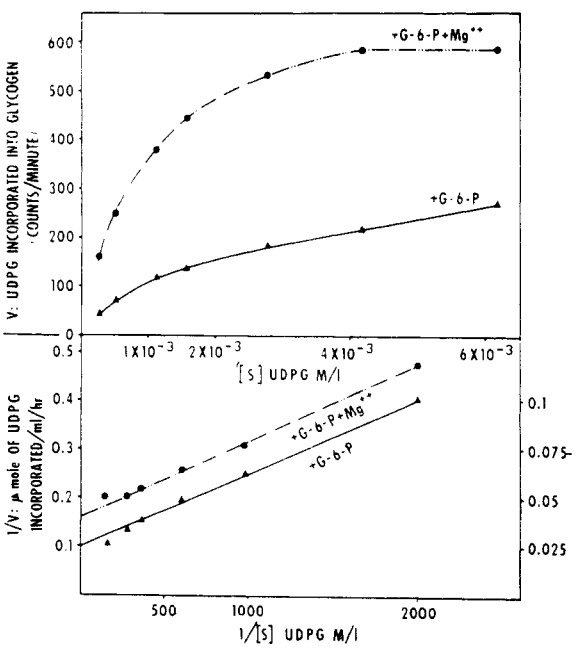

FIG. 2.—UDPG dependence of frog transglucosylase. In the reciprocal plot, the right scale relates to the plots of activity in the presence of glucose-6-P plus Mg^{++} and the left scale the plots of activity in the presence of glucose-6-P alone.

TABLE III
KINETIC CONSTANTS FOR UDPG-GLYCOGEN TRANSGLUCOSYLASE[a]

	K_m (M UDPG)		V (μmole/mg Prot/hr)	
Enzymes	+G-6-P	+G-6-P+Mg^{++}	+G-6-P	+G-6-P+Mg^{++}
Frog	1.8×10^{-3}	1.1×10^{-3}	22.7	54.2
Toadfish	2.5×10^{-4}	1.5×10^{-4}	10.6	14.3

[a] Constants were determined in the presence of glucose-6-P.

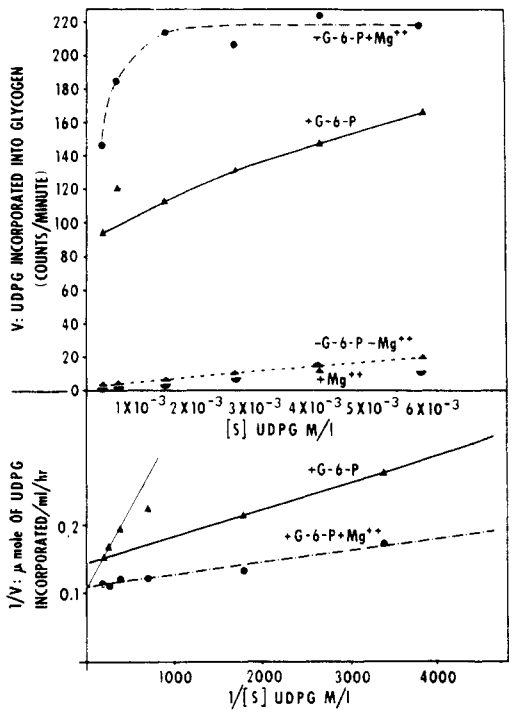

FIG. 3.— UDPG dependence of transglucosylase from toadfish.

manner which does not appear related to the glucose-6-P content.[5]

It is shown here that a marked species variation exists in the type of glycogen-synthesizing enzyme. Surveys on the zoological scale would be of great interest and would serve perhaps to clarify some of the mechanistic points not known as yet. It is of interest to note that Dubowitz and Pearse (1960) found histochemical differences in the enzyme complement of the muscle fibers of toad and fish as compared with those of rat, pigeon, and humans.

[5] Low activity in the absence of added glucose-6-P was observed in toadfish and frog muscle homogenates. This activity was lost mainly after the first centrifugation (Table II, Step II) in the case of the frog and after the second centrifugation (Table I, Step III) in the case of the toadfish. In the latter case it appears reasonable that the loss may be due to the endogenous glucose-6-P present in the supernatant fluid that was removed. In the former case the loss may be explained either by the removal of glucose-6-P that was trapped or bound with the protein precipitate or by the removal of a glucose-6-P independent activity present in small amounts.

Trivelloni (1960) and Hess and Pearse (1961) recently noted that the enzyme in locust is not stimulated by added glucose-6-P. The latter authors found by histochemical methods that the mammalian enzyme was stimulated by glucose-6-P. These findings, together with the present report which demonstrates an absolute requirement for this co-factor in the frog, suggest that in nature the enzyme exists in a form that requires glucose-6-P for its activity, as well as in a form that apparently does not require this co-factor.

With regard to the unusual kinetic curve of activity in the presence of glucose-6-P in toadfish enzyme preparations, studies by Reiner (1959) indicate that this may be explained by the behavior of enzymes with more than one active site in the molecule. Further studies with more purified preparations may clarify this question.

The species variation reported here and also the existence of two separate activities of UDPG-glycogen transglucosylase demonstrated in the preceding paper explain some of the controversial reports on the stimulation of this enzyme by glucose-6-P and Mg^{++} (Leloir and Goldemberg, 1960; Leloir *et al.*, 1959; Hauk and Brown, 1959).

ACKNOWLEDGMENT

The authors wish to express their thanks to Mr. Norman E. Brown and to Miss Helene Sasko for their technical assistance in these studies.

REFERENCES

Dubowitz, V., and Pearse, A. G. E. (1960), *Nature 185*, 701.
Hauk, R., and Brown, D. H. (1959), *Biochim. et Biophys. Acta 33*, 556.
Hess, R., and Pearse, A. G. E. (1961), *Enzymol. Biol. Clin. 1*, 15.
Larner, J., Villar-Palasi, C., and Richman, D. J. (1959), *Arch. Biochem. Biophys. 86*, 56.
Leloir, L. F., Olavarria, T. M., Goldemberg, S. H., and Carminatti, M. (1959), *Arch. Biochem. Biophys. 81*, 508.
Leloir, L. F., and Goldemberg, S. H. (1960), *J. Biol. Chem. 235*, 919.
Reiner, J. M. (1959), Behavior of Enzyme Systems, Minneapolis, Burgess Publishing Company, Chap. 4.
Rosell-Perez, M., and Larner, J. (1961), Abstracts of Papers, 140th Meeting of the American Chemical Society, 12 D.
Rosell-Perez, M., Villar-Palasi, C., and Larner, J (1962), *Biochemistry 1*, 763.
Trivelloni, J. C. (1960), *Arch. Biochem. Biophys. 89*, 149.