PHOSPHOGLUCOMUTASE AND PHOSPHOHEXOSE ISOMERASE IN TUNICATES*

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The tunicates, sessile marine animals of the subphylum Urochorda (1), are unique in the animal kingdom in that they synthesize cellulose (2). An investigation of the pattern of carbohydrate metabolism in these animals has been undertaken as a first step in the direction of studying the biosynthesis of cellulose. The present paper deals with some of the properties of phosphoglucomutase and phosphohexose isomerase in the tissues of Molgula (the sea-squirt).

Materials and Methods

α-Glucose-1-phosphate (G-1-P) was prepared from potato starch by incubation with potato phosphorylase and inorganic phosphate. A potassium salt recrystallized three times was used. β-Glucose-1-phosphate was kindly supplied by Dr. Frank J. Reithel. Fructose-6-phosphate (F-6-P) was purchased from the Nutritional Biochemicals Corporation. Glucose-6-phosphate (G-6-P) was prepared by crystallization of the barium salt produced on incubation of barium F-6-P with a dialyzed extract of rabbit muscle or with purified rabbit muscle isomerase, according to an unpublished method of Horecker. Recrystallization was accomplished by suspending the salt in a small volume of water, removing barium with H₂SO₄, and then neutralizing to pH 8.2 with Ba(OH)₂. Two such recrystallizations were used for purification. Adenosinetriphosphate (ATP) was purchased from the Pabst Laboratories. Glucose, fructose, sucrose, and cellobiose of c.p. grade were purchased from the Pfanstiehl Chemical Company. Versene (the disodium salt of ethylenediaminetetraacetic acid), analytical grade, was a gift from the Bersworth Chemical Company. Samples of Molgula were collected from a float in the harbor at Harwichport, Massachusetts, or were purchased from the Marine Biological Laboratory, Woods Hole, Massa-

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1 Personal communication from Dr. E. W. Sutherland.
2 Personal communication from Dr. B. L. Horecker.
3 The commonest species in this area is Molgula manhattensis, but no attempt was made to identify the species used.
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chusetts. Other materials and analytical methods used were described previously (3). All incubations were carried out at 30°.

Preparation of Extracts of Molgula—The live animals were used within 24 hours of collection. The tunic was incised and the body extruded and separated from the tunic, and the latter was then usually discarded. The body cavity was opened and the viscera removed as thoroughly as possible. The remaining tissue was rinsed in distilled water and then used for preparation of the extracts. From 100 animals about 75 ml. of loosely packed tissue, chiefly muscle, were usually obtained. To this were added 10 ml. each of cold 0.5 M KCl, 0.5 M KHCO₃, 100 ml. of cold distilled water, and enough sand to make a loose paste, which was then pounded for several minutes in a chilled mortar, and left at 3° for 30 minutes with occasional grinding. The solid was removed by high speed centrifugation in a Servall SS-1 centrifuge, and then reextracted with a mixture of 1 ml. of KCl, 1 ml. of KHCO₃, and 50 ml. of water. Usually a third extraction was carried out in the same way. The extracts were combined (volume, 250 ml.) and dialyzed overnight against 10 liters of distilled water at 3°. The resulting solutions contained 0.1 to 0.3 per cent protein. The extracts were frozen when not in use, and were found to lose activity slowly over a period of a few months when kept in this way. Storage at 3° resulted in a much more rapid loss of enzymatic activity.

Phosphoglucomutase—Phosphoglucomutase activity was invariably present. The test system was based on that described by Najjar (4). An enzyme solution was prepared containing 100 µM of tris(hydroxymethyl) aminomethane (Tris), 50 µM of cysteine, 50 µM of MgCl₂, and 3.0 ml. of dialyzed extract to give a final volume of 5.90 ml. and a pH of 7.6. Aliquots of 1.00 ml. were added to a series of tubes each containing 0.10 ml. of a 0.02 M solution of G-1-P. The reaction was stopped by addition of 5.0 ml. of 6 per cent perchloric acid, and the extent of the reaction was measured by determination of the decrease in acid-labile phosphate (inorganic phosphate liberated in 6 minutes at 100° in 1 N H₂SO₄). At the same time, similar experiments were carried out in which MgCl₂ or cysteine or both were omitted. The result of such an experiment is shown in Fig. 1, A. It can be seen that full activity is not obtained unless both cysteine and Mg²⁺ are added. In the absence of these two activators the activity was greatly diminished but was never entirely abolished, even on prolonged dialysis against several changes of water. In the presence of 0.005 M Versene, however, there was no conversion of acid-labile phosphate to an acid-stable form, indicating that a tightly bound divalent cation present in the extract was activating the enzyme.

The residual activity was eliminated as follows: to 75 ml. of the dialyzed extract were added 8.3 ml. of a solution of Versene (0.10 M, pH 7.6) and the solution was then dialyzed for 6 hours against 3.5 liters of 0.01 M NaCl at
3°. The enzyme activity was tested as described above. In the absence of Mg\textsuperscript{++} and cysteine, and when cysteine was added alone, the enzyme was inactive, but activity was restored by the addition of Mg\textsuperscript{++}. In tests of other divalent cations as possible activators, cysteine was found to cause precipitation of some of these, and the effect of cysteine on the Versene-treated extract was investigated. Whereas cysteine was required for full activity before treatment with Versene, after such treatment cysteine not only was without enhancing effect in the presence of Mg\textsuperscript{++} but actually showed small but definite inhibition. This is shown in Fig. 1, B. Consequently, cysteine was omitted in the later experiments. The activation of the enzyme by Mg\textsuperscript{++} is shown in Fig. 2. There is a broad optimal range between 10\textsuperscript{-2} and 10\textsuperscript{-3} M. Several other divalent cations were tested. Of these, Ba\textsuperscript{++} was found to be definitely stimulatory with a sharp optimum at 3 \times 10\textsuperscript{-3} M (Fig. 3, A), while Mn\textsuperscript{++}, Co\textsuperscript{++}, Ni\textsuperscript{++}, Cu\textsuperscript{++}, Zn\textsuperscript{++}, and Ca\textsuperscript{++} at concentrations of 2 \times 10\textsuperscript{-2} M were inactive.\textsuperscript{4} The possibility that Ba\textsuperscript{++} and Mg\textsuperscript{++} might activate in different ways was investigated by carrying out the reaction\textsuperscript{4} with the Versene-treated extract in the presence of different mixtures of Ba\textsuperscript{++} and Mg\textsuperscript{++} at a total concentration of 3 \times 10\textsuperscript{-3} M. The result of such an experiment is shown in Fig. 3, B. There appears to be neither inhibition nor reinforcement of the effect of one ion by the other.

The affinity of the enzyme for Mg\textsuperscript{++} and G-1-P (Fig. 4) was determined

\textsuperscript{4} In these experiments the hydrolysis of glucose-1-phosphate was carried out in 1 N perchloric acid.
by the method of Lineweaver and Burk (5). The locations of the best straight lines for the points obtained were calculated by the method of least squares. $K_m$ for Mg$^{++}$ was found to be $1.2 \times 10^{-4}$ and for G-1-P was $4.8 \times 10^{-4}$.

**Fig. 2.** Activation of phosphoglucomutase by Mg$^{++}$. The incubation mixtures contained G-1-P ($2 \times 10^{-3}$ M) and Tris ($2 \times 10^{-2}$ M), pH 7.6. A Versene-treated extract was used and cysteine was omitted.

**Fig. 3.** Activation of phosphoglucomutase by Mg$^{++}$ and Ba$^{++}$. Incubation mixtures without cysteine. In A, the single symbol marked Mg represents the activity of the same enzyme preparation in the presence of Mg$^{++}$ ($3 \times 10^{-3}$ M) with no added Ba$^{++}$. In B, the activating effect of Mg$^{++}$-Ba$^{++}$ mixtures is shown; total concentration of divalent cation = $3 \times 10^{-3}$ M.

The effect of varying pH was determined by carrying out the reaction in solutions buffered with Tris-succinate mixtures. Tris and succinic acid were mixed in equimolar proportions and a series of buffer solutions was prepared by adjusting the pH with KOH. The actual pH of the incubation mixtures was determined with a glass electrode. This experiment (Fig. 5) showed an optimal range between pH 7.0 and 7.5.
Najjar (4) has reported that crystalline phosphoglucomutase of rabbit muscle is inhibited by fluoride in a way which suggests the formation of an inactive complex of Mg++, G-1-P, and fluoride ion. He reports the inhibition constant for this reaction to be $1.7 \times 10^{-12}$. Similar experiments were carried out with the *Molgula* phosphoglucomutase. With concentrations of $\text{Mg}^{++} = 10^{-3}$ M and $\text{G-1-P} = 1.44 \times 10^{-3}$ M, $10^{-3}$ M fluoride was found to produce 28 per cent inhibition and $2 \times 10^{-3}$ M fluoride to produce 46 per cent inhibition. The inhibition constants for these concentrations were $3.7 \times 10^{-12}$ and $6.8 \times 10^{-12}$, respectively, indicating an effect of the same order of magnitude as that described by Najjar.

The absence of cysteine from the reaction mixtures made it possible to study the role of protein -SH groups in the active site of the enzyme. The enzyme activity was not affected when $3 \times 10^{-3}$ M iodoacetate was present in the incubated mixtures, nor when the enzyme was incubated in the presence of $4 \times 10^{-3}$ M iodoacetate for 15 minutes before addition of substrate to start the reaction (Fig. 6).

In the preparation of phosphoglucomutase from rabbit muscle (4) a large volume of protein solution is heated to 65° without destroying the desired enzymatic activity. Phosphoglucomutase of yeast shows similar stability under these conditions. The phosphoglucomutase of *Molgula* is more labile. A 5 ml. portion of the extract was adjusted to pH 5, heated to 63°, and cooled rapidly in ice, the time from the start of heating until

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A. J. Guarino and H. Z. Sable, unpublished experiments.
the temperature returned to 10° or lower being about 5 minutes. Under these conditions a moderately large precipitate formed, but most of the phosphoglucomutase activity remained in the solution. When larger quantities of extract were heated, however, the total heating and cooling time was prolonged to about 15 minutes, a period which still was shorter than that used in the preparation of the enzyme from rabbit muscle. This longer heating period led to complete inactivation of the Molgula phosphoglucomutase. The enzyme of Molgula is therefore considerably more heat-labile than the corresponding enzyme of rabbit muscle and yeast.

![Fig. 5](image)

**Fig. 5.** Variation of phosphoglucomutase activity with pH. G-1-P, 1.1 \times 10^{-3} M; Mg^{++}, 4.7 \times 10^{-3} M. Tris and succinate, each 1.6 \times 10^{-2} M.

![Fig. 6](image)

**Fig. 6.** Lack of effect of iodoacetate on phosphoglucomutase. Incubation mixture without cysteine. Mg^{++}, 1.7 \times 10^{-3} M. ○, no iodoacetate; ○, 3 \times 10^{-3} M iodoacetate present in incubation mixture; ×, enzyme solution preincubated in 4 \times 10^{-4} M iodoacetate for 15 minutes before addition of G-1-P.

That the product of the reaction was actually glucose-6-phosphate was established by analysis of the incubated mixtures with *Zwischenferment*. The usual incubation was carried out, deproteinization being accomplished by addition of 0.5 volume of 2.5 per cent HgCl₂ in 0.5 M HCl. Excess mercury was removed with H₂S and the filtrates aerated and neutralized. Phosphate analysis indicated the formation of 1.51 \muM of an acid-stable phosphate ester. Analysis with *Zwischenferment* indicated the presence of 1.14 \muM of G-6-P, which accounted for 76 per cent of the acid-stable ester. Since the *Zwischenferment* preparation used was practically free of phosphohexose isomerase and the *Molgula* extract was rich in isomerase (see below), the acid-stable ester formed from G-1-P must be G-6-P.
When inorganic phosphate was incubated with the Molgula extracts, there was no formation of acid-stable phosphate. Likewise, ribose-1-phosphate and β-glucose-1-phosphate were not acted upon by the extracts. An attempt was made to prepare enzymatically active extracts from the integument. The inner surfaces of a large number of tunics were separated from the outer fibrous layer and extracted as described above. The resulting solutions were incubated with α- and β-glucose-1-phosphate, but did not catalyze the formation of acid-stable esters.

Phosphohexose Isomerase—The difference between the amount of acid-stable phosphate ester formed and the amount of G-6-P found on analysis with Zwischenferment suggested the presence of a highly active phosphohexose isomerase. Direct evidence for the presence of this enzyme was obtained by incubation of F-6-P with 0.5 ml. aliquots of the Molgula extract. The reaction was stopped with perchloric acid and aliquots of the protein-free filtrates were analyzed by the resorcinol test of Roe (6), equilibrium being reached when about 65 per cent of the fructose-reactive material initially present had been converted to an unreactive form. When G-6-P was incubated with the extract, fructose-reactive material appeared until about 35 per cent of the G-6-P was converted (Fig. 7).

Prolonged dialysis of the extracts led to complete inactivation of the isomerase without affecting the phosphoglucomutase. No evidence could

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Fig. 7. Phosphohexose isomerase in dialyzed extract. Upper curve, Tris, 0.03 M, pH 7.6; F-6-P, 2 \times 10^{-3} M. Lower curve, Tris, 0.04 M, pH 7.6; G-6-P, 3 \times 10^{-3} M.

\* In these analyses we have taken account of the fact that F-6-P gives considerably less color than an equimolar amount of free fructose. We have used the factor 0.75 to correct for this difference.
be found for metal activation or for the presence in the extracts of a dialyzable cofactor for the phosphohexose isomerase reaction.

The extracts were tested for hexokinase in the usual way (7), but no evidence was found for the phosphorylation of glucose by ATP, as measured by reducing power of Ba(OH)$_2$-ZnSO$_4$ filtrates. The extracts did not catalyze the hydrolysis or arsenuyolysis of cellobiose.

**DISCUSSION**

Using rabbit muscle phosphoglucomutase, Sutherland (8) found that cysteine could be replaced by various metal-binding agents and that pre-incubation with cysteine decreased enzymatic activity. This indicated that cysteine probably was not concerned with the regeneration or protection of protein —SH groups required for enzyme action. From the present work it is apparent that, once the enzyme solution has been treated with a metal-binding agent to remove contaminating cations, cysteine is not only unnecessary but actually is slightly inhibitory, probably because it forms chelate compounds with the activating cation, and thus effectively removes an appreciable amount of it. Krimsky and Racker (9) found that they could dispense with cysteine in the oxidation of glyceraldehyde-3-phosphate by the dehydrogenase of rabbit muscle if they added Versene or KCN to the protein solution during isolation of the enzyme; under these circumstances the enzyme was obtained with the necessary protein —SH groups already in the reduced form. The lack of inhibition of phosphoglucomutase by iodoacetate, however, indicates that protein —SH groups are not necessary parts of the catalytically active site of this protein.

Phosphoglucomutase has been shown to be activated by $\alpha$-glucose-1,6-diphosphate (10-12). No special precautions (such as fractional crystallization or ion exchange chromatography) were taken to remove any hexose diphosphate from the G-1-P used, and no tests were made to detect the presence of glucose-1,6-diphosphate in the extracts or in the G-1-P. It is reasonable to assume that the mechanism of the phosphoglucomutase reaction is the same regardless of the source of the enzyme, but, in the absence of direct tests, no conclusions can be drawn.

**SUMMARY**

Soluble protein extracts of the marine organism *Molgula* contain phosphoglucomutase and phosphohexose isomerase.

The phosphoglucomutase requires the presence of Mg$^{++}$ or Ba$^{++}$. Other divalent cations do not activate this enzyme. After treatment with a metal-binding agent, the phosphoglucomutase is fully active in the absence of cysteine and is not affected by iodoacetate. The pH optimum and the affinity constants for Mg$^{++}$ and G-1-P have been determined.
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