The Mechanism of Utilization of Polyphosphate by Polyphosphate Glucokinase from Propionibacterium shermanii

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Catherine A. Pepin and Harland G. Wood
From the Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106

The phosphorylation of glucose by polyphosphate glucokinase with both long- and short-chain polyphosphates has been shown to occur by either a nonprocessive mechanism, i.e. with repeated association and dissociation of the polyphosphate from the enzyme after each phosphorylation or by a quasiprocessive mechanism in which several phosphorylations occur prior to the release of polyphosphate and the reassociation with the enzyme. In contrast, the phosphorylation of ADP to ATP by polyphosphate kinase is by a strictly processive mechanism; the phosphorylation occurs without release of the polymer from the enzyme prior to termination of the reaction (Robinson, N. A., Clark, J. E., and Wood, H. G. (1987) J. Biol. Chem. 262, 5216–5222). The demonstration that the mechanism is quasiprocessive or nonprocessive was accomplished by electrophoresis using a variety of concentrations of polyacrylamide gels which made it possible to detect the intermediate sizes formed during the reactions. It also has been shown that all chains longer than about 100 are used simultaneously, but with chains of less than 100 residues, there is preferential utilization of the longest chains. Thus a narrow range of sizes is formed from a heterogeneous mixture of long chains. It is this formation of the narrow range of sizes that makes it possible to use polyphosphate glucokinase for the determination of the average size of long chains (Pepin, C. A., Wood, H. G., and Robinson, N. A. (1986) Biochem. Int. 12, 111–123).

Polymers of orthophosphate which contain phosphonohyde linkages thermodynamically equivalent to the high energy phosphates of ATP. They have been detected in a wide variety of sources from bacteria to mammals (1). Our laboratory has undertaken the study of polyphosphate-utilizing enzymes found in Propionibacterium shermanii, specifically poly(P) glucokinase which catalyzes the phosphorylation of glucose without the involvement of ATP (Reaction 1) and poly(P) kinase which catalyzes the synthesis and utilization of poly(P) (Reaction 2).

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\text{Glucose} + \text{poly(P)}_n \rightarrow \text{glucose-6-phosphate} + \text{poly(P)}_{n-1} \tag{1}
\]

\[
\text{ATP} + \text{poly(P)}_n \rightleftharpoons \text{ADP} + \text{poly(P)}_{n+1} \tag{2}
\]

Previously, we have investigated the mechanism of poly(P) glucokinase by gel electrophoresis to determine the change in the size of the poly(P) during the course of the reaction (2). With long-chain poly(P) (Maddrell salt, having an average chain length of about 450 residues), no intermediate sizes were observed prior to a chain length of about 100. It was concluded that there is repeated phosphorylation of glucose without release of the poly(P) from the enzyme until it reaches about 100 residues. The Km for poly(P) by poly(P) glucokinase was determined using a wide span of sizes of poly(P). A remarkable decrease in Km was observed with increase in chain length, the Km being 2000-fold lower with poly(P) of an average chain length of 724 than with an average chain length of 30. There was a gradual increase in Km with decrease in the size of poly(P) and then, at a chain length of about 100, a very rapid increase. This change in Km is in accord with the observed accumulation of poly(P) at a chain length of about 100. It is considered that the short chains cannot compete with long chains that have a high affinity (low Km) for the active site on the enzyme and therefore the short chains accumulate and the long chains are used preferentially. When poly(P) of chain lengths shorter than 100 residues were used as substrates, it was found that there is a progressive decrease in the size of the poly(P) and if a mixture of sizes were used, the longer chains were used preferentially.

A limitation of this process was the use of only 15% polyacrylamide gels for the electrophoresis. It was realized that there is limited resolution of long-chain poly(P) with this gel and therefore it was considered that the formation of intermediate sizes from long chains prior to chain length 100 might have escaped detection (2). Clark and Wood (3) have demonstrated that different sizes of long-chain polyphosphates can be resolved and sized by electrophoresis in decreasing concentrations of polyacrylamide and agarose gels. We have now applied these methods in our investigations. We find that the mechanism is not strictly processive with long chains; it is either quasiprocessive or nonprocessive. We define a strictly processive process as one in which following initiation, the polymer is not released from the enzyme until the reaction is terminated and a nonprocessive process as one in which the poly(P) is released from the enzyme after each phosphorylation of glucose to glucose 6-phosphate. Between these two extremes is the quasiprocessive mechanism in which the poly(P) is released from the enzyme after more than one phosphorylation and the released poly(P) then reassociates with the enzyme with continuation of the reaction. This dissociation-reassociation could occur several times prior to the final termination.

EXPERIMENTAL PROCEDURES

Materials—Acrylamide A grade (two times crystallized) was from Accurate Chemical and Scientific Corp. (Westbury, NY); N,N'-methylenebisacrylamide was from Bio-Rad and xylene cyanol FF was from Eastman Kodak; bromphenol blue from Fisher; and toluidine blue O, glucose-6-phosphate dehydrogenase, NADP, sodium poly-
phosphate glass type 75*, and Maddrell salt (insoluble long-chain poly(P), P-8635) were from Sigma. Maddrell salt was solubilized using Van Wazer's method (5). Reagent grade phenol was distilled before use. The poly(P) glucokinase was a partially purified preparation from *P. shermanii* (2). All chemicals were reagent grade.

Poly(P) Glucokinase Assay—The formation of glucose 6-phosphate was determined spectrophotometrically. The assay mixture (500 μl) contained 7.6 mM MgCl₂, 7.6 mM glucose, 150 mM Tris-HCl, pH 7.5, 0.5 mM NADP, 0.36 units of glucose-6-phosphate dehydrogenase and polyphosphate glucokinase. The reaction was initiated with 10 μl of 0.05 mM poly(P) type 75* (1.7 mM final concentration on the basis of total poly(P)). The reaction was monitored at 340 nm using a Gilford spectrophotometer. One unit of activity is defined as micromoles of glucose 6-phosphate produced per min.

Polycyramide Gel Electrophoresis—A variety of polycyramide slab gels were used which contained a 20:1 ratio of acrylamide to bisacrylamide. The 15, 10, 4, and 2% (w/v) polyacrylamide gels were either 0.5 or 1.8 × 180 × 400 mm; the 4% and the 2% polycyramide gels both contained 0.5% agarose; see Ref. 3 for specific directions for the preparation of mixed polycyramide and agarose gels. To remove contaminating ions, the 15% and 10% polycyramide gels were pre-electrophoresed at 800 volts and the 4% and 2% polycyramide gels with 0.5% agarose at 100 volts for 1 h. Unless otherwise indicated, the electrophoresis buffer was 90 mM Tris borate, pH 8.3, with 2.7 mM EDTA. Additionally, urea was omitted from the gels unless otherwise noted.

Isolation of Poly(P) Containing a Narrow Range of Sizes—Two independent procedures were used for isolation of discrete sizes of poly(P). A 200-ml Sepharose 6B column was equilibrated and eluted in 145 mM NaCl, 50 mM Tris, pH 7.6, and 4 mM EDTA. Solubilized Maddrell salt (1.8 g) was dissolved in 2 ml of water and loaded onto the column. The average chain length of Maddrell salt is 450 residues (2). Fractions were collected and concentrations were determined by the toluidine blue 0 assay (6). Five μg of poly(P) from each fraction was loaded onto a 4% (201) polyacrylamide gel with 0.5% agarose and standards for estimation of the size (3). A second method involved electrophoresis on a preparative 2% polyacrylamide gel containing 0.5% agarose (3.5 × 160 × 400 mm); see Clark and Wood (3) for details. The size of each isolated polyphosphate fraction was determined by electrophoresis along with standards (3).

Isolation of Poly(P) from Reactions—The isolation from reactions with poly(P) glucokinase and preparation of the poly(P) for gel electrophoresis were done as described previously (2).

RESULTS

The experiment of Fig. 1, Panel A, was done with poly(P) with an average chain length of 700. During the reaction, samples were removed, the poly(P) was isolated and then electrophoresed into a 2% polycyramide gel, which gives linearities of migrations between chain lengths 780 and 380 (Panel D). Lane 1 was from zero time and lanes 3–11 with poly(P) from the reaction at 5–50 min. It is evident that a large span of sizes of poly(P) were formed ranging down to chain lengths of 320 or less. Clearly, the mechanism is not strictly processive.

The experiment in Panel B was done with poly(P) with an average size of 230. A 10% polycyramide gel was used which gives linearity from 210 to 60 residues (3). Lane 2 was from zero time and lanes 3 to 12 was poly(P) from the reaction at 3–20 min. It is apparent that with time a wide variety of sizes were formed and that there was an accumulation of the shortened poly(P) of chain length about 100 during the reaction.

It had been observed previously (2) with polyphosphates of chain length less than 100 that the longer chains are used preferentially prior to the utilization of shorter chains. The experiments of Fig. 2 were done to determine whether with long-chain poly(P) there is preferential use of the shorter chains. Poly(P) with average chain lengths of 700 and 210 were added simultaneously as substrates in the reaction. For this evaluation, four concentrations of polycyramide were used which give linearity over a range of sizes (3). In each case, lane 1 is the zero time and lanes 2–10 are the poly(P) isolated from the reaction at intervals from 8 to 71 min. It is clear that with all concentrations of polycyramide that both sizes of poly(P) were used simultaneously. It is also evident that electrophoresis in more than one concentration of polycyramide is required to obtain a clear picture of what is happening with the different sizes of poly(P). For poly(P) of chain length 700, the 2% polycyramide with linearity from 780 to 380 (Panel D) is most informative; it shows that the concentration of the longer chains are decreasing and a large span of sizes are formed. With the other concentrations of polycyramide (Panels A, C, and D), little information was obtained concerning the poly(P) in which the long chains remain stacked at the top of the gel.

The formation of intermediate sizes from poly(P) (320) is apparent in all the gels but only the 10 and 15% gels are appropriate for the evaluation of the accumulated shortened poly(P). Utilization of the chains shortened to approximately 100 residues occurred only when the longer chains had been completely consumed; see lanes 9 and 10 of Panel A.

The experiment of Fig. 3 was with poly(P) of average chain length 700 and 52 as substrates. Aliquots of the poly(P) from the reaction were electrophoresed at intervals from 20 to 120 min into a 15% polycyramide gel. It is seen that the poly(P) formed from the poly(P) accumulates at a chain length of about 100 and only after all the long-chain poly(P) (lane 12) has been utilized is the poly(P) of chain length 100 utilized. After it is utilized, then the utilization of the poly(P) of chain length 52 becomes active (lane 13).

**DISCUSSION**

It is clear from the experiments of Figs. 1 and 2 that intermediate sizes of poly(P) are formed by poly(P) glucoki-
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The reaction mixture contained 7.6 mM glucose, 150 mM Tris-HCl, pH 7.5, 0.0528 units of poly(P) glucokinase, and 440 ng of each of the two poly(P) substrates of average chain lengths 700 and 210 in a volume of 14.4 ml. Four 300-μl aliquots were removed at each time point and prepared for gel electrophoresis. Electrophoresis was with four different percentages of polyacrylamide gels. Lane 1 was from zero time and lanes 2-10 with poly(P) isolated after 8, 16, 24, 30, 38, 46, 62, and 71 min, respectively. Polyacrylamide gels of 15, 10, and 4% with 0.5% agarose and 0.3% agarose were used for Panels A, B, C, and D, respectively. The running buffer for the 4% and 2% mixed polyacrylamide gels contained Tris/aceta-
tate/NaCl/EDTA (40:20:182 mM), pH 7.5, 0.52 units of poly(P) glucokinase, and 120 ng of each poly(P) species.

FIG. 2. Demonstration with poly(P) of average chain lengths 700 and 210 that both are utilized simultaneously. The reaction mixture contained 7.6 mM glucose, 150 mM Tris-HCl, pH 7.5, 0.0528 units of poly(P) glucokinase, and 440 ng of each of the two poly(P) substrates of average chain lengths 700 and 210 in a volume of 14.4 ml. Four 300-μl aliquots were removed at each time point and prepared for gel electrophoresis. Electrophoresis was with four different percentages of polyacrylamide gels. Lane 1 was from zero time and lanes 2-10 with poly(P) isolated after 8, 16, 24, 30, 38, 46, 62, and 71 min, respectively. Polyacrylamide gels of 15, 10, and 4% with 0.5% agarose and 0.3% agarose were used for Panels A, B, C, and D, respectively. The running buffer for the 4% and 2% mixed polyacrylamide gels contained Tris/aceta-
tate/NaCl/EDTA (40:20:182 mM), pH 8.0.

FIG. 3. Demonstration that poly(P) of chain length 700 is used prior to that of chain length 52. The reaction mixture contained 7.6 mM glucose, 150 mM Tris-HCl, pH 7.5, 7.6 mM MgCl₂, 0.312 units of poly(P) glucokinase, and 120 ng of each poly(P) species in a volume of 7.8 ml. Aliquots (330 μl) were removed at each time point, the poly(P) was isolated and electrophoresed into a 15% (201) polyacrylamide gel (0.3-mm thick) containing agar. Lane 1 contained the marker dye bromphenol blue and lane 2 was zero time. Lanes 3-13 were with poly(P) from 20, 40, 60, 80, 85, 90, 95, 100, 105, 110, and 115 min, respectively.

Considered previously (2) with 15% polyacrylamide gels, that chain lengths of 200 would be identified. However, it is evident from Panel D of Fig. 2 that the concentration of poly(P) of this chain length is so low with long chains that they would escape detection.

When poly(P) of average chain length of 210 is included as a substrate, this chain length is used simultaneously with the poly(P) of chain length 700 by a quasiprocessive or nonprocessive process (Fig. 2). However, the poly(P) of average chain length of 210 (Fig. 2) is used more rapidly than the poly(P₀); this is in agreement with the fact that the V₅₀ of shorter poly(P) is larger than that of longer chains (2). In contrast, chain lengths shorter than 100 are utilized only after the longer chains are consumed (Fig. 3). The necessity of using a series of concentrations of polyacrylamide for the detection of intermediate sizes of poly(P) formed is clear from the results presented in Figs. 1, 2, and 3.

The above results are in accord with those obtained with end-labeled poly(P) that was synthesized from a 32P short-chain primer and unlabeled ATP using poly(P) kinase; see Fig. 3 of Ref. 7. With poly(P) glucokinase, when about 20% of the end-labeled poly(P) was utilized, all the radioactivity was removed from the poly(P) but with poly(P) kinase, during the phosphorylation of ADP, the radioactivity persisted until all the poly(P) was utilized. In a quasi- or nonprocessive process, since the poly(P) dissociates and reassociates repeatedly during the utilization of the poly(P), the ends of the poly(P) polymer are removed at random from all the molecules present. Therefore, radioactivity is removed from all the molecules prior to their complete utilization. In contrast, by a strictly processive process (7, 8) a molecule of poly(P), once attached to the enzyme, is not released until the poly(P) is completely utilized. Thus, the remaining molecules retain their radioactivity as demonstrated with the poly(P) kinase. The use of end-labeled poly(P), therefore, provides a convenient tool for differentiating between a strictly processive and quasi- or nonprocessive processes.

The fact that poly(P) glucokinase removes phosphates only from the ends of the poly(P) chain and the shortened poly(P) accumulates as discrete bands has made it possible to use poly(P) glucokinase to determine the average size of the poly(P) (9). This method of sizing is quite accurate up to a chain length of about 450 but the error becomes large with longer chain lengths. Recently, a method for sizing poly(P)
has been devised which is applicable to longer chains of poly(P) (3). This method involves electrophoresis of the poly(P) in a series of polyacrylamide gels that give linear migration of various sizes of poly(P). The migration of the unknown is compared on a series of gels with the migration of standards containing a narrow range of sizes of poly(P). The procedure has been described for preparing and sizing the poly(P) standards (3). Not only does this procedure provide a method for determining the average size of the poly(P), with only 2–20 µg of poly(P), it also provides a measure of the range of sizes present in the unknown. This is the only method thus far described, which provides a measure of the range of sizes. These procedures provided the basis for our present studies.

It was not possible by the present procedures to differentiate between a quasi- and nonprocessive mechanism, i.e., whether there is one or more phosphorylations of glucose prior to the release of the poly(P) from the enzyme. With short chains of less than 100 it is possible to establish some limitation to the number of phosphorylations that could have occurred prior to each release from the enzyme. It has been repeatedly observed that poly(P) is formed which by gel electrophoresis gives only 5 or 6 bands, each of which differs from another by only 1 phosphoryl residue; see Fig. 2A of Ref. 2. It, therefore, is evident that a quasiprocessive mechanism could not have been involved in which more than 7 phosphorylations occurred prior to release from the enzyme. If so, bands would be formed which were separated from the main group of bands. If a short-chain poly(P), which consisted of only 1 chain length were available, it should be possible to determine whether poly(P) was formed with a gap of more than one chain length from the parent compound and thus whether the mechanism is quasi- or nonprocessive.

An interesting feature of the utilization of long-chain poly(P) is that a wide span of chain lengths is converted to a very narrow range of sizes when the lengths have been reduced to short chains, see Fig. 2, A and B. This reduction in the span of sizes could occur by preferential utilization of the longer chains as occurs with short chains. The fact that the 32P is removed from the end-labeled [32P]poly(P) consisting of a large span of sizes, when only 20% of the poly(P) is utilized, shows, however, that the 32P was removed from all poly(P) standards with only 1 chain length from the parent compound and thus whether there is one or more phosphorylations of glucose, whereas in the glucose grown cells, it is used to phosphorylate glucose and is thereby shortened and the amount is reduced (11). Knowing the properties of the poly(P) kinase and poly(P) glucokinase, some predictions can be made about the poly(P), if, in fact, poly(P) kinase is the source of the poly(P) and it is utilized by the poly(P) glucokinase. By the strictly processive mechanism, long-chain poly(P) would be formed by poly(P) kinase and by the quasi- or nonprocessive process, short chains would be produced by the poly(P) glucokinase which in turn would be used as primers for the synthesis of the poly(P) by the poly(P) kinase. Therefore, it is predicted that the poly(P) of glucose grown cells consists of a mixture of long-chain poly(P) similar to that found in lactate grown cells (presumably produced by poly(P) kinase) and of short chains which are formed by the poly(P) glucokinase. Experiments are underway to determine if this is the case.

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REFERENCES