Kinetic Studies on Muscle Glycogen Synthase*

(Received for publication, July 1, 1974)

EDUARDO SALSAS† AND JOSEPH LARNER
From the Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22903

Using the I form of rabbit muscle glycogen synthase essentially free of glycogen, the kinetics and mechanism of action was investigated. No evidence for an exchange between \(^{14}C\) UDP and UDP-glucose was found. The bisubstrate kinetics of the enzyme for UDP-glucose and glycogen, as well as for UDP-glucose and maltose, was determined. An intersecting pattern in the double reciprocal plot (velocity versus substrate concentration) suggestive of a sequential mechanism (ordered or random) was found in all cases. The \(K_m\) for UDP-glucose (45 to 48 mM) was the same with either maltose or glycogen as acceptor. The \(K_m\) for maltose (230 mM) and for glycogen (1.5 \(\mu\)g/ml) differed.

Rabbit skeletal muscle glycogen synthase was described as having a bisubstrate kinetic pattern suggesting a ping-pong mechanism (1). It was pointed out that the data were compatible with a stable enzyme-substrate intermediate being formed during the reaction and that therefore an exchange between \(^{14}C\) UDP and UDP-glucose should be tested in the absence of glycogen.

Using rabbit skeletal muscle glycogen synthase, we have found no evidence for such an exchange. Accordingly, the bisubstrate kinetics of this enzyme for UDP-glucose and glycogen was reinvestigated and we report here the presence of an intersecting pattern in the double reciprocal plot of velocity versus concentration of substrates suggestive of a sequential mechanism. In addition, to avoid the problem of using glycogen as one of the substrates since it is not molecularly defined, we have used maltose as acceptor with essentially the same results.

EXPERIMENTAL PROCEDURES

Enzyme—Glycogen synthase I form was prepared from rabbit skeletal muscle according to Smith et al. (2) with some minor modifications that will be described under "Results." The I form contains essentially no detectable carbohydrate (less than 1% respect to protein) and shows no activity in the absence of added acceptor.

Enzyme Assay for Kinetics Experiments—The velocity of the reaction was determined as a function of UDP-glucose and glycogen (or maltose) concentrations. For each set of concentrations, the time course of the reaction was determined separately, and the velocity was calculated as the slope of the resulting straight line. In this way, the changing backgrounds encountered with varying UDP-glucose and glycogen concentrations were avoided. Special care was taken to assure that less than 10% of the total substrate was depleted during the reactions (usually 1 to 5%).

The concentration of UDP-glucose used ranged from 5 \(\times\) 10\(^{-4}\) M to 2 \(\times\) 10\(^{-4}\) M (specific activity, 20,000 cpm/nmol). The concentration of glycogen, when used, ranged from 1 to 20 \(\mu\)g/ml. The concentration of maltose, when used, ranged from 45 to 900 mM. In addition, the reaction mixtures contained 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, and 2 mM Na\(\text{SO}_4\). The enzyme was appropriately diluted with a buffer (50 mM Triss-HCl (pH 7.8), 5 mM EDTA, and 50 mM mercaptoethanol) and preincubated for 10 min at 30°.

The reaction was begun by adding the enzyme. At the appropriate times, aliquots were removed from the reaction mixtures and treated as follows. When glycogen was the acceptor, the aliquots were spotted on filter papers and processed according to the method of Thomas et al. (3). When maltose was the acceptor, the aliquots were deionized by means of a mixed bed ion exchanger and counted (4). Duplicates were run for each point.

The data were plotted as the reciprocal of initial velocity versus reciprocal of the concentration of the variable substrate (primary plots). Secondary plots were made by plotting the slopes or the y intercepts of the primary plots versus reciprocal concentration of the second substrate, which is called the changing fixed substrate (5).

Analytical Methods—Protein was determined by the method of Lowry et al. (6). Carbohydrate content was determined by the phenol-sulfuric acid method of Dubois et al. (7) or in the presence of glycogen by enzymatic determination of the glucose after acid hydrolysis, using hexokinase and glucose-6-P dehydrogenase.

Affinity Chromatography—\(\alpha\)-Aminomalyl Sepharose for hydrophobic chromatography was prepared according to Shaltiel and Er-El (8).

Materials—Maltose (H\(\text{IO}\)) grade HHH from Hayashibara Co., Ltd. (Japan) was a gift of Dr. S. Hizukuri. All other materials were purchased from conventional sources.

RESULTS AND DISCUSSION

Purification of Glycogen Synthase—Fully converted glycogen synthase I form was purified from rabbit skeletal muscle by the procedure described previously (2), with the following modifications. The high speed centrifugation step normally following the first ethanol precipitation was omitted. In order to prevent the aggregation of the enzyme when it is free of glycogen (9), the \(\alpha\)-amylase digestion was carried out at room temperature.
temperature, as described previously, but in the presence of 25% glycerol.

The presence of glycerol (25 or 50%) and/or a high salt concentration (0.5 M KCl) effectively prevents the loss of enzyme activity due to the aggregation and may even partially reverse it (Table I).

The last step in the purification procedure was Sepharose 4B gel filtration. We have now found that this can be substituted by hydrophobic chromatography using ω-aminoalkyl Sepharose according to the method of Shaltiel and Er-El (8). We have used two ω-aminoalkyl Sepharoses, namely the 4- and 5-carbon derivatives. With the purified enzyme, the Sepharose-NH-(CH2)4-NH4+ rather than the 4-carbon analog, was found to be most suitable. With the 4-carbon derivative, the enzyme was only retarded (Fig. 1A), whereas with the 5-carbon derivative the enzyme was effectively bound (Fig. 1B).

Isotopic Exchange between [14C]UDP and UDP-Glucose—In order to test the possibility of a glucosyl intermediate being formed during the reaction, 1.5 nmol of [14C]UDP (0.2 μCi) and 1 μmol of UDP-glucose were incubated with 50 milliunits of glycogen synthase I essentially free of glycogen in 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.5) in a total volume of 1 ml. At the end of 2 or 4 hours of incubation at 30°C, trichloroacetic acid was added to a final concentration of 5%. The incubation reaction mixture was centrifuged, and the supernatant was extracted with ether several times until the pH reached 5.5. The reaction mixture was lyophilized. The lyophilized material was redissolved in water, UDP was added to a final concentration of 1 mM, and the mixture was submitted to chromatography on Whatman No. 3MM paper in ammonium acetate-ethanol (3:7, v/v, pH 7.5) (3) containing 20 mM EDTA in order to minimize hydrolysis of UDP-glucose. Table II shows that both at 2-hour and 4-hour intervals, essentially no radioactivity of UDP was incorporated into UDP-glucose, thus demonstrating no detectable exchange under these conditions.

Because this experiment was negative and we do not have further information about the extent of exchange that should be expected under those conditions, we cannot discard completely the possibility of a glucosyl intermediate, although it seems unlikely.

Initial Velocity Experiments with UDP-Glucose and Glycogen as Substrates—The double reciprocal plot of velocity versus concentration of substrate with UDP-glucose as the variable substrate at different fixed glycogen concentrations shows a series of intersecting lines (Fig. 2). The secondary plot of the intercepts and slopes (Fig. 3) obtained at the different glycogen concentrations is linear and allows the calculation of a $K_m$ value of 1.5 μg/ml for glycogen. Calculation of the $K_m$ for glycogen in terms of the nonreducing end termini, assuming that 9% of the glucosyl units are present as nonreducing end termini, gives the value of $8 \times 10^{-2}$ μM.

The double reciprocal plot with glycogen as a variable substrate at different fixed levels of UDP-glucose shows again a
TABLE II
Isotopic exchange between [14C]UDP and UDP-glucose

An incubation mixture containing 1.5 mM [14C]UDP (0.2 μCi), 1 μM UDP-glucose, 50 milliunits of glycogen synthase I essentially free of glycogen, and 50 mM 2-(N-morpholine)ethane sulfonic acid (pH 6.5) in a total volume of 1 ml was incubated at 30° during 2 or 4 hours. Trichloroacetic acid was then added to a final concentration of 5%, the incubation reaction mixture was centrifuged, the supernatant was extracted with ether several times until the pH reached 5.5, and the mixture was lyophilized. The lyophilized material was redissolved in water, UDP was added to a final concentration of 1 mM, and the mixture was chromatographed on Whatman No. 3MM paper in ammonium acetate-ethanol (3:7, v/v, pH 7.5) containing 20 mM EDTA. The radioactivity found in the UDP and UDP-glucose positions is shown.

| Incubation time | UDP-glucose | UDP-glucose |
|-----------------|-------------|-------------|
| 2 hours         | 10,110 cpm  | 55          |
| 4 hours         | 9,190 cpm   | N.D.*       |

*Not detectable over background which was 40 cpm.

Fig. 1. Double reciprocal plots of velocity versus UDP-glucose concentration at various fixed levels of glycogen for the glycogen synthase. The concentrations of UDP-glucose used were 5 μM (●), 10 μM (■), 20 μM (▲), and 50 μM (▼).

Fig. 2. Double reciprocal plots of velocity versus UDP-glucose concentration at various fixed levels of glycogen for the glycogen synthase. The concentrations of glycogen used were 1 μg/ml (○), 5 μg/ml (△), and 10 μg/ml (▽).

Fig. 3. Secondary plots of the intercepts and slopes from the double reciprocal plot of Fig. 2.

series of intersecting lines (Fig. 4). The secondary plot of the intercepts and slopes obtained at the different UDP-glucose concentrations is also linear, and the V_{max} obtained is in good agreement with the one obtained in Fig. 3. A K_{m} of 45 μM for UDP-glucose can be calculated.

The problem with enzymes acting on polysaccharides is the difficulty in defining molecularly the substrate (in this case, glycogen) and the fact that the reaction always proceeds in the presence of the product of the reaction which also serves as substrate.

In the case of phosphorylase, it has been indicated (10) that it would be necessary to consider two complexes of enzyme and polysaccharide. In one complex, the binding would be for degradation, whereas in the other complex, the binding would be for chain elongation. In such a case, the rate equations are somewhat different from the common two substrate systems, and for some mechanisms could present a pattern of lines in the double reciprocal plot (10) different from the usual patterns (5, 11).

In the case of glycogen synthase, the product again is expected to form a complex with the enzyme, but depending on the nature of the kinetic mechanism, this complex can either immobilize the enzyme in a non-useful form or be useful for the next catalytic cycle, and therefore not inhibit the reaction. Particularly, in an ordered sequential mechanism with UDP-glucose binding first and in a ping-pong mechanism (for which the only possibility is UDP-glucose binding first), a complex of enzyme and glycogen (involving in any way the active center) will produce an inhibition. The extent of this inhibition will depend on the relative values of the kinetic constants of the steps involved. On the other hand, in a random mechanism or in an ordered sequential mechanism with glycogen binding first and the glycogen product released last, most likely no inhibition will be seen because the enzyme is not able to bind the glycogen in any way different from that for elongation.

Initial Velocity Experiments with UDP-Glucose and Maltose as Substrates—A simple and expeditive way to circumvent the outlined difficulties encountered with glycogen is to use small oligosaccharides such as maltose and maltotriose as acceptors. Both of them, as well as glucose (12), can be acceptors for the glycogen synthase catalyzed reaction, although at a much lower rate (13). If the concentration of acceptor is high enough, the reaction is single step; thus when maltose is used, only maltotriose is found as product; whereas when maltotriose is used, only maltotetrasi is found as product. All of the radioactivity is found in the nonreducing termini. This fact has been proven in the case of maltotriose formation by means of metaperiodate oxidation and separation of the formic acid formed. Of the total radioactivity, one-sixth is recovered as formic acid.

When the bisubstrate kinetic analysis is done using UDP-
glucose and maltose, the double reciprocal plots show intersecting patterns (Figs. 5 and 6). As expected, the concentrations of acceptor necessary to achieve measurable velocities are much higher than those of glycogen in terms of nonreducing ends. Saturation with the maltose acceptor was not achieved, the main limitation being the viscosity and ultimately the solubility of the maltose.

The secondary plots are linear, and a $K_m$ of 230 mM for maltose and a $K_m$ of 48 mM for UDP-glucose can be calculated from them. Therefore, while the $K_m$ for the acceptor changes markedly with its complexity, the $K_m$ for UDP-glucose is the same with maltose or glycogen as acceptors.

**GENERAL DISCUSSION**

The $I$ form of glycogen synthase more readily aggregates than the $D$ form and is more cold-sensitive when freed of glycogen (9). We have found that glycerol and high salt concentrations prevent and even partially reverse this aggregation.

The absence of isotopic exchange between [$^{14}$C]UDP and UDP-glucose suggests that no glucosyl enzyme intermediate is formed. This fact rules out a Ping Pong Bi Bi mechanism for glycogen synthase. It is worthwhile to note that the test for the back reaction was run at pH 6.7 instead of pH 7.8. It has been pointed out (14) that the synthase reaction liberates a proton, the neutralization of which displaces the equilibrium further to the right. We considered it necessary to minimize this factor to have a better chance of seeing the exchange reaction should it occur.

In agreement with the lack of a demonstrable exchange reaction, when a careful reinvestigation of the bisubstrate kinetics was undertaken, we found an intersecting pattern, compatible with a sequential (ordered or random) mechanism. Nevertheless, in certain experiments (not shown) a curvature in the double reciprocal plot was apparent. In such cases, an apparent parallelism of the lines was seen near the y axis. This parallelism led us to consider as a working hypothesis that the pattern of addition of glycosyl residues to the glycogen nonreducing terminals could change in relation to the relative concentrations of both substrates, glycogen and UDP-glucose. In that way, at high UDP-glucose concentrations, the pattern would be close to a single chain elongation. The glycogen chain would not need to leave the active center of the enzyme, and the release of 1 or more UDP residues before all the molecules of UDP-glucose were added would give a pattern close to parallelism. We have chemically proven that this is not the case. Thus, only 1 or 2 glucose residues added successively to the same branch, and most probably only 1, is compatible with these results.

Another explanation for the curvature found only with some preparations of enzyme would be the presence of two forms of the enzyme (15-17), one of them arising from limited proteolysis of the other. In fact, evidence has been found that indicates that this actually happens during the standard purification procedures of both forms of the enzyme.

In any event, the present studies indicate that the kinetic mechanism is compatible with a sequential mechanism for glycogen synthase acting either on glycogen or maltose as acceptor. After these studies were completed, two publications on the subject have appeared. Plesner et al. (18) working with glycogen synthase $D$ from human polymorphonuclear leukocytes showed that the enzyme has a rapid equilibrium random Bi Bi mechanism, in which the attachment of the activator glucose-6-P was a necessary prerequisite for the addition of the substrate UDP-glucose. Huang and Cabib (19) observed biphasic curves in the double reciprocal plots of reaction rate versus UDP-glucose concentration. Convergent patterns of lines on double reciprocal plots was observed in all cases. Thus, these results are in general agreement with those we obtained with the rabbit muscle enzyme.

**REFERENCES**

1. Brown, N. E., and Larner, J. (1971) *Biochim. Biophys. Acta* 242, 69-90
2. Smith, C. H., Villar-Palasi, C., Brown, N. E. Schledner, K. K., Rosenkranz, A. M., and Larner, J. (1972) *Methods Enzymol.* 28, 530-539
3. Thomas, J. A., Schledner, K. K., and Larner, J. (1968) *Anal. Biochem.* 25, 485-499
4. DeWulf, H., Stalmans, W., and Hers, H. G. (1970) *Eur. J. Biochem.* 15, 1-8
5. Cleland, W. W. (1970) in *The Enzymes* (Dyer, P. D., ed) Vol. II, pp 1-65, Academic Press, New York
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275
7. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* 28, 350-356
8. Shaltiel, S., and Er-El, Z. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 778-781
9. Smith, C. H., and Larner, J. (1972) *Biochim. Biophys. Acta* 254, 224-228
10. Chao, J., Johnson, G. F., and Graves, D. J. (1969) *Biochemistry* 8, 1459-1466
11. Cleland, W. W. (1963) *Biochim. Biophys. Acta* 67, 188-196
12. Salsas, E. (1974) *Fed. Proc.* 33, 1238

---

*Y. Takada and J. Larner, manuscript in preparation.*
13. Goldemberg, S. H. (1962) *Biochim. Biophys. Acta* 56, 357-359
14. Larner, J., Villar-Palasi, C., and Richman, D. J. (1959) Ann. N.Y. Acad. Sci. 82, 345-353
15. Piras, R., Rothman, I. R., and Cabib, E. (1968) *Biochemistry* 7, 56-66
16. Thomas, J. A., and Larner, J. (1973) *Biochim. Biophys. Acta* 293, 62-72
17. Schlender, K. K., and Larner, J. (1973) *Biochim. Biophys. Acta* 293, 73-83
18. Plesner, I., Plesner, I. W., and Esmann, V. (1974) *J. Biol. Chem.* 249, 1119-1125
19. Huang, K.-P., and Cabib, E. (1974) *J. Biol. Chem.* 249, 3851-3857
