Kinetic Mechanism of Rabbit Muscle Glycogen Synthase I†

Allen M. Gold

ABSTRACT: The kinetic mechanism of rabbit muscle glycogen synthase I was investigated by determining isotope-exchange rates at chemical equilibrium between uridine diphosphoglucose (UDPG) and glycogen and between UDPG and uridine 5′-diphosphate (UDP). The rates were followed simultaneously by use of UDPG labeled with ^{14}C in the glucose moiety and with ^{3}H in the uracil group. They were found to be independent of the concentrations of glycogen and the UDPG–UDP pair, averaging 6×10^{-9} mol min^{-1} mg^{-1}, with a ratio of UDPG–glycogen exchange to UDPG–UDP exchange of 0.85–0.95. The conclusion is that glycogen synthase has a rapid equilibrium random bi bi mechanism. The previously reported slow activation of glycogen-free synthase in the presence of glycogen was examined kinetically. The activation rate appears to be independent of glycogen concentration over a wide range, while the maximum activation is related to the third or fourth root of the glycogen concentration. This suggests that the slow bimolecular reaction mechanism proposed for human polymorphonuclear leucocyte glycogen synthase I [Sølling, H., & Esmann, V. (1977) *Eur. J. Biochem. 81*, 129] does not apply to rabbit muscle synthase I. The rate of exchange of glycogen molecules in the complex between glycogen and rabbit muscle synthase I under conditions where the enzyme is catalytically active was estimated by a novel method. The enzyme–glycogen complex was treated with [*glucose*-^{14}C]UDPG and glycogen of different molecular weight. The distribution of isotope between the two forms of glycogen was determined after their separation by agarose gel chromatography. A rate constant of 0.3 min^{-1} was estimated for the exchange. It can be calculated, on the basis of the specific activity of the enzyme (20 μmol min^{-1} mg^{-1}) and its action pattern, that hundreds of individual chains in the glycogen molecule must be available to the enzyme during the average lifetime of the complex. A mechanism is proposed for this process.

Glycogen synthase (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) catalyzes the rate-limiting step of glycogen synthesis in mammals; it is finely regulated by a phosphorylation–dephosphorylation mechanism and metabolite concentrations which act in concert with the glycogen degradative machinery (Stalmans & Hers, 1973; Hers, 1976). The kinetic mechanism of glycogen synthase has been obscured by two factors. One is that glycogen is both substrate and product, which complicates the interpretation of steady-state initial velocity and product inhibition studies. Glycogen cannot be varied as substrate without simultaneous variation as product and vice versa, while uridine 5′-diphosphate (UDP) used as an inhibitor is capable of taking part in a back-reaction which compromises analysis of the kinetics. Fortunately, in this case the equilibrium constant is far in the direction of UDP, and it is not likely that the back-reaction can seriously influence the characteristic product inhibition patterns for any kinetic mechanism.

The second factor complicating the kinetics is the slow formation of stable complexes between glycogen synthase and glycogen. Activation of glycogen-free enzyme during incubation with glycogen has been observed with rabbit muscle glycogen synthase I (Nimmo et al., 1976), porcine brain synthases D and I (Rottenberg et al., 1972; Passonneau et al., 1975), and the synthase I from human polymorphonuclear leucocytes (Sølling & Esmann, 1977a). Sølling & Esmann (1977b) have done a careful study of the latter enzyme and found that activation involves formation of an enzyme–glycogen complex. Kinetics of activation were consistent with a model in which glycogen synthase I and glycogen combine in a slow bimolecular reaction. Given these properties it is clear that many kinetic experiments reported in the past where glycogen is the varied substrate are of questionable validity.

The most complete study of the kinetic mechanism of a glycogen synthase was carried out by Plesner et al. (1974) on the glycogen synthase D from human polymorphonuclear leucocytes. Initial velocity experiments with uridine diphosphoglucose (UDPG) and glycogen, when plotted as $1/v$ vs. $1/S$, gave a pattern of straight lines intersecting on the $1/S$ axis. UDP inhibited competitively with respect to UDPG and noncompetitively with respect to glycogen. Glucose 6-phosphate, the activator, gave a pattern of lines intersecting on the $1/v$ axis when UDPG was the varied substrate and a set of lines intersecting on the $1/S$ axis when glycogen was the varied substrate. These data were interpreted as indicating a partially random rapid equilibrium mechanism in which substrates bind in random order, but glucose 6-phosphate must bind to the enzyme before UDPG can bind. Initial velocity and product inhibition experiments were carried out with rat liver glycogen synthase D by McVerry & Kim (1974) with results similar to those described above; however, these experiments alone are insufficient to justify their conclusion that the mechanism is random. Salsas & Larner (1975a) carried out initial velocity experiments with rabbit muscle glycogen synthase I using both glycogen and maltose as acceptors and obtained results similar to those of Plesner et al. (1974).

Several groups have examined the possibility of glycogen synthase catalyzing isotope exchange between UDP and UDPG in the absence of glycogen. Neither the rabbit muscle enzyme (Kornfeld & Brown, 1962; Salsas & Larner, 1975a) nor the rat liver enzyme (Steiner et al., 1965; McVerry & Kim, 1974) could be shown to catalyze exchange.

The conclusions of Plesner et al. (1974) with respect to the kinetic mechanism of the leucocyte synthase are the result of a closely reasoned argument involving a number of assumptions. Furthermore, it requires that the "action pattern" of the enzyme be of the multichain type. That is, the number of glucosyl groups that are transferred to an acceptor chain before the enzyme moves to another chain should be exactly

† From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received January 22, 1980.* This material is based upon work supported by the National Science Foundation under Grant No. PCM77-00174.

0006-2960/80/0419-3766$01.00/0

1. This has been examined, using chemical methods, by Parodi et al. (1970) and by Salsas & Larner (1975b). Under the most favorable conditions the lowest value for the number of residues transferred successively to the same chain is 1.4–1.7 (Salsas & Larner, 1975b). Such a result is inconsistent with a true rapid-equilibrium mechanism.

In the present work the kinetic mechanism of rabbit muscle glycogen synthase is examined by means of isotope exchange rate studies at chemical equilibrium (Boyer, 1959; Boyer & Silverstein, 1963; Cleland, 1967) and by other methods. The former technique provides a relatively rigorous means of determining steady-state kinetic mechanisms. Only two exchanges are possible in the glycogen synthase reaction: exchange of the elements of UDP with UDPG and exchange of the glucosyl group of UDPG with glycogen. It was possible to examine both of these exchange rates simultaneously as a function of concentrations of reactants; the conclusion is that the kinetic mechanism is indeed rapid equilibrium random bi bi.

The time-dependent activation of glycogen-free synthase I by glycogen has been reconfirmed and examined in some of its details. Results were significantly different from those of Sølling & Esmann (1977b) but are not adequate to propose an alternate model.

Because of the slow complex formation between glycogen synthase and glycogen, it is of interest to determine the rate at which the enzyme–glycogen complex exchanges glycogen molecules under conditions where the enzyme is actively catalyzing transglucosylation. A method based on the separation of high and low molecular weight forms of glycogen by chromatography on agarose gel was devised to carry out this measurement. A $t_{1/2}$ of 2 min, at pH 7.0 and 30 °C was estimated. Comparison of this value with the specific activity and action pattern of the enzyme leads to the conclusion that the enzyme is able to add glucose residues to the termini of several hundred chains of the polysaccharide molecule before it dissociates and binds to another. A mechanism for this process is suggested.

Materials and Methods

UDPG, rabbit liver glycogen (type III), oyster glycogen (type II), pyruvate kinase, nucleoside diphosphate kinase, UDPG pyrophosphorylase, inorganic pyrophosphatase, and porcine pancreatic amylase (type 1-A) were obtained from Sigma Chemical Co. Calf intestinal alkaline phosphatase is a product of Boehringer, Mannheim. [*glucose*-^{14}C(U)]UDPG, 229 mCi/mmol, was obtained from New England Nuclear; [5-^{3}H]uridine 5′-triphosphate, 20 Ci/mmol, and [5′-^{3}H]uridine 5′-diphosphate, 11 Ci/mmol, are products of Schwarz/Mann. Sepharose 4B is a product of Pharmacia.

[*uracil-5-*^{3}*H*]*UDPG*. [^{3}H]UTP (100 μCi) (without carrier) was incubated with 10 mM Tris-HCl, pH 7.0, 1 mM $MgSO_4$, 1 mM glucose 1-phosphate, 1 enzyme unit (EU) of UDPG pyrophosphorylase, and 1 EU of inorganic pyrophosphatase in a final volume of 50 μL at 37 °C for 30 min. The mixture was applied, in a 3-cm strip, to a PEI-cellulose thin-layer sheet (J. T. Baker Chemical Co.) that had been prewashed with 0.9 M acetic acid and developed with 0.9 M acetic acid–0.3 M LiCl. The area containing product was scraped off and extracted with a small volume of 1 M NaCl; the nucleotide was adsorbed to 5 mg of Norit A, washed with water, eluted with 0.2 N NH_3–EtOH (1:1), dried over H_2SO_4, and taken up in 0.2 mL of 1 mM Tris-HCl, pH 7.0.

Glycogen Synthase I. The glycogen-free enzyme was prepared by the method of Soderling et al. (1970) from a frozen glycogen-pellet preparation described by DeLange et al. (1968) and Krebs et al. (1964). The preparation was carried out with 500 g of muscle tissue obtained from a single animal. The following changes in procedure were introduced: (1) The rabbit was killed by an intracardiac injection of 5 mL of 6% pentobarbital, and the back and leg muscle tissue was excised immediately. (2) Phenylmethanesulfonyl fluoride, 1×10^{-4} M, was introduced into the defrosted glycogen preparation and again before addition of amylase. (3) Porcine pancreatic amylase was substituted for human salivary amylase. (4) Combined fractions from Sepharose 4B chromatography were concentrated by dialysis against solid sucrose (Schwarz/Mann, ultrapure) followed by dialysis against 50 mM β-glycerophosphate, 2 mM EDTA, 40 mM mercaptoethanol, and 10% sucrose, pH 7.0. This preparation was distributed among a number of small plastic tubes and stored at −20 °C. It could be defrosted and refrozen several times without loss of activity. The protein concentration was 0.75 mg/mL based on an $A_{280}^{1\%}$ of 13.4 (Nimmo et al., 1976), and the specific activity was 21 μmol min^{-1} mg^{-1} by the assay described below. Only 3% activity could be detected in the absence of added glycogen.

Glycogen. Low molecular weight glycogen was prepared by dissolving 100 mg of oyster glycogen in 2 mL of 0.010 N HCl and heating at 100 °C for 30 min. This solution was applied to a 1.4 × 26 cm column of Sepharose 4B and eluted with distilled water. Polysaccharide was determined by the method of Dubois et al. (1956). A group of fractions clearly distinct from those in which rabbit liver glycogen would be eluted were combined and concentrated. Two such preparations were combined and rechromatographed. An average molecular weight of ~600 000 was estimated by comparison with dextran standards. High molecular weight glycogen was prepared by chromatographing rabbit liver glycogen and selecting only the peak fractions. Oyster glycogen solutions were deionized by stirring with Dowex-501 mixed-bed resin, and those for use in isotope-exchange experiments were also passed through Dowex 1-X8 chloride (200–400 mesh).

Glycogen Synthase Assay. A modification of the method of Leloir & Goldemberg (1960) was used. Enzyme was preincubated for 30 min at 37 °C in a solution containing 0.10 M Tris-HCl, pH 7.8, 4 mM EDTA, 20 mM mercaptoethanol, 40 mM Na_2SO_4, 0.4 mg/mL bovine albumin, and 1.5 mg/mL oyster glycogen. The assay was started by adding 25 μL of this solution to 25 μL of 20 mM UDPG and incubating the mixture at 37 °C for 10 min. The reaction was terminated by heating at 100 °C for 30 s. Blanks were prepared by omitting glycogen synthase or by stopping the reaction immediately. Various aspects of the assay were modified, as noted, in different experiments.

UDP produced in the reaction was determined by treating the reaction mixture with 25 μL of a solution containing 4 mM phosphoenolpyruvic acid (monocyclohexylammonium salt), 4 mM ATP, and 0.16 M KCl and with 25 μL of a solution of pyruvate kinase (1.0 mg/mL), nucleoside diphosphate kinase (10 EU/mL), and 50 mM $MgSO_4$. After the mixture was incubated for 10 min at 37 °C, the resulting pyruvate was determined by treatment with 0.200 mL of 0.4 mM 2,4-dinitrophenylhydrazine in 0.6 M HCl and, after another 10-min incubation, with 0.200 mL of 2.5 M NaOH and 0.20 M EDTA. Absorbance was determined at 460 nm; calibration gave a factor of ~27 absorbance units/μmol.

Activation of Glycogen Synthase I by Glycogen. Preincubation was carried out as described for the enzyme assay except that all solutes were present at half the concentrations indicated (except where noted) and glycogen and time were varied. The UDPG solution contained the same solutes as the preincu-

bation solution (except enzyme), so the concentrations in the final assay solution were the same as described for the enzyme assay.

Equilibrium Isotope Exchange. Enzyme was preincubated similarly to the enzyme assay; however, the oyster glycogen concentration was as noted in Table I, the pH was 7.0, and the incubation was carried out for 90 min at 30 °C. The enzyme concentration ranged from 80 μg/mL at the highest UDP concentration to 8 μg/mL at the lowest. The enzyme solution was then added to an equal volume of solution containing glycogen, UDP, UDPG, [^{14}C]UDPG, and [^{3}H]UDPG so as to give the final concentrations of these reactants indicated in Table I. The total volume of the reaction mixture was 0.300 mL when UDP was 1 mM or 10 mM and 0.150 mL when UDP was ~100 mM. Incubation was carried out at 30 °C. Aliquots of 50 μL (25 μL when UDP was 100 mM) were removed immediately and after three equal intervals (10–30 min) and heated at 100 °C for 30 s to stop the reaction; they were then stored in ice until processed. Enzyme concentrations and incubation times were adjusted so that between 3 and 20% of the isotopes exchanged at the longest time point. Approximately 40000 cpm of ^{14}C and 100000 cpm of ^{3}H were present in each aliquot, regardless of the concentrations of UDP and UDPG.

Samples were treated with 50 μL of calf intestinal alkaline phosphatase in 10 mM $MgSO_4$. When UDP was 1 mM, the phosphatase solution contained 1 EU, and incubation was carried out for 30 min; for 10 mM UDP, 4.3 EU was used for 30 min; for 100 mM UDP, 11.6 EU was used for 60 min. All incubations were at 37 °C and were stopped by heating at 100 °C for 30 s. No significant degradation of UDPG occurs under these conditions. The samples were then applied to small columns containing ~0.3 mL of Dowex 1-X8 chloride (200–400 mesh) and washed through quantitatively with water. Volumes of ~1.5 mL were collected in preweighed tubes and weighed to determine volumes. One milliliter was added to 10 mL of Aquasol 2 (New England Nuclear) and assayed in ^{14}C and ^{3}H channels in a Beckman LS-100C liquid scintillation spectrometer. Activities of the individual isotopes in the sample were calculated and multiplied by the volume to give the total activity. The total isotope initially present in UDPG was determined by adding a 50-μL (or 25-μL) aliquot of the original reaction mixture directly to a weighed tube, adding ~1.5 mL of water, and proceeding as described above.

The four aliquots from each reaction mixture gave excellent linearity in time for exchange of both isotopes, even when as much as 20% exchange occurred. Rates, in terms of fractional conversion per minute, were calculated by a least-squares treatment; coefficients of determination, r^2, were never smaller than 0.990, corresponding to a standard deviation of 7% in the rate, and the majority were larger than 0.998, corresponding to a standard deviation of less than 3%. Isotope-exchange rates were obtained by multiplying by the concentration of UDPG and dividing by the concentration of enzyme. Control experiments were carried out to show that little labeled glycogen or UDP was lost in processing. The addition of amylase before treatment with Dowex 1-X8 resulted in only a 2–3% increase in the recovery of ^{14}C, while a control with UDP omitted indicated that ~95% of both isotopes was transferred to products by glycogen synthase.

Exchange of Glycogen in the Enzyme–Glycogen Complex. Preincubation was carried out similarly to the assay; however, oyster glycogen was replaced by either 4.3 mg/mL high molecular weight glycogen or 11.9 mg/mL low molecular weight glycogen (see above), the pH was 7.0, and preincubation was continued at 30 °C for 60 min. Enzyme was 25 μg/mL or 2.5 μg/mL. Enzyme–glycogen solution (120 μL) was added to 120 μL of 0.20 mM UDPG containing [*glucose*-^{14}C(U)]-UDPG (210000 cpm) and either no glycogen or the concentrations of high or low molecular weight glycogen indicated above. After incubation at 30 °C for 3 or 30 min, the reaction was stopped by heating at 100 °C for 1 min, and the solution was passed through a Dowex 1-X8 column as described above. A small aliquot was assayed for ^{14}C to determine total incorporation.

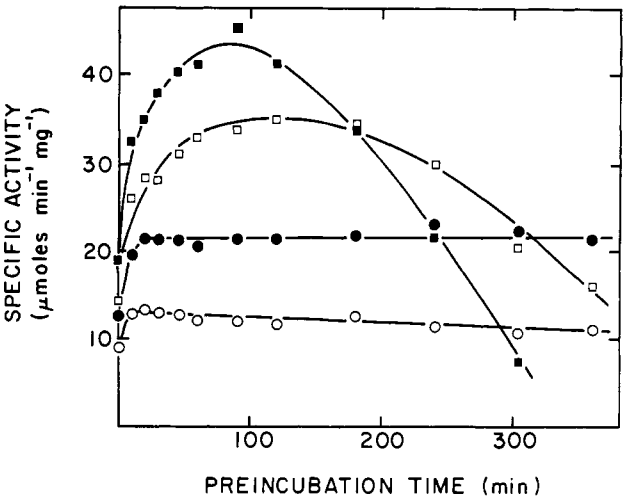

FIGURE 1: Activation of glycogen synthase I by glycogen at 37 °C, pH 7.8, as a function of glycogen concentration and time. The enzyme concentration is 6.2 μg/mL in the preincubation solution. Oyster glycogen concentrations during preincubation and assay are (○) 0.050 mg/mL, (●) 0.50 mg/mL, (□) 5.0 mg/mL, and (■) 25 mg/mL. Assays were incubated for 5 min at 37 °C.

Each product was concentrated by evaporation, applied to a 1.0 × 25 cm column of Sepharose 4B, and eluted with water at a flow rate of ~6 mL/h. Fractions of ~1 mL were collected and assayed for ^{14}C as described above and for polyglucose by the method of Dubois et al. (1956). The specific activity, counts per minute per micromole of hexose, remained fairly constant over fractions 8–11 (containing the high molecular weight glycogen) and fractions 13–18 (containing the low molecular weight glycogen).

Alkaline Phosphatase Assay. The method of Garen & Levinthal (1960) using *p*-nitrophenyl phosphate at pH 8.0 and 25 °C was used.

Nucleotide Assay. Concentrations of uridine nucleotides were determined from A_{260} by using a molar extinction coefficient of 10000.

Results

Activation of Glycogen Synthase I by Glycogen. When glycogen-free synthase was preincubated with oyster glycogen at 37 °C and pH 7.8, activity increased significantly with time (Figure 1). The extent of activation was dependent upon the glycogen concentration up to at least 25 mg/mL. Enzyme inactivation was observed and appeared to increase with increasing glycogen concentration. At a lower temperature, 30 °C, similar effects occurred; however, lower maximum activities were observed, and the rates of activation and inactivation were reduced. Activities at the beginning of preincubation appeared to be substantial, suggesting that some of the enzyme was active immediately, but at least a part of this activity must be the result of enzyme activation during the 5-min assay.

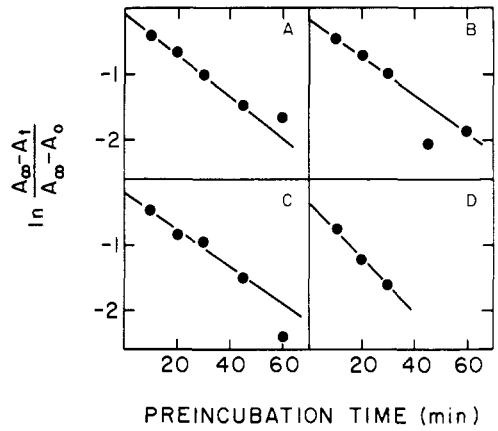

FIGURE 2: Activation of glycogen synthase I at 30 °C, pH 7.8, as a function of glycogen concentration and time. The data are plotted as a first-order reaction according to the equation $\ln [(A_\infty - A_t)/(A_\infty - A_0)] = -kt$, where A_∞ is the maximum activity observed, A_0 is the activity at $t = 0$, and A_t is the activity at time t. (A) Glycogen is 25 mg/mL; $k = 0.031$ min^{-1}. (B) Glycogen is 5.0 mg/mL; $k = 0.029$ min^{-1}. (C) Glycogen is 0.50 mg/mL; $k = 0.028$ min^{-1}. (D) Glycogen is 0.050 mg/mL; $k = 0.042$ min^{-1}.

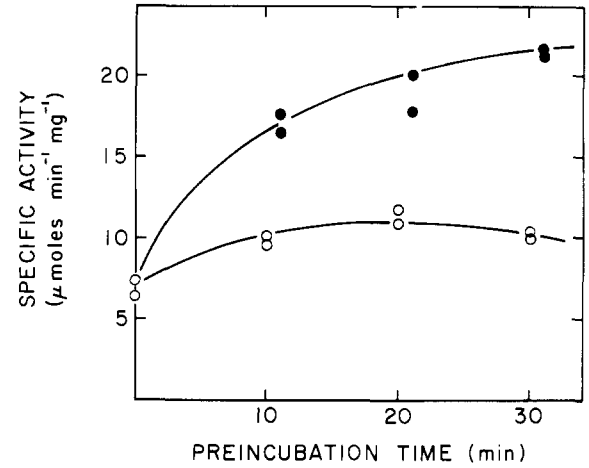

FIGURE 3: Activation of glycogen synthase I at 37 °C, pH 7.8, as a function of protein concentration and time. Conditions and solute concentrations are similar to those for preincubation in the enzyme assay, but the oyster glycogen concentration is 0.50 mg/mL during preincubation and 0.25 mg/mL during assay. The enzyme is preincubated at concentrations of 23 μg/mL (○) or 3.8 μg/mL (●). The higher concentration enzyme is diluted to the lower concentration just prior to assay, so that assay conditions are equivalent in the two series. Zero-time points are common to both curves. Assays were incubated for 5 min at 37 °C.

An estimate of the rates of activation can be made by plotting the early time data at 30 °C as a first-order reaction. The result, shown in Figure 2, indicates that the activation rate is essentially independent of the glycogen concentration. Increasing the enzyme concentration appears to have a negative effect on the extent of activation. Figure 3 illustrates an experiment in which enzyme at two concentrations was incubated with a low glycogen concentration (0.5 mg/mL) at 37 °C and diluted so that all assays contained the same amount of enzyme protein and glycogen. Other experiments confirm the conclusion that there is slightly greater activation at lower enzyme concentrations when the glycogen concentration is 5 mg/mL.

Equilibrium Isotope Exchange Rates. Rates of exchange of the glucose group of UDPG into glycogen and the uracil group into UDP were determined simultaneously by using UDPG labeled uniformly in the glucose residue with ^{14}C and in position 5 of the uracil group with ^{3}H. After incubation with enzyme, glycogen, and UDP under conditions of approximate chemical equilibrium, aliquots of the reaction mixture were quenched by heating and treated with alkaline phosphatase to convert the UDP quantitatively into uridine. Passage of the mixture through a small column of Dowex 1-X8 chloride removed UDPG, and the eluate was assayed for ^{14}C and ^{3}H by means of liquid scintillation spectrometry.

The equilibrium constant for the mammalian glycogen synthase reaction (eq 1) is not known. However, the standard

$$\text{UDPG} + G_n \rightleftharpoons \text{UDP} + G_{n+1} \qquad (1)$$

free energy of hydrolysis of the glucosidic bond of UDPG has been estimated as −8 kcal/mol at pH 7.4 (Neufeld & Hassid, 1963). Based on the assumption that the standard free energy of hydrolysis of a terminal α-1,4-glucosyl residue of glycogen is −4 kcal/mol and that the pK_a of UDP is 6.5 (Bock, 1960), an equilibrium constant of 400 can be calculated for reaction 1 at pH 7.0 and 25 °C.

Fox et al. (1976) have determined a pH-independent equilibrium constant of 45.8 ± 4.5 for the *Escherichia coli* glycogen synthase reaction, which uses ADPG as substrate, at 37 °C. This represents the ratio $ADP^{2-}/ADPG^{2-}$. By use of 6.4 as the pK_a of ADP (Bock, 1960), the equilibrium constant is 230 at pH 7.0.

Several experiments were carried out to determine what effect the ratio of UDP/UDPG has on the exchange rates; they were done at a concentration of 7.15 mg/mL glycogen and both 1.0 and 10 mM UDP by using concentrations of UDPG corresponding to equilibrium constants of 200 and 400. In each case the ^{14}C and ^{3}H exchange rates, expressed in terms of the percent of total isotope exchanged per minute, were unaffected by the concentration of UDPG. This indicates that exchange rates are proportional to the concentration of UDPG under the conditions of these experiments. A UDP/UDPG ratio of 300 was arbitrarily selected for all experiments reported in this work.

Results of several experiments are given in Table I. Rates within a particular series, e.g., experiment 5, where glycogen was fixed and UDP and UDPG were varied over a concentration range of 100, may exhibit small, apparently erratic, variations. The ratios of UDPG–glycogen exchange rate to UDPG–UDP exchange rate should have high reliability because the two rates were determined simultaneously on the same reaction mixture. These ratios tend to be in the range of 0.85–0.95, with a few as low as 0.82; there is no clear evidence for the suppression of one or the other exchange rate as either UDP–UDPG or glycogen is raised to high concentrations.

Another group of experiments was carried out by using maltoheptaose as activator and substrate. Preincubation was with 100 mM maltoheptaose, and the exchange was carried out with 1.00 mM UDP and 3.33×10^{-3} mM UDPG. When exchange was conducted with 50 mM maltoheptaose, the ratio of exchange rates was 1.000 and the rate was 5.50×10^{-3} μmol min^{-1} mg^{-1}; when maltoheptaose was 100 mM, the ratio was 1.007 and the rate was 13.6×10^{-3} μmol min^{-1} mg^{-1}.

Exchange of Glycogen in the Enzyme–Glycogen Complex. Glycogen synthase is known to be strongly bound to glycogen both in vivo and in vitro, and there is evidence that the activation of glycogen-free synthase by preincubation with glycogen involves complex formation between the enzyme and the polysaccharide (Sølling & Esmann, 1977b). An experiment was designed to test whether the complex formed during preincubation is the initial acceptor of glucosyl residues from UDPG and whether enzyme molecules are able to dissociate and recombine with other polysaccharide molecules under

Table I: Equilibrium Isotope Exchange Rates in the Glycogen Synthase Reaction at 30 °C, pH 7.0

expt	glycogen preincubn (mg/mL)	glycogen exchange (mg/mL)	UDP (mM)	UDPG (μM)	exchange rates [(μmol min^{-1} mg^{-1}) × 10^3] ^{14}C	^{3}H	ratio[a]
1	1.0	0.25	1.0	3.30	6.29	7.11	0.89
	1.0	3.8	1.0	3.30	6.32	7.23	0.87
	1.0	17.4	1.0	3.30	6.44	7.19	0.90
	1.0	60.3	1.0	3.30	6.05	6.74	0.90
2	1.0	0.50	10.0	34.0	4.38	5.36	0.82
	1.0	6.8	10.0	34.0	3.99	4.75	0.84
	1.0	60.6	10.0	34.0	6.12	6.56	0.93
3	1.0	7.6	106[b]	330	5.14	5.84	0.88
4	1.0	7.6	94[b]	340	4.60	5.32	0.86
	1.0	60.6	86[b]	340	5.14	5.43	0.95
5	14.3	7.2	1.00	3.30	5.42	6.27	0.86
	14.3	7.2	10.0	33.0	6.17	7.18	0.86
	14.3	7.2	101[b]	330	4.66	5.32	0.88
6	14.3	7.2	0.200	0.67	4.96	5.23	0.95
	14.3	7.2	1.00	3.30	6.00	6.32	0.95

[a] The ratio is UDPG–glycogen exchange rate/UDPG–UDP exchange rate. [b] Determined on an aliquot of the reaction mixture from A_{260}.

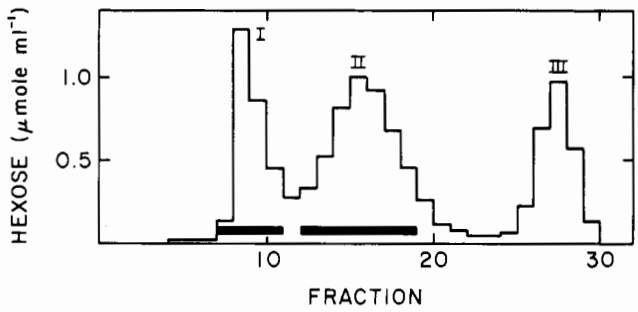

FIGURE 4: Separation of high and low molecular weight glycogens by chromatography on Sepharose 4B. Experimental details are described under Materials and Methods. This sample contained 0.5 mg of high molecular weight glycogen (I), 1.4 mg of low molecular weight glycogen (II), and 0.4 mg of sucrose (III) from the glycogen synthase concentrate. The heavy bars indicate the fractions whose ^{14}C activities were added to calculate the total isotope in the two glycogen forms (Table II).

assay conditions. This was accomplished by utilizing high and low molecular weight forms of glycogen which could be separated by chromatography on Sepharose 4B. Rabbit liver glycogen was used as the high molecular weight form; the low molecular weight form was oyster glycogen partially degraded by acid hydrolysis. Both were initially fractionated by chromatography on Sepharose 4B. Separation of these materials is shown in Figure 4. The approximate kinetic equivalence of the two polysaccharides was demonstrated by preincubating the enzyme at concentrations between 25 and 1.6 μg/mL with 4.3 mg/mL high molecular weight glycogen or with 11.9 mg/mL low molecular weight glycogen for a period of 30 min at 30 °C. The enzyme activities were almost exactly the same with the two polysaccharides. In addition, the time courses of activation were virtually identical when the enzyme was 6 μg/mL.

The experiment was carried out by preincubating each of the polysaccharides with glycogen synthase and adding these to solutions containing UDPG with ^{14}C in the glucosyl group and either no polysaccharide or the other molecular weight form. After 3 min the reactions were stopped. At the same time, experiments were carried out by using 10% of the enzyme and incubating 10 times as long with UDPG. In each case approximately half of the isotope had been transferred to polysaccharide. The solutions were then chromatographed on Sepharose 4B, and the individual fractions were analyzed for polysaccharide and isotope (Figure 4); the results are shown in Table II. When no additional polysaccharide is present

Table II: Distribution of Transferred Glucose Residues between High and Low Molecular Weight Glycogens

no.	glycogen in preincubn	glycogen added with [^{14}C]-UDPG	incubn time with [^{14}C]-UDPG (min)	total ^{14}C transfer (%)	cor isotope[a] high M_r glycogen (%)	cor isotope[a] low M_r glycogen (%)
1	high M_r	none	3	54	100	0
2	high M_r	low M_r	3	53	77	23
3	high M_r[b]	low M_r	30	56	52	48
4	low M_r	none	3	57	0	100
5	low M_r	high M_r	3	55	24	76
6	low M_r[b]	high M_r	30	57	44	56

[a] Calculated from the sum of cpm in 0.50-mL aliquots of fractions 8–11 for high M_r glycogen and fractions 13–19 for low M_r glycogen. Results were corrected for 3% spillover of high M_r glycogen into fractions 13–19 and 11% spillover of low M_r glycogen into fractions 8–11. [b] These reactions contained only 2.5 μg/mL enzyme during preincubation, as opposed to 25 μg/mL in the others, and were allowed to react with [^{14}C]UDPG for a proportionately longer time.

in the assay, most of the isotope is recovered in fractions corresponding to the main peak of the polysaccharide present during preincubation (97% for the high molecular weight form and 89% for the low molecular weight form). When the data are corrected for this partial spillover into the wrong fractions, it can be seen that in the experiments carried out over 30 min the isotope approaches an equal division between the two polysaccharides, but in the 3-min experiments the distribution is ~75% in the preincubated polysaccharide and ~25% in the glycogen added in the assay.

Discussion

These experiments confirm the slow activation of rabbit muscle synthase I in the presence of glycogen; however, the details differ significantly from the more extensive work on human leucocyte synthase I (Sølling & Esmann, 1977b). Leucocyte synthase I is activated at a rate proportional to the concentration of glycogen, while the present experiments show an approximately constant rate at glycogen concentrations from 0.05 to 25 mg/mL. Furthermore, the final activities observed in incubations of leucocyte synthase fit their model in that the fraction of glycogen-bound enzyme depends upon the first power of the glycogen concentration, while the present experiments indicate a dependence upon the third or fourth root of the glycogen concentration. Possibly the inactivation

of synthase observed here influences this result, but the difference seems too large to attribute to this cause alone. Although the present experiments are not extensive enough to justify proposing a mechanism for activation, it is clear that the rabbit muscle enzyme does not fit the model that accommodates the leucocyte enzyme so well.

A similar slow activation of rabbit muscle phosphorylase *a* by glycogen was investigated by Metzger et al. (1967). The initial rate of activation was independent of the glycogen concentration, while the ultimate extent of activation depended on the concentration. They concluded that phosphorylase *a* tetramer cannot bind to glycogen but must first dissociate to the dimer which then binds rapidly. A similar model may apply to rabbit muscle glycogen synthase I, which has been shown to exist as a tetramer (Nimmo et al., 1976). Although Metzger et al. (1967) did not examine the effect of enzyme concentration, their proposed mechanism is compatible with decreased activation at higher enzyme concentration, as observed in this work (Figure 3).

An experiment was designed to estimate the rate at which the glycogen synthase–glycogen complex exchanges one glycogen molecule for another. The result in Table II indicates that when enzyme bound to either high or low molecular weight glycogen is treated with the other polysaccharide and [^{14}C]UDPG, the isotope distribution between the two polysaccharides approaches 50% of its final value in 3 min. The experiment is difficult to interpret exactly because UDPG was converted 53–57% to UDP, which is an inhibitor competitive with respect to UDPG (McVerry & Kim, 1974; Plesner et al., 1974); therefore, reaction with UDPG was proceeding faster at the beginning when the enzyme was bound largely to the preincubated polysaccharide. It is clear that the polysaccharide molecules bound to the enzyme during preincubation are the initial sites of glucose incorporation and that the enzyme is capable of slowly exchanging this polysaccharide molecule for others under the reaction conditions.

The rate constant for exchange was estimated roughly by a trial and error method involving numerical integration. It is necessary to assume that exchange is a first-order process which is not affected by the changing concentrations of UDP and UDPG and eventually reaches an equilibrium in which the concentrations of the two kinds of enzyme–polysaccharide complexes are equal. It is helpful to assume that K_m for UDPG is approximately equal to K_I for UDP and that the initial concentration of UDPG (0.2 mM) is relatively large compared to K_m. The reported values are as follows: K_m for UDPG with rabbit muscle synthase I is 0.05 mM (Salsas & Larner, 1975a); K_I for UDP with leucocyte synthase D is 0.05 mM (Plesner et al., 1974) and with rabbit liver synthase D is 0.2 mM [from the data of McVerry & Kim (1974)]. This calculation leads to a value of 0.3 min^{-1} for the rate constant for exchange of glycogen molecules under the conditions of the experiment; a similar value may be estimated by inspection of the data. Values of 2 min for the half-time of the exchange reaction and 3 min for the average lifetime of the enzyme–glycogen complex can be calculated from the rate constant.

A striking comparison emerges if we consider the specific activity of the enzyme, 20 μmol min^{-1} mg^{-1}, under conditions similar to those under which the exchange of polysaccharide molecules is estimated. Based on the assumptions that the subunit molecular weight is 88 000 (Nimmo et al., 1976), that all subunits are functioning simultaneously, and that the synthase used here is pure, the minimum turnover number (catalytic center activity) is 1760 min^{-1}. Approximately 5000 glucose residues are incorporated into the polysaccharide molecule during the 3-min average lifetime of the enzyme–glycogen complex. If only one chain terminus in the glycogen molecule were available to the active site, this would require a chain elongation of 5000 before the enzyme could begin to act on another chain. In fact, action patterns of 1.4–1.7 (Salsas & Larner, 1975b) and ~9 [estimated from the data of Parodi et al. (1970) for rabbit muscle synthase] have been determined by chemical methods; even accepting the highest value, we calculate 550 to be the minimum number of chains in the glycogen molecule that is accessible to the bound enzyme before it dissociates. If substantial errors in the rates used in this calculation are assumed, it appears that the most conservative estimate requires that the enzyme be able to migrate freely over the surface of a glycogen molecule so as to act on hundreds of chain termini before dissociating.

Rabbit muscle glycogen synthase is tetrameric (Nimmo et al., 1976) and must contain at least four binding sites for individual chains in glycogen. This suggests a mechanism for the rapid exchange of individual chains without dissociation. If the enzyme binds several chains simultaneously, it may be possible for single binding sites to dissociate from one chain and bind to an adjacent chain; in this way the enzyme can "walk" over the glycogen molecule and act on any accessible chain without undergoing overall dissociation. Multiple binding sites also offer a potential explanation of the high affinity of glycogen synthase for glycogen compared to its poor binding to linear maltodextrins (Goldemberg, 1962; Larner et al., 1976); a similar explanation for the polysaccharide binding properties of muscle phosphorylase *a* has been presented on the basis of quantitative studies (Hu & Gold, 1975).

Because of the large equilibrium constant of the glycogen synthase reaction, it is practical to follow the isotope-exchange reactions in only one direction: from UDPG to UDP or glycogen. The same factor keeps exchange essentially linear in time over a substantial part of the reaction. If this were not the case, the heterogeneous nature of glycogen would cause deviations from linearity and complicate the calculation of rates for UDPG–glycogen exchange. Since the equilibrium constant for the glycogen synthase reaction (eq 1) has not been determined directly, it was necessary to estimate it from free-energy considerations. This gave a value twice that measured directly for the ADPG-dependent *E. coli* glycogen synthase reaction (Fox et al., 1976). An average value of 300 was used for these experiments. The exact value of the equilibrium constant is probably not of great consequence in the experiments, since the ratio of UDP to UDPG in the range of concentrations used here does not affect the relative rates of the two exchange reactions or the reaction rate in terms of fractional exchange per minute.

Reactant concentrations in the exchange reactions can be varied in such a way as to maintain chemical equilibrium. Since the equilibrium constant contains only the concentrations of UDP and UDPG, these could be varied over a range of 0.2–100 mM (for UDP), keeping their ratio constant. Glycogen could be varied independently from 0.25 to 61 mg/mL. Although there was some variation of rates in runs done at the same time (Table I), it appears that all the rates are approximately the same and all conditions studied must be giving a maximum velocity for exchange. The significant observation is that the UDPG–glycogen exchange rate is almost always 85–95% of the UDPG–UDP exchange rate in the same run. Equality of all exchange rates is characteristic of rapid equilibrium mechanisms where the interconversion of central complexes is rate limiting for all exchanges as well as for the overall reaction. Ordered and random mechanisms

which are not rapid equilibrium should show complete or partial inhibition of some exchanges relative to others as the concentrations of appropriate substrate–product pairs are raised to high levels.

The deviation of the exchange rates from equality is greater than would be expected from their standard deviations (3–7%) and is consistently in the same direction. It is likely that the difference between the rates is the result of a systematic error in which some glycogen is retained by the Dowex 1-X8 used to remove labeled UDPG. Several experiments gave ratios of 0.95 for the exchange rates; when one considers the fact that treatment of reaction mixtures with amylase results in a 2–3% increase in the recovery of the isotope in glycogen, this ratio is close enough to be unity.

Other possible sources of error are unlikely because use of maltoheptaose as activator and substrate instead of glycogen resulted in exchange ratios of almost exactly 1. The kinetic mechanism with maltoheptaose is strictly rapid equilibrium, consistent with its multichain action pattern (Goldemberg, 1962; Brown et al., 1965). It is noteworthy that the rate of exchange with maltoheptaose is approximately doubled by increasing its concentration from 50 to 100 mM, indicating that maltoheptaose is far from saturating at the higher concentration. The exchange rate at 100 mM maltoheptaose is double that with glycogen, so the maximum exchange rate is likely to be considerably greater than the maximum rate with glycogen. This agrees with observations that the catalytic V_{max}'s of some maltodextrins are greater than the V_{max} of glycogen (Goldemberg, 1962; Larner et al., 1976), although the differences in exchange rates seem much greater.

The reported action pattern of this enzyme (Parodi et al., 1970; Salsas & Larner, 1975b) is inconsistent with the proposed mechanism. It is possible that the slightly reduced rate of UDPG–glycogen exchange, compared to the UDPG–UDP exchange, is a reflection of the action pattern (or vice versa). This would mean that the mechanism is not strictly rapid equilibrium, but the rate of dissociation of individual chain termini from the active site approaches the rate of interconversion of central complexes, so a small fraction of the labeled glucose residues is converted back to UDPG. This is unlikely because the minimum turnover number for isotope exchange is $\sim 0.5\ min^{-1}$, with the criteria described above, which is close to the rate at which the enzyme–glycogen complex exchanges glycogen molecules. Since the enzyme is capable of acting upon hundreds of chain termini during the average lifetime of the complex, there must be a similar number of chains available during one isotope-exchange turnover, and the probability of a labeled glucose residue being converted back to UDPG is small. A final possibility is that the conclusions of Salsas & Larner (1975b) are in error, and the action pattern is strictly multichain.

Since the kinetic mechanism with glycogen as substrate is of the rapid-equilibrium type, results of initial velocity and product inhibition experiments require that there be kinetically significant binary complexes of the enzyme with glycogen, UDPG, and UDP and ternary complexes with glycogen–UDPG and glycogen–UDP. The kinetic mechanism may be considered rapid equilibrium random bi bi; however, it must be recognized that it is not possible to distinguish this mechanism from a rapid equilibrium ordered mechanism with dead-end binary complexes by any of the methods described here (Frieden, 1976; Purich et al., 1977).

An apparent contradiction exists in designating a kinetic mechanism "rapid equilibrium" when it can be shown that a particular substrate or product dissociates slowly. In the case of glycogen synthase, or any enzyme that acts on a polymer containing numerous reactive sites, the kinetic rapid equilibrium condition is satisfied if a large pool of reactive groups in a single substrate molecule exchanges within the enzyme active site rapidly compared to the rate of interconversion of central complexes. The fact that true dissociation of the enzyme–substrate complex is slow does not negate the rapid equilibrium character of the kinetic mechanism.

References

Bock, R. M. (1960) *Enzymes, 2nd Ed. 2*, 3.

Boyer, P. D. (1959) *Arch. Biochem. Biophys. 82*, 387.

Boyer, P. D., & Silverstein, E. (1963) *Acta Chem. Scand. 17*, S195.

Brown, D. H., Illingworth, B., & Kornfeld, R. (1965) *Biochemistry 4*, 486.

Cleland, W. W. (1967) *Annu. Rev. Biochem. 36*, 77.

DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., & Krebs, E. G. (1968) *J. Biol. Chem. 243*, 2200.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.

Fox, J., Kawaguchi, K., Greenberg, E., & Preiss, J. (1976) *Biochemistry 15*, 849.

Frieden, C. (1976) *Biochem. Biophys. Res. Commun. 68*, 914.

Garen, A., & Levinthal, C. (1960) *Biochim. Biophys. Acta 38*, 470.

Goldemberg, S. H. (1962) *Biochim. Biophys. Acta 56*, 357.

Hers, H. G. (1976) *Annu. Rev. Biochem. 45*, 167.

Hu, H.-Y., & Gold, A. M. (1975) *Biochemistry 14*, 2224.

Kornfeld, R., & Brown, D. H. (1962) *J. Biol. Chem. 237*, 1772.

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., & Fischer, E. H. (1964) *Biochemistry 3*, 1022.

Larner, J., Takeda, Y., & Hizukuri, S. (1976) *Mol. Cell. Biochem. 12*, 131.

Leloir, L. F., & Goldemberg, S. H. (1960) *J. Biol. Chem. 235*, 919.

McVerry, P. H., & Kim, K.-H. (1974) *Biochemistry 13*, 3505.

Metzger, B., Helmreich, E., & Glaser, L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 994.

Neufeld, E. F., & Hassid, W. Z. (1963) *Adv. Carbohydr. Chem. 18*, 309.

Nimmo, H. G., Proud, C. G., & Cohen, P. (1976) *Eur. J. Biochem. 68*, 21.

Parodi, A. J., Mordoh, J., Krisman, C. R., & Leloir, L. F. (1970) *Eur. J. Biochem. 16*, 499.

Passonneau, J. V., Schwartz, J. P., & Rottenberg, D. A. (1975) *J. Biol. Chem. 250*, 2287.

Plesner, L., Plesner, I. W., & Esmann, V. (1974) *J. Biol. Chem. 249*, 1119.

Purich, D. L., Allison, R. D., & Todhunter, J. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 753.

Rottenberg, D. A., Passonneau, J. V., & Lust, W. D. (1972) *Biochem. Biophys. Res. Commun. 48*, 1192.

Salsas, E., & Larner, J. (1975a) *J. Biol. Chem. 250*, 3471.

Salsas, E., & Larner, J. (1975b) *Mol. Cell. Biochem. 7*, 195.

Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A., & Krebs, E. G. (1970) *J. Biol. Chem. 245*, 6317.

Sølling, H., & Esmann, V. (1977a) *Eur. J. Biochem. 81*, 119.

Sølling, H., & Esmann, V. (1977b) *Eur. J. Biochem. 81*, 129.

Stalmans, W., & Hers, H. G. (1973) *Enzymes, 3rd Ed. 9*, 309.

Steiner, D. F., Younger, L., & King, J. (1965) *Biochemistry 4*, 740.