Biosynthesis of Bacterial Glycogen. Purification and Properties of the *Escherichia coli* B ADPglucose:1,4-α-D-glucan 4-α-Glucosyltransferase†

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ABSTRACT: The *Escherichia coli* B glycogen synthase has been purified to apparent homogeneity with the use of a 4-aminobutyl-Sepharose column. Two fractions of the enzyme were obtained: glycogen synthase I with a specific activity of 380 μmol mg^{-1} and devoid of branching enzyme activity and glycogen synthase II having a specific activity of 505 μmol mg^{-1} and containing branching enzyme activity which was 0.1% of the activity observed for the glycogen synthase. Only one protein band was found in disc gel electrophoresis for each glycogen synthase fraction and they were coincident with glycogen synthase activity. One major protein band and one very faint protein band which hardly moved into the gel were observed in sodium dodecyl sulfate gel electrophoresis of the glycogen synthase fractions. The subunit molecular weight of the major protein band in sodium dodecyl sulfate gel electrophoresis of both glycogen synthase fractions was determined to be 49 000 ± 2 000. The molecular weights of the native enzymes were determined by sucrose density gradient ultracentrifugation. Glycogen synthase I had a molecular weight of 93 000 while glycogen synthase II had a molecular weight of 200 000. On standing at 4 °C or at −85 °C both enzymes transform into species having molecular weights of 98 000, 135 000, and 185 000. Thus active forms of the *E. coli* B glycogen synthase can exist as dimers, trimers, and tetramers of the subunit. The enzyme was shown to catalyze transfer of glucose from ADPglucose to maltose and to higher oligosaccharides of the maltodextrin series but not to glucose. 1,5-Gluconolactone was shown to be a potent inhibitor of the glycogen synthase reaction. The glycogen synthase reaction was shown to be reversible. Formation of labeled ADPglucose occurred from either [^{14}C]ADP or [^{14}C]glycogen. The ratio of ADP to ADPglucose at equilibrium at 37 °C was determined and was found to vary threefold in the pH range of 5.27–6.82. From these data the ratio of ADP^{2-} to ADPglucose at equilibrium was determined to be 45.8 ± 4.5. Assuming that $\Delta F°$ of the hydrolysis of the α-1,4-glucosidic linkage is −4.0 kcal the $\Delta F°$ of hydrolysis of the glucosidic linkage in ADPglucose is −6.3 kcal.

The synthesis of α-1,4-glucosidic linkages of glycogen is considered to be catalyzed by ADPglucose:1,4-α-glucan 4-α-glucosyltransferase (EC 2.4.1.21, bacterial glycogen synthase) in bacteria (Preiss, 1969) and by UDPglucose:glycogen 4-α-glucosyltransferase (EC 2.4.1.11) in mammals and in yeast (Stalmans and Hers, 1973). While information on subunit structure (Soderling et al., 1970; Smith et al., 1971; Lin and Segal, 1973; Issa and Mendicino, 1973; McVerry and Kim, 1974; Huang and Cabib, 1974) and on kinetic mechanism (Plesner et al., 1974; Huang and Cabib, 1974; Salsas and Larner, 1975) is available on the animal and yeast glycogen synthases, little is known about either for the bacterial glycogen synthases. Thus a study of the *Escherichia coli* B glycogen synthase was initiated to determine its physical, chemical, and kinetic properties. Previous reports were concerned with partial purification of the enzyme (Fox et al., 1972), preliminary kinetic studies (Preiss and Greenberg, 1965), and with the interaction of partially purified *E. coli* B glycogen synthase with branching enzyme (Fox et al., 1973) in a reaction catalyzed by high concentrations of citrate in the presence of small concentrations of α-glucan primer.

This paper reports on the purification of the *E. coli* B glycogen synthase to apparent homogeneity with the aid of 4-aminoalkyl-Sepharose (Shaltiel and Er-El, 1973), the molecular weight of the native enzyme and of its subunits, and on some kinetic properties of the enzyme. The reaction catalyzed by the synthase has also been shown to be reversible and a value for the equilibrium constant has been obtained.

Experimental Section

Materials

ADP[^{14}C]glucose was prepared enzymatically from [U-^{14}C]glucose (Preiss and Greenberg, 1972). Maize amylopectin (amylose free) was purchased from Calbiochem, Los Angeles, Calif.; maltose from Mann Research Laboratories, New York, N.Y.; rabbit liver glycogen from Sigma, St. Louis, Mo.; potato amylose (average DP-300) from Nutritional Biochemical Co., Cleveland, Ohio; hog pancreatic α-amylase (DFP-treated; 640 U/mg) from Worthington, Freehold, N.J. Malto oligosaccharides [degree of polymerization 2–9] were prepared from amylose by partial acid hydrolysis and isolated by paper chromatography (Ough, 1964). *E. coli* B glycogen was isolated as previously described (Preiss et al., 1975). 3-Aminopropyl-, 4-amino-

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butyl- and 6-aminohexyl-Sepharose columns were prepared as described by Shaltiel and Er-El (1973). Other reagents were obtained commercially at the highest purity available.

Methods

Maintenance and Growth of Organisms. The *E. coli* B strain used was a mutant, SG 3, derepressed in levels of ADPglucose pyrophosphorylase and glycogen synthase (Govons et al., 1969). It was maintained on nutrient broth agar slants. Cells were grown in 100 l. of media, pH 7.0, containing 0.0625 M KH_2PO_4, 0.063 M K_2HPO_4, 0.6% yeast extract, and 0.4% glucose in a 130-l. New Brunswick fermenter for 16 h at 37 °C. The cells were harvested in a Sharples ultracentrifuge and stored at −85 °C.

Paper chromatography was done on Whatman No. 1 paper for 48 h in butan-1-ol–pyridine–water (6:4:3, v/v), solvent A, or for 18 h in 95% ethanol–1 M ammonium acetate (pH 3.8) (5:2, v/v), solvent B, or for 24 h in isobutyric acid–1 M NH_3–0.1 M EDTA (pH 7.3) (100:66:1), solvent C. Thin-layer chromatography on polyethylenimine-cellulose was done as described by Randerath and Randerath (1967).

Assay of Bacterial Glycogen Synthase. Incorporation of Glucose into α-Glucan. Assay A. The reaction mixture contained 140 nmol of ADP[^{14}C]glucose (500 cpm/nmol), 10 μmol of bicine[1] buffer (pH 8.5), 5 μmol of potassium acetate, 2 μmol of GSH, 0.1 μmol of magnesium acetate, 100 μg of bovine plasma albumin, 0.5 mg of rabbit liver glycogen, and enzyme in a total volume of 0.2 ml. After incubation for 15 min at 37 °C the [^{14}C]glucose incorporated into methanol-insoluble polysaccharide was determined by the method of Preiss and Greenberg (1965). Glycogen synthase fractions were diluted into a buffer solution containing 0.05 M Hepes–NaOH buffer (pH 7.0) containing 5 mM dithiothreitol and 100 μg/ml of bovine serum albumin before assay.

Assay A was used to measure glycogen synthase activity during the purification procedure. Magnesium acetate was not required for activity but increased the extent of reaction that was linear with time and protein concentration. It was omitted, however, in the equilibrium and kinetic studies. One unit of glycogen synthase activity is defined as 1 μmol of glucose transferred in 1 min under the conditions of assay A.

Incorporation of Glucose into Oligosaccharides. Assay B. The reaction mixture was the same as assay A except various oligosaccharides (maltose, maltotriose, etc.) were substituted for glycogen. After incubation for 15 min at 37 °C, the reaction was stopped by heating for 1 min at 100 °C and 1 ml of slurry containing 200 mg of anion-exchange resin [Dowex 1-X-8, Cl^- form; 200–400 mesh] was added to adsorb the remaining ADP[^{14}C]glucose. After filtration through Whatman No. 1 filter paper, the radioactivity in 0.5 ml of the filtrate was determined in a liquid scintillation counter (Ozbun et al., 1972).

Reversibility of the Glycogen Synthase Reaction. Assay Using [^{14}C]ADP. The reaction mixture contained 20 μmol of buffer, 1.0 μmol of [^{14}C]ADP (1005 cpm/nmol), 0.4 mg of rabbit liver glycogen, 2.5 μmol of potassium acetate, 1 μmol of GSH, and enzyme in a volume of 0.2 ml. The reaction was incubated at 37 °C for 2 to 60 min and then terminated by heat denaturation at 100 °C for 1 min. Bicine buffer (pH 8.0), 20 μmol, $MgCl_2$, 1.0 μmol, and *E. coli* alkaline phosphatase, 2.2 units, were added and the reaction mixture (now 0.25 ml in volume) was incubated for 1 h at 37 °C. The phosphatase quantitatively converted the labeled ADP to adenosine and this allowed better separation of the product ADPglucose from the labeled substrate. EDTA, 2 μmol, was added and the reaction mixture streaked out on Whatman No. 1 paper strips 1.5 cm wide. The papers were chromatographed in solvent C with ADPglucose and ADP as standards. The radioactive compounds (ADPglucose and adenosine) were located with a strip counter, cut out, and counted in a liquid scintillation counter.

Assay Using [^{14}C]Glycogen. The reaction mixture contained 7.5 μmol of potassium acetate buffer (pH 5.6), 800 nmol of ADP, 10 μg of [^{14}C]glycogen (2.13×10^5 cpm), and 2.6 units of glycogen synthase in a volume of 0.06 ml. The reaction was incubated for 30 min and terminated by heat denaturation for 1 min at 100 °C. The mixture was chromatographed on Whatman No. 1 paper in solvent B overnight with an ADPglucose standard. The radioactive spots (glycogen at the origin and ADPglucose) were located with a strip counter, cut out, and counted in a liquid scintillation counter.

Preparation of [^{14}C]Glycogen. The [^{14}C]glycogen used in the reversal experiment was synthesized using ADP[^{14}C]glucose (specific activity 6870 cpm/nmol) and glycogen synthase I (Bio-Gel P200 fraction). The reaction mixture contained 4.2 μmol of ADP[^{14}C]-glucose, 1 mg of rabbit liver glycogen, 100 μg of bovine serum albumin, 2 μmol of GSH, 15 μmol of potassium acetate, 30 μmol of bicine buffer (pH 8.5), 5 μmol of magnesium acetate, and 0.32 μg of enzyme in a volume of 0.72 ml. The mixture was incubated for 40 min at 37 °C and then 2 ml of absolute methanol plus 1 drop of 20% KCl were added. After standing overnight the glycogen precipitate was centrifuged for 10 min at 16 000g and washed twice with 75% aqueous methanol containing 1% KCl. The glycogen precipitate was dissolved in 0.5 ml of H_2O.

Protein Determination. Proteins were assayed by the method of Lowry et al. (1951).

Assay of Branching Enzyme Activity [EC 2.4.1.18]. The stimulation caused by branching enzyme of the catalysis of synthesis of 1,4-α-glucan from glucose-1-P by rabbit muscle phosphorylase (Illingworth et al., 1961; Brown et al., 1961) was the basis of the assay and was a modification of the procedure previously published by Brown and Brown (1966). The reaction mixture contained in a volume of 0.2 ml, 0.1 M sodium citrate (pH 7.0), 1 mM AMP, 50 mM [^{14}C]glucose-1-P (5.0×10^4 cpm/μmol), 40 μg of crystalline rabbit muscle phosphorylase A (Worthington Biochemical Corporation, Freehold, N.J., or Sigma, St. Louis, Mo.), and branching enzyme. The reaction was initiated by addition of glucose-1-P and incubated at 30 °C. Aliquots were taken at 60, 90, and 120 min and incorporation of label into glucan was assayed as previously described (Hawker et al., 1974). A unit of enzyme activity is defined as 1 μmol of glucose incorporated per min under the above conditions. Controls containing no branching enzyme or branching enzyme (heat denatured at 100 °C for 1 min) incorporated less than 20 nmol of glucose into the glucan fraction. The control values were subtracted from the values obtained from reaction mixtures containing active branching enzyme.

Assay of α-Amylase [EC 3.2.1.1] Activity. α-Amylase

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; bicine, *N*,*N*′-bis(2-hydroxyethyl)glycine.

activity was determined by the method of Bernfeld (1951).

Electrophoresis. Disc gel electrophoresis was performed on 8% polyacrylamide gel using the Ornstein-Davis Tris-glycine buffer system (Ornstein, 1964; Davis, 1964). Gel electrophoresis was also performed on 10% polyacrylamide gels in sodium dodecyl sulfate (Weber and Osborn, 1969). Protein bands were located by staining with Coomassie Blue (Chrambach et al., 1967).

Location of Glycogen Synthase Activity in Polyacrylamide Gels. The detection of glycogen synthase activity in gels was based on iodine complex formation with the glucan product. Bicine buffer (pH 8.0), 100 μmol, potassium acetate, 50 μmol, magnesium acetate, 1 μmol, bovine serum albumin, 1 mg, GSH, 20 μmol, rabbit liver glycogen 5 mg or amylose 500 μg, and ADPglucose, 2 μmol, were mixed together in a volume of 2 ml and incubated with the gels for 2–4 h at room temperature. The reaction mixture was then removed and the gels were immersed in a 0.05 N HCl solution containing 0.2% KI and 0.02% I_2. A brownish-blue band was detected on the gel. When ADPglucose was omitted from the reaction mixture no band formation was observed.

Ultracentrifugation in Sucrose Density Gradients. Sucrose density centrifugation was carried out according to the method of Martin and Ames (1961). Linear sucrose gradients (4.3 ml) were prepared by mixing 5% (w/v) sucrose and 25% (w/v) sucrose solutions both containing 50 mM Hepes buffer (pH 7.0) and 5 mM dithiothreitol. The gradients were layered with 90 μl of the above buffer solution containing either 3.2 μg of glycogen synthase fraction I or 5 μg of glycogen synthase fraction II with 50 μg of beef heart lactate dehydrogenase, and 50 μg of beef liver malate dehydrogenase. In a few experiments 100 μg of rabbit muscle pyruvate kinase was also added as a molecular weight standard. Centrifugation was carried out for 16–20 h and 7-drop fractions were collected. Lactate dehydrogenase activity was assayed by the method of Kornberg (1955), malate dehydrogenase activity was determined by the method of Ochoa (1955), and pyruvate kinase activity was determined by the method of Bücher and Pfleiderer (1955). Glycogen synthase activity was measured as described above (assay A).

Determination of Polyglucose in Glycogen Synthase Fractions. Glycogen synthase fraction I and glycogen synthase fraction II, 200 μg, were diluted to 1 ml and dialyzed against 4 l. of H_2O for 12 h. The dialyzed fractions were placed into a fresh 4 l. of H_2O and dialyzed for another 12 h. The change of dialyzing medium was repeated three more times. The dialyzed fractions were made 2 N HCl with a 6 N HCl solution and hydrolyzed for 2 h in a boiling water bath. The hydrolyzed fractions were concentrated to 0.5 ml by flash evaporation and then lyophilized. The fractions were dissolved in 1.0 ml of H_2O and the pH was adjusted to pH 7.0 with a 0.1 N NH_3 solution. The concentration of glucose was determined by measuring the amount of TPNH formed when the neutralized fractions were added to reaction mixtures containing yeast hexokinase and glucose-6-P dehydrogenase (Bergmeyer et al., 1974).

Results

Purification of Glycogen Synthase

(1) Extraction of the Enzyme and Isolation of the 79 000g Particulate Fraction. The glycogen synthase was purified through the DEAE-cellulose chromatography as previously described (Fox et al., 1972) except with the modifications described below. *E. coli* B strain SG 3 cells, 954 g, were suspended in 1700 ml of a cold 50 mM glycylglycine buffer (pH 7.0) containing 5 mM dithiothreitol and passed through a Manton-Gaulin homogenizer (Charm and Matteo, 1971) three times at 7 000 psi pressure. The temperature of the extract was not allowed to increase above 15 °C. The crude extract was centrifuged at 10 000 rpm for 15 min and the supernatant fluid was further centrifuged at 78 000*g* for 90 min. The 78 000*g* precipitate, which contained 88% of the glycogen synthase activity, was suspended in 1300 ml of 0.05 M potassium phosphate buffer (pH 6.8) containing 5 mM dithiothreitol and 0.1 M NaCl.

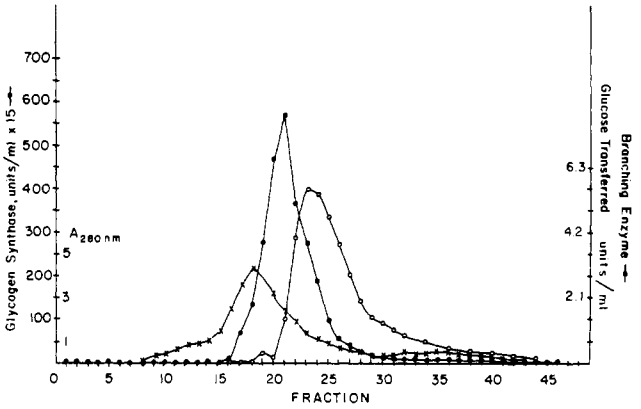

FIGURE 1: Chromatography of glycogen synthase on DEAE-cellulose 52 (Whatman). The procedure is described in the text. DEAE-cellulose fractions 17–20 are glycogen synthase fraction 1 and fractions 21–25 are glycogen synthase fraction 2. (x) Absorbance at 280 nm. The fraction volumes were 40 ml.

(2) α-Amylase Treatment of the 78000g Precipitate. This step was done as previously described (Fox et al., 1972). This treatment solubilized the enzyme by degrading the particulate glycogen fraction.

(3) Ammonium Sulfate Fractionation. The glycogen synthase fraction from the α-amylase treatment step was fractionated with ammonium sulfate as previously described (Fox et al., 1972).

(4) DEAE-cellulose Chromatography Step. The DEAE-cellulose chromatography step was done as previously described (Fox et al., 1972) except that branching enzyme activity was assayed for in the fractions. The column was 2.5 × 40 cm and was equilibrated with 0.05 M Tris-Cl buffer (pH 7.5) containing 2.5 mM dithiothreitol and 5% sucrose. The enzyme was eluted with a linear gradient composed of the above Tris-Cl, dithiothreitol–sucrose buffer in the mixing chamber, and 1 l. of the same buffer containing 0.6 M NaCl in the reservoir chamber. Figure 1 shows that there is partial separation of glycogen synthase from branching enzyme. Glycogen synthase fractions 17–20 contain very little branching enzyme. Thus glycogen synthase fractions 17–20, hereafter called glycogen synthase fraction I (DEAE fraction I in Table I), were pooled and concentrated by ultrafiltration and $(NH_4)_2SO_4$ fractionation as previously described (Fox et al., 1972). Fractions 21–25 which contained branching enzyme were pooled and concentrated in the same manner and are hereafter designated glycogen synthase fraction II (DEAE fraction II in Table I). The two fractions were further purified separately. Over 95% of the α-amylase activity remaining with the glycogen synthase fractions after ammonium sulfate fractionation was not adsorbed onto the DEAE-cellulose column and appeared in

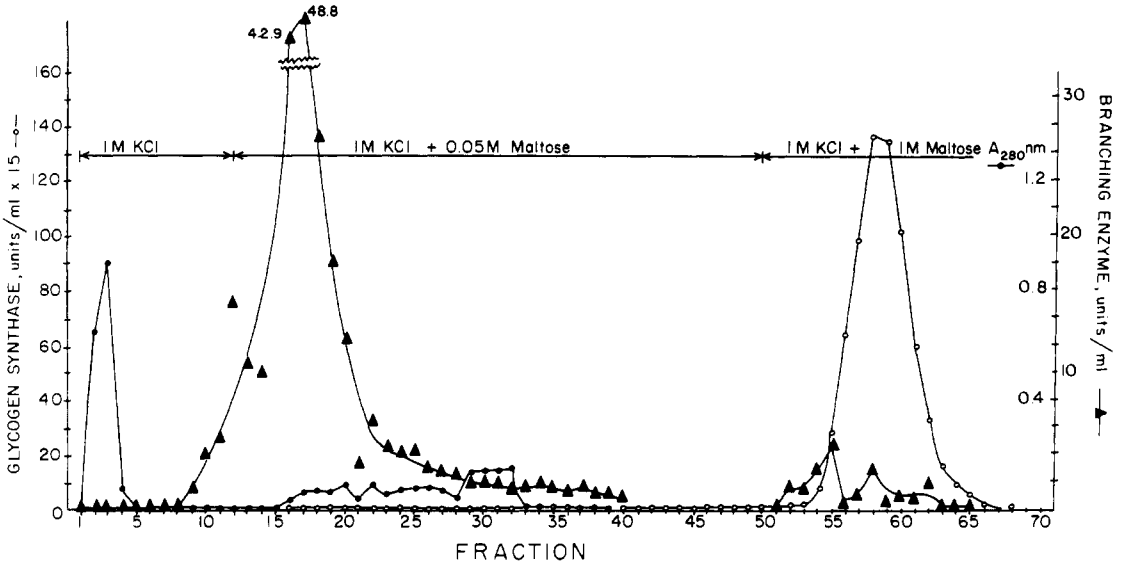

FIGURE 2: Chromatography of glycogen synthase II on 4-aminobutyl-Sepharose. The procedure is described in the text. (●) Absorbance at 280 nm. Fractions 55–62 containing glycogen synthase activity were pooled and concentrated. The volume of fractions 1–49 was 100 ml and the volume for fractions 50–70 was 50 ml.

Table I: Purification of *E. coli* Glycogen Synthase.[a]

Step	Vol (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg)
1. Crude extract	1 760	57 200	12 067	0.21
1. 70 000*g* precipitate	1 300	8 590	10 667	1.25
2. α-Amylase treatment + 70 000*g* supn't	5 130	3 680	8 000	2.17
3. $(NH_4)_2SO_4$	74.5	2 160	5 960	2.75
4. DEAE fraction I	16	706	1 320	1.87
DEAE fraction II	12.5	389	2 487	6.4

[a] The units of branching enzyme activity in DEAE fraction II were 187. DEAE fraction I contained less than 0.006 unit of branching enzyme activity.

Table II: Purification of Glycogen Synthases I and II.

Step	Vol (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Branching Enzyme (units)
Glycogen Synthase I					
4. DEAE fraction I	16	706	1,320	1.87	
5. 4-Aminobutyl-Sepharose	4.1	3.5	679	193	
6. Bio-Gel P200	2.5	1.30	494	380	
Glycogen Synthase II					
4. DEAE fraction II	12.5	389	2487	6.4	187
5. 4-Aminobutyl-Sepharose	4.5	3.83	1247	273	1.83
6. Bio-Gel P200	2.3	2.37	1198	505	1.45

the pass through and wash fraction when assayed by the method of Bernfeld (1951).

(*5*) *Chromatography on 4-Aminobutyl-Sepharose.* Glycogen synthase fraction I, 16 ml, was adsorbed onto a 4-aminobutyl-Sepharose column (2.5 × 39 cm resin bed volume, 192 ml) equilibrated with 0.05 M Hepes buffer (pH 7.0) containing 5% sucrose and 2.5 mM dithiothreitol. The column was washed successively with 800 ml of the Hepes–sucrose–dithiothreitol buffer, with 1200 ml of the Hepes–sucrose–dithiothreitol buffer containing 1 M KCl, and with 1800 ml of the Hepes–sucrose–dithiothreitol buffer containing 0.05 M maltose and 1 M KCl. The enzyme was eluted with 1200 ml of the Hepes–sucrose–dithiothreitol buffer containing 1 M maltose plus 1 M KCl. The fractions containing synthase activity were pooled and concentrated to 4 ml with an Amicon ultrafiltration apparatus using a PM-30 membrane filter and then dialyzed against 2 l. of 0.05 M Hepes buffer (pH 7.0) containing 10% sucrose and 5 mM dithiothreitol.

Glycogen synthase fraction II was purified in the same manner except 4500 ml of the buffer solution containing 0.05 M maltose and 1 M KCl were used. Figure 2 shows that almost all of the branching enzyme activity is eluted with the buffer solution containing 0.05 M maltose and 1 M KCl.

(*6*) *Chromatography on Bio-Gel P200.* Glycogen synthase fraction I was passed through a 1.6 × 30 cm (resin bed volume 45 ml) column of Bio-Gel P200 equilibrated with 0.05 M Hepes buffer (pH 7.0) containing 5% sucrose and 5 mM dithiothreitol. The same buffer was used for elution. The flow rate of the column was 10 ml/h and 1.8-ml fractions were collected. The fractions containing glycogen synthase activity (5–9) were pooled and concentrated by ultrafiltration as described above.

Glycogen synthase fraction II was purified on Bio-Gel P200 in the same manner. Tables I and II summarize the purification of the two synthase fractions. Glycogen synthase fraction I was devoid of branching enzyme activity while glycogen synthase II, having a higher specific activity than synthase fraction I, contained a very small amount of branching enzyme activity. When 7 units of the Bio-Gel P200 fraction of either glycogen synthase I or II are incubated in the standard reaction mixture (assay A) with or without 40 mM NaCl, there was quantitative incorporation of the glucose from ADPglucose into glycogen within 1 h. No loss of radioactivity from the alcohol-insoluble product was observed even after 22 h incubation indicating that

Table III: Activities of Oligosaccharide Acceptors of Glycogen Synthase.[a]

	Glucose Transferred (nmol)
Maltose, 25 mM	2.0
Maltose, 250 mM	18.8
Maltotriose, 25 mM	13.5
Maltotetraose, 25 mM	21.9
Maltoheptaose, 25 mM	24.5
Rabbit liver glycogen, 2.5 mg/ml	25.1

[a] The enzyme used was glycogen synthase II (0.004 μg) and the procedure is described in the text (assay B).

there was no α-amylase present in the synthase fractions. No adenylate kinase activity (Colowick, 1955) was found in either fraction. The final yield of synthase activity summing both fractions was 14%. The enzyme fractions lose 50% activity in 2 weeks if stored at 4 °C but retain 100% activity for at least 1 year if stored at −85 °C.

Glucan was detected in both glycogen synthase fractions. There was 0.52 mg of anhydroglucose units per mg of protein in synthase fraction I and 1.63 mg of anhydroglucose units per mg of protein in synthase fraction II. Chromatography of the acid-hydrolyzed fractions (see Methods) in solvent A and detection of reducing sugars with $AgNO_3$ (Trevelyan et al., 1950) showed only one spot which cochromatographed with glucose.

Gel Electrophoresis of Glycogen Synthase Fractions. The Bio-Gel P200 fractions of glycogen synthases I and II showed only one protein staining band after disc gel electrophoresis in the Davis Tris-glycine system (Davis, 1964). About 20–24 μg of protein was used for the analyses. This protein band corresponded to the activity band observed after I_2 staining (see Methods). Electrophoresis of 20 μg of protein of the synthase fractions on sodium dodecyl sulfate gels according to the procedure of Weber and Osborn (1969) showed one major protein band with a very faint slower moving band that hardly migrated into the gel. The electrophoretic mobilities of the synthase subunit in the sodium dodecyl sulfate gels were compared with standard proteins electrophoresed at the same time on separate gels. The standard proteins were myoglobin, conconavalin, beef liver malate dehydrogenase, beef heart lactate dehydrogenase, rabbit muscle aldolase, ovalbumin, and rabbit muscle pyruvate kinase. The molecular weight values are those listed by Darnell and Klotz (1975). A plot of the distances of migration relative to the tracking dye (R_m) vs. the logarithm of the molecular weights of the standards gave a straight line. The R_m obtained for both synthase fractions was 0.27 which corresponded to a molecular weight of 49 000. The average molecular weight of the glycogen synthase subunit from four different preparations of the synthase fractions was 49 000 ± 2 000.

Ultracentrifugation in Sucrose Density Gradients. Glycogen synthase I migrated faster than malate dehydrogenase and slower than lactate dehydrogenase in sucrose gradient ultracentrifugation. The apparent molecular weight for synthase I was determined to be 92 700 ± 2 000 using lactate dehydrogenase and malate dehydrogenase as references for molecular weights (140 000 and 72 000, respectively). Glycogen synthase II moved faster than lactate dehydrogenase in sucrose gradient ultracentrifugation. Using lactate dehydrogenase and malate dehydrogenase as standards for molecular weight values the apparent molecular weight for synthase II was determined to be 200 000 ± 5 000. Glycogen synthase I, therefore, appears to be in the dimer form and synthase II appears to be in the tetrameric form. Aged preparations of glycogen synthase I or II when centrifuged in sucrose gradients yield three activity peaks with molecular weights of 98 000, 135 000, and 185 000, respectively (results not shown). Thus the enzyme can exist in active form as the dimer, trimer, or tetramer of the subunit.

Kinetic Properties of the Glycogen Synthase

pH Optimum. It was previously reported that the *E. coli* B glycogen synthase has a broad pH optimum from pH 7.0 to 9.5 (Preiss and Greenberg, 1965). The highly purified glycogen synthases I and II also had the same broad pH optimum. Moreover synthase I and II activity in reaction mixtures containing 0.05 M bicine buffer (pH 8.5) is twofold greater than when Tris-acetate or Tris-Cl (pH 8.5) is the buffer. The activity of the synthases with reaction mixtures containing Hepes buffer (pH 7.0), Mes buffer (pH 6.0), Mes buffer (pH 5.5), sodium acetate buffer (pH 5.5), or sodium acetate buffer (pH 5.0) was 95, 50, 14, 39, and 1.5%, respectively, of the activity observed with bicine buffer at pH 8.5.

Kinetic Constants. The K_m values of ADPglucose, *E. coli* B glycogen, rabbit liver glycogen, and soluble potato amylose were 35 μM, 274 μg/ml, 298 μg/ml, and 833 μg/ml, respectively, for glycogen synthase I. Essentially the same values were obtained for glycogen synthase II. About 10% of the glycosyl residues of glycogen are terminal and they are presumably the acceptors of glucose from ADPglucose. Thus the K_m values of rabbit liver glycogen and *E. coli* B glycogen may be expressed in terms of glucose terminal units as 0.18 and 0.17 mM, respectively. In these terms the K_m of soluble amylose (degree of polymerization = 300) is 0.017 mM.

Activity with Glucose and Oligosaccharides. Glucose and oligosaccharides of the maltodextrin series were tested for their ability to act as acceptors (Table III). Glucose up to a concentration of 1.4 M was found to be inactive as an acceptor. No activity was seen for glucose even if the amount of enzyme used in the experiment described in Table III was increased 50-fold.

Activity, however, was seen for maltose, maltotriose, maltotetraose, and maltoheptaose. At relatively low concentrations the higher oligosaccharides maltoheptaose and maltotetraose are more active and even approach the activity observed with 2.5 mg/ml of glycogen. The filtrates obtained from the Dowex 1-X8 (Cl^-) treatments were chromatographed in solvent A with the appropriate oligosaccharide standards to determine the nature of the products formed. The chromatograms were counted in a paper strip counter to locate the radioactive products. In each case the product formed was the next higher oligosaccharide in the maltodextrin series; i.e., maltotriose, maltopentaose, and maltooctaose were formed from maltose, maltotetraose, and maltoheptaose, respectively.

Inhibition by 1,5-D-Gluconolactone. Figure 3 shows that 1,5-D-gluconolactone is an effective inhibitor of glycogen synthase I. A Dixon plot gives a K_i value for gluconolactone of 0.38 mM under the conditions of the experiments. Glycogen synthase II was also inhibited by 1,5-D-gluconolactone.

Reversibility of the Glycogen Synthase Reaction. Formation of radioactive ADPglucose from ADP and glycogen was demonstrated using either [^{14}C]ADP or [^{14}C]glycogen

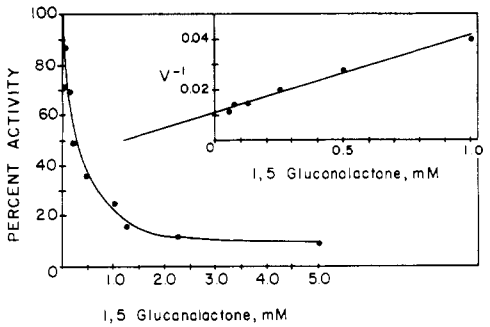

FIGURE 3: Effect of 1,5-D-gluconolactone on glycogen synthase I. The reaction mixtures and conditions are described as assay A except that 1,5-D-gluconolactone was added as indicated and Hepes buffer (pH 7.0) was used instead of bicine buffer. One hundred percent activity is the activity observed in the absence of inhibitor (10.8 nmol of glucose transferred).

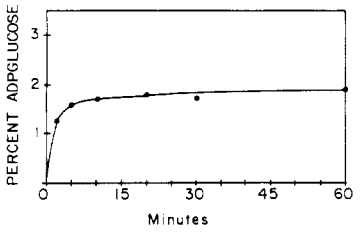

FIGURE 4: Formation of [^{14}C]ADPglucose from [^{14}C]ADP. The reaction mixture contained 1.17 μmol of ADP (specific activity = 890 cpm/nmol), sodium acetate buffer (pH 5.67), and 2.6 units of glycogen synthase II. The rest of the reaction mixture and procedure is described in the Methods section.

(Table IV). The reaction required ADP and glycogen and was inhibited by α-amylase. The radioactive product formed from either [^{14}C]glycogen or [^{14}]ADP was eluted from paper with H_2O and was verified to be ADPglucose by cochromatography with authentic ADPglucose in solvents B and C on Whatman No. 1 paper and in thin-layer chromatography on polyethylenimine-cellulose plates in 0.25 M LiCl and in 1.0 M LiCl (Randerath and Randerath, 1967). The isolated radioactive ADPglucose formed from either [^{14}C]glycogen or [^{14}C]ADP was reactive in the ADPglucose pyrophosphorylase reaction. Incubation with homogeneous *E. coli* B enzyme (Haugen et al., 1974) plus $MgCl_2$ and PP_i yielded a radioactive spot from the product formed from [^{14}C]glycogen which cochromatographed with glucose-1-P in solvent A. The product formed from [^{14}C]ADP gave rise to a radioactive product which cochromatographed with ATP when incubated with PP_i, $MgCl_2$, and ADPglucose pyrophosphorylase. The formation of labeled ATP and glucose-1-P required $MgCl_2$ and PP_i.

Equilibrium Constant of Glycogen Synthase Reaction. The equilibrium constant of the synthase reaction was determined using [^{14}C]ADP. Figure 4 shows the formation of ADPglucose with time. Addition of 2.6 more units of enzyme after 30 min of incubation did not increase the amount of ADPglucose formed from ADP. The average percent conversion in three experiments was 1.72 ± 0.08%. Using this value the equilibrium ratio of ADP to ADPglucose was 58 ± 2.5 at pH 5.67. Table V shows the equilibrium ratios of ADP to ADPglucose of the glycogen synthase reaction determined at pH values of 5.27-6.82. The equilibrium ratio increases about threefold as the pH of the reaction mixture increases in the above range.

Table IV: Reversibility of Glycogen Synthase Reaction.[a]

Conditions	ADP Glucose Formed: From [^{14}C]Glycogen (cpm)	ADP Glucose Formed: From [^{14}C]ADP (nmol)
Complete reaction	24 600	22
−ADP	1 717	
−Glycogen		2.1
Heat denatured enzyme	100	0.3
+α-Amylase, 12 units plus 12.5 mM KCl	250	0.9

[a] The reaction mixtures and procedure for the above experiments are described in the Methods section. The buffer used in the experiment with [^{14}C]ADP was sodium acetate (pH 5.67). The amount of ADP used was 1.17 μmol and its specific activity was 890 cpm/nmol. Glycogen synthase II, 2.6 units, was used for both experiments.

Table V: The Effect of pH on the Equilibrium Ratio of ADP to ADPglucose in the Glycogen Synthase Reaction.

Buffer	pH	ADPglucose Formed (%)	ADP/ADPglucose
Acetate	5.27	1.77	55.5
Acetate	5.67	1.72	58.1
Mes	6.06	1.49	66.1
Mes	6.46	1.11	89.1
Hepes	6.82	0.66	151

Discussion

The use of 4-aminobutyl-Sepharose resin has enabled us to purify the *E. coli* B glycogen synthase to a high degree of purity. The DEAE-cellulose fractions were purified 45- to 100-fold with the aid of the 4-aminobutyl-Sepharose resin. Rabbit skeletal muscle glycogen synthase had been previously purified using 4-aminobutyl-Sepharose as an adsorbent (Shaltiel and Er-El, 1973; Salsas and Larner, 1975) and was eluted either with a NaCl gradient (0.05-0.4 M) or with 0.5 M KCl. The *E. coli* B glycogen cannot be eluted even with 2 M KCl or by a 1 M maltose solution. It is eluted with a solution containing both 1 M maltose and 1 M KCl. No other oligosaccharides have been tried in place of maltose but 1 M KCl solutions containing either 20 mM ADP, a competitive inhibitor of the glycogen synthase (Preiss and Greenberg, 1965), or 1 M glucose do not elute the *E. coli* B glycogen synthase from the aminoalkyl-Sepharose column. The tight binding of the *E. coli* B glycogen synthase to the aminoalkyl-Sepharose column and the specific elution by a 1 M maltose–1 M KCl solution suggest that retention of synthase is due to other interactions in addition to ionic interactions. On the basis of their results, Er-El et al. (1972) and Shaltiel and Er-El (1973) have postulated hydrophobic bonding as a possible interaction between proteins and aminoalkyl arms.

Other affinity resins have been tried in the attempt to purify the *E. coli* B glycogen synthase. 3-Aminopropyl-Sepharose retained only 60% of the glycogen synthase added and gave poor resolution of the enzyme from branching enzyme. 6-Aminohexyl-Sepharose bound the glycogen synthase tightly and gave excellent resolution from branching enzyme. However, the recovery of the synthase was poor and large volumes of the 1 M maltose plus 1 M KCl solution were required for elution. The glycogen synthase could also

be adsorbed strongly on the affinity resins, ADP-hexanolamine-Sepharose (Barker et al., 1972) and concanavalin A-Sepharose (Pharmacia). As with the 4-aminobutyl-Sepharose affinity resin a 1 M KCl buffer solution could not elute the enzyme from the above two resins. The 1 M KCl solution containing 1 M maltose was able to elute the glycogen synthase but with low yields (~30%) and there was inconsistent and poor resolution of the enzyme from the branching enzyme.

The specific activity values of 380 and 505 μmol mg^{-1} are much higher than those reported for the mammalian glycogen synthases which are in the range of 5–35 μmol mg^{-1} (Soderling et al., 1970; Smith et al., 1971; Lin and Segal, 1973; Issa and Mendicino, 1973; McVerry and Kim, 1974) and for the yeast enzyme which was reported to be 93 μmol mg^{-1} (Huang and Cabib, 1974). Based on the disc gel and dodecyl sulfate gel experiments, the synthase fractions may be considered to be essentially pure. Glycogen synthase II, however, had a trace of branching enzyme activity which was about 0.1% of the glycogen synthase activity.

Of interest is the comparison of the similarities and differences of the bacterial glycogen synthase with the mammalian glycogen synthase. The specificity of the two enzymes with respect to sugar nucleotide donor is different. The subunit molecular weight of the glycogen synthase from mammals and yeast has been reported to be from 77 000 to 100 000 (Soderling et al., 1970; Smith et al., 1971; McVerry and Kim, 1974; Huang and Cabib, 1974). The *E. coli* glycogen synthase appears to have a subunit molecular weight about one-half the size of the animal and yeast enzyme. The active oligomeric forms of the *E. coli* glycogen synthase appear to be the dimer, trimer, and tetramer. After purification, glycogen synthase I appears to be in the dimer form (average mol wt 93 000) and glycogen synthase II is in the tetrameric form (mol wt 200 000). On standing at 4 °C or at −85 °C both glycogen synthase fractions rearrange into the dimeric, trimeric, or tetrameric forms. Different aggregate forms have also been reported for the mammalian and yeast enzymes. The predominant active form for the rabbit skeletal muscle glycogen synthase I (Soderling et al., 1970), the swine kidney I form (Issa and Mendicino, 1973), and the yeast glycogen synthase D form (Huang and Cabib, 1974) have been reported to be the tetramer while rabbit skeletal muscle glycogen synthase D (Brown and Larner, 1971) and rat liver glycogen synthase D (Lin and Segal, 1973) active forms have been shown to exist predominantly as trimers. Aggregation of mammalian glycogen synthases into various oligomeric forms under different conditions has been reported for the rat liver enzyme (Sanada and Segal, 1971; McVerry and Kim, 1972) and for the rat and rabbit muscle glycogen synthases (Staneloni and Piras, 1971; Smith and Larner, 1972). Thus the tendency to aggregate into various active molecular sizes is a property of both animal and bacterial glycogen synthases.

1,5-D-Gluconolactone has been shown to be a potential inhibitor of glycosidases (Leaback, 1968) and polysaccharide phosphorylases (Tu et al., 1971). Recently McVerry and Kim (1974) have shown that 1,5-D-gluconolactone was an inhibitor of the rat liver glycogen synthase reaction. The inhibition by the lactone has been suggested to be due to the high degree of similarity between the inhibitor and the glucosyl moiety in the transition state of the reaction. The lactone exists in a half-chain conformation and thus resembles the oxonium ion which was proposed by Mayer and Larner (1959) to be an intermediate for amylase catalysis. This intermediate may be a common transition state for all glycosyl transferases. Figure 3 shows that 1,5-D-gluconolactone is an effective inhibitor of the *E. coli* B glycogen synthase. Thus the *E. coli* B ADPglucose specific glycogen synthase is very similar to the UDPglucose specific glycogen synthase and other glycosidases and transglycosylases with respect to 1,5-D-gluconolactone inhibition.

p-Chloromercuribenzoate has also been reported to be a potent inhibitor of both bacterial (Preiss and Greenberg, 1965; Greenberg and Preiss, 1965) and rabbit skeletal muscle glycogen synthases (Kornfeld and Brown, 1962). The rat liver glycogen synthase has also been reported to be readily inactivated by the sulfhydryl group reagents, *p*-hydroxymercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) (Ernst and Kim, 1973; Lin and Segal, 1973).

The rabbit muscle glycogen synthase has been reported to be able to utilize both glucose and maltose as glucosyl acceptors from UDPglucose in place of glycogen (Salsas and Larner, 1975). Previous results had shown that maltose was an acceptor of glucose from ADPglucose in the *E. coli* and *Arthrobacter viscosus* glycogen synthase reactions (Preiss and Greenberg, 1965; Greenberg and Preiss, 1965). Table III shows that other oligosaccharides of the maltodextrin series are also active. At lower concentrations the maltodextrins of higher degree of polymerization are more effective acceptors of glucose than maltose or maltotriose. In contrast to the rabbit muscle glycogen synthase system glucose up to a concentration of 1.4 M is not an acceptor of glucose from ADPglucose in the *E. coli* B glycogen synthase reaction.

The equilibrium ratio of ADP to ADPglucose for the *E. coli* B glycogen synthase has been determined (Figure 4 and Table V) and is dependent on pH in the range of 5.2–6.8 (Table V). This is most probably due to formation of ADP^{2-} during the reaction. The pK_a of the primary phosphate group of ADP at 0.1 ionic strength and 25 °C is 6.4 (Bock, 1960), and the amount of ADP^{2-} which is formed in the glycogen synthase reaction that dissociates to ADP^{3-} is dependent on the pH of the reaction mixture. If it is assumed that the pK of ADP is 6.4 under the conditions of the experiments in Table V the equilibrium ratio of ADP^{2-} to ADPglucose can be calculated. The ADP^{2-}/ADPglucose ratio remains approximately constant (from 41.4 to 51.7) in the range of pH 5.27–6.82 while the total ADP to ADPglucose ratio increases from 55.5 to 151. The average equilibrium ratio of ADP^{2-} to ADPglucose in this range is 45.8 ± 4.5. The constancy of this equilibrium ratio suggests that ADP^{2-} is the only reactive species in the glycogen synthase reaction.

The equilibrium of the glycogen synthase reaction, ADPglucose + $(G)_n \leftrightarrows (G)_{n+1}$ + ADP, may be depicted as $K_{eq} = (ADP)(G)_{n+1}/(ADPglucose)(G)_n$, where K_{eq} is the equilibrium constant, $(G)_n$ is the polyglucose primer molecule, and $(G)_{n+1}$ is the polyglucose primer molecule where one glucosyl residue has been added. Since $(G)_n$, $(G)_{n+1}$, or even $(G)_{n+x}$ can act as either acceptors or donors of glucosyl residues, K_{eq} would not be affected by the concentration of primer and the ratio of ADP to ADPglucose would be a measure of K_{eq} (for discussion of the negligible effect of primer on the equilibrium of the polysaccharide phosphorylase reaction, a reaction similar to the above glycogen synthase reaction, see Brown and Cori, 1961). Thus K_{eq} of the glycogen synthase reaction is equal to the ratio of ADP^{2-} to ADPglucose and is 45.8 ± 4.5. The average $\Delta F°$ of the reaction at 37 °C may then be calculated to be −2.3

± 0.06 kcal from the equation, $\Delta F^\circ = -RT \ln K_{eq}$. If it is assumed that the ΔF° of hydrolysis of the newly formed α-1,4-glucosidic linkage is −4 kcal then the ΔF° of hydrolysis of the glucosidic linkage of ADPglucose may be calculated to be −6.3 kcal.

The ΔF° of hydrolysis of the glucosidic linkage of UDPglucose has been determined to be −8 kcal on the basis of the sucrose phosphorylase (EC 2.4.1.7) and sucrose synthase (EC 2.4.1.13) reaction equilibrium constants (Hassid and Doudoroff, 1950; Cardini et al., 1955; Neufeld and Hassid, 1963). This value is based on the equilibrium constant for the sucrose synthase reactions at pH 7.4 and does not take into account that a proton is liberated from UDP at pH 7.4 (Leloir et al., 1960; Oesper, 1951).

The equilibrium constant for the mammalian glycogen synthase has not been determined. However, demonstration of its reversibility has been reported by Kornfeld and Brown (1962) and McVerry and Kim (1974).

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A Kinetic Study of the Subunit Dissociation and Reassembly of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The kinetics of dissociation and reassembly of rabbit skeletal muscle phosphofructokinase has been studied using fluorescence, stopped-flow fluorescence and enzyme activity measurements. The dissociation of the fully active tetramer in 0.8 M guanidine hydrochloride (0.1 M potassium phosphate, pH 8.0) occurs in three kinetic phases as measured by changes in the protein fluorescence emission intensity: dissociation of tetramer to dimer with a relaxation time of a few milliseconds; dissociation of dimer to monomer with a relaxation time of a few seconds; and a conformational change of the monomer with a relaxation time of a few minutes. All three phases exhibit first-order kinetics; ATP (0.05 mM) retards the second step but does not influence the rate of the other two processes. The rate of the second process increases with decreasing temperature; this may be due to the involvement of hydrophobic interactions in the stabilization of the dimeric enzyme. A further unfolding of the monomer polypeptide chain occurs at higher guanidine concentrations, and the relaxation time associated with this process was found to be 83 ms in 2.5 M guanidine, 0.1 M potassium phosphate (pH 8.0) at 23 °C. The phosphofructokinase monomers were reassembled from 0.8 M guanidine chloride by 1:10 dilution of the guanidine hydrochloride concentration and yielded a protein with 70–94% of the original activity, depending on the protein concentration. The reactivation process follows second-order kinetics; ATP (5 mM) increases the rate of reactivation without altering the reaction order, while fructose 6-phosphate does not influence the rate of reaction. The rate-determining step is probably the association of monomers to form the dimer.

The experimental and theoretical aspects of folding and unfolding processes of single chain globular proteins are currently under intensive investigation in a number of laboratories (cf. Anfinsen and Scheraga, 1975; Pace, 1975; Tsong, 1973; Ikai and Tanford, 1973; Wetlaufer and Ristow, 1973; Tsong et al., 1972; Tanford, 1968, 1970). Several recent studies have shown that, after extensive denaturation in high concentrations of urea or Gdn·HCl,[1] oligomeric proteins may also regain substantial enzymatic activity (Teipel and Koshland, 1971a,b; Bornmann et al., 1974; London et al., 1974). The reactivation of multisubunit proteins represents an extension of the basic chain folding process in that reassembly of fully folded or partially folded protomers must be considered. In the case of rabbit muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), the dissociation process can be isolated from the gross unfolding of the polypeptide chains (Parr and Hammes, 1975). Thus dissociation–reassociation phenomena under the influence of a denaturant such as Gdn·HCl may be studied without the added complication of extensive protomer unfolding and refolding.

Rabbit muscle phosphofructokinase is composed of identical subunits (Pavelich and Hammes, 1973; Coffee et al., 1973), and aggregates smaller than the tetramer possess little activity (Pavelich and Hammes, 1973; Lad et al., 1973). Furthermore, dissociation–reassociation equilibria appear to be relevant to the regulation of enzymatic activity (Hofer and Krystek, 1975; Lad et al., 1973; Hill and Hammes, 1975).

In this work some of the kinetic parameters for dissociation and reassembly of phosphofructokinase are reported. On addition of the phosphofructokinase tetramer to 0.8 M Gdn·HCl, three transitions well resolved in time are observed. The first transition has a relaxation time of a few milliseconds and is related to the dissociation of tetramer to dimer. The second and third transitions have relaxation times of a few seconds and a few minutes, respectively, and represent dissociation of dimer to monomer followed by a conformational alteration of the monomer. These results are in agreement with the mechanism proposed previously for the equilibrium denaturation of phosphofructokinase by Gdn·HCl (Parr and Hammes, 1975).

Under suitable conditions, the monomer units may be reassembled to yield almost fully active enzyme. This renaturation process is found to obey second-order kinetics. The presence of ATP accelerates the regain of activity, and the monomer-to-dimer association appears to be the rate-limiting step in reactivation.

Experimental Section

Reagents. The ATP, fructose 6-phosphate, aldolase, α-glycerophosphate dehydrogenase, triosephosphate isomer-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid.