Biosynthesis of Bacterial Glycogen. Purification and Properties of the *Escherichia coli* B α-1,4-Glucan:α-1,4-Glucan 6-Glycosyltransferase†

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ABSTRACT: The *Escherichia coli* B branching enzyme has been purified to near homogeneity with the use of 4-aminobutyl-Sepharose columns. Two fractions of the enzyme were obtained; branching enzyme I, found in the 70 000*g* supernatant fraction, had a specific activity of 460 μmol mg^{-1} and branching enzyme II, co-purified with glycogen synthase, had a specific activity of 299 μmol mg^{-1}. Both fractions contained low activities of glycogen synthase which were 0.3 and 1.0% the activity of branching enzymes I and II, respectively. Two major protein bands were observed for each branching fraction in disc gel electrophoresis and they were coincident with branching activity. One major protein band and up to three faint bands were observed in sodium dodecyl sulfate gel electrophoresis of the branching enzyme fractions. The subunit molecular weight of the major band of both fractions was 84 000 ± 2000. The molecular weights of the native enzymes as determined by sucrose density gradient ultracentrifugation were 95 000 for a major species and 178 000 for a minor species of branching enzyme I and 87 000 for branching enzyme II. Thus, the active form of the *E. coli* B branching enzyme is primarily monomeric but dimers of the subunit can exist. Purified branching enzyme showed little activity on either *E. coli* or rabbit liver glycogen as indicated by a low decrease of absorption of their I_2 complex spectra and average unit chain length. Branching of various amylose and amylopectin fractions resulted in changes in the absorption and wavelength maximum of the I_2 complex absorption spectra and in the average unit chain length values. However, the profiles of the unit chains of the debranched glucan products on Sephadex G-50 were more heterogeneous than unit chains of native glycogens. Branching activities of 1 and 20 times that of phosphorylase or *E. coli* glycogen synthase stimulated incorporation of glucose into α-glucan. However, the products formed in reactions where the activity ratio was 1:1 were more linear than amylopectin as indicated by their I_2 complex spectra and their low solubility. Only the product formed in reactions where the activity ratio was 20:1 resembled native glycogens as indicated by iodine staining, average unit chain lengths, and unit chain profiles on Sephadex G-50. Molecular weights of these synthetic glycogens were similar to native glycogens and were greater than 10^7.

The synthesis of α-1,6-glucosidic linkages of glycogen in bacteria and in animals is considered to be catalyzed by α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase (EC 2.4.1.18, branching enzyme). While information on the mechanism of branching of amylose and amylopectin by both animal (Krisman, 1962a; Larner, 1955) and bacterial (Walker and Builder, 1971; Sigal et al., 1965; Zevenhuizen, 1964) branching enzymes is available, these investigations provide only a basis for examining the specificity and other properties of branching enzymes, but do not yield insights into the reaction mechanisms. The mechanisms of animal branching enzymes have been studied by the combined action of branching enzyme and phosphorylase (Cori and Cori, 1943) or glycogen synthase (Parodi et al., 1969) or by the branching of polysaccharides enlarged by the action of phosphorylase (Verhue and Hers, 1966) or by animal glycogen synthase (Brown and Brown, 1966b). However, little is known of the mechanism of branching in bacteria. Similarly, little is known of the subunit structure of either group of branching enzymes. Thus, a study of *Escherichia coli* B branching enzyme was initiated to determine its physical properties and initiate studies into the branching mechanism. Previous reports were concerned with the stimulation of partially purified *E. coli* B glycogen synthase by branching enzyme in a reaction catalyzed by high concentrations of citrate in the presence of low concentrations of α-glucan primer (Fox et al., 1973).

This paper reports on the purification of the *E. coli* B branching enzyme to apparent homogeneity with the aid of 4-aminobutyl-Sepharose (Shaltiel and Er-El, 1973), the molecular weight of the native enzyme and its subunits, and the interaction of branching enzyme with phosphorylase and glycogen synthase in the formation of branched α-glucan.

Experimental Section

Materials

ADP [^{14}C]glucose was prepared enzymatically from [U-^{14}C]glucose (Preiss and Greenberg, 1972). Maize amylopectin (amylose free) and potato amylose (amylopectin free) were purchased from Calbiochem, Los Angeles, Calif.; potato amylose (average dp = 300) was from Nutritional Biochemical Co., Cleveland, Ohio; rabbit liver glycogen and rabbit muscle phosphorylase *a* were from Sigma, St. Louis, Mo.; hog pancreatic α-amylase (iPr_2FP[1]-treated, 640 U/mg) was from Worthington, Freehold, N.J.; and pullulanase was from Boehringer-Mannheim Corp., New York, N.Y. *E. coli* glycogen was isolated as previously described (Preiss et al., 1975). Isoamylase isolated from *Cytophaga* (Gunja-Smith et al., 1970) was a gift from Dr. Z. Gunja-Smith of the Department of Biochemistry, University of Miami School of Medicine, Miami, Fla. Glycogen synthase was purified as previously described (Fox et al., 1976). 4-Aminobutyl-Sepharose columns

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[1] Abbreviations used are: iPr_2FP, diisopropyl fluorophosphate; glucose-1-P, glucose 1-phosphate; Tris, tris(hydroxymethyl)aminomethane; TPNH, reduced triphosphopyridine nucleotide; DEAE, diethylaminoethyl.

were prepared as described by Shaltiel and Er-El (1973). Other reagents were obtained at the highest purity available.

Methods

Maintenance and Growth of Organisms. The *E. coli* B strain used was a mutant, AC70R1, derepressed in levels of ADPglucose pyrophosphorylase, glycogen synthase, and branching enzyme (Haugen et al., 1974). 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% acetate in a 130-L New Brunswick fermentor for 16 h at 37 °C. The cells were harvested in a Sharples ultracentrifuge and stored at −85 °C.

Assay of Bacterial Branching Enzyme; Assay A. The basis of the assay is the stimulation by branching enzyme of the incorporation of glucose from glucose-1-P into α-glucan catalyzed by rabbit muscle phosphorylase (Illingworth et al., 1961; Brown et al., 1961). 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*, and branching enzyme. The reaction was initiated by addition of glucose-1-P and the incubation was at 30 °C. Aliquots were taken at 30, 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 incorporation into glucan per min under the above conditions. Controls containing no branching enzyme or heat-denatured branching enzyme (1 min at 100 °C) incorporated less than 20 nmol of glucose into the glucan fraction. Control values were subtracted from the values obtained from reaction mixtures containing active branching enzyme. Column fractions were assayed by halving the reaction volume and incubating for 120 min.

Decrease of Absorption of the Amylose-I_2 Complex; Assay B. Reaction mixtures containing 60 μg of amylose, 10 μmol of sodium citrate (pH 7.0), and branching enzyme per 0.1-mL volume were incubated at 30 °C and 50-μL aliquots sampled at timed intervals. Water (0.35 mL) and 2.6 mL of iodine reagent were added. Iodine reagent was made daily from 0.5 mL of stock solution (0.26 g of I_2 and 2.6 g of KI in 10 mL of water) mixed with 0.5 mL of 1 N HCl and diluted to 130 mL (Krisman, 1962b). The absorbance of the iodine-glucan complex was measured at 660 nm.

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

Assay for Glycogen Synthase Activity. The incorporation of glucose into α-glucan from ADP-glucose was measured by the procedure of Fox et al. (1976).

Assays for α-Amylase and R-Enzyme. The assays of these enzymes were done as described by Drummond et al. (1972).

Electrophoresis. Disc gel electrophoresis was performed on 6% polyacrylamide gels using the Ornstein-Davis Tris-glycine buffer system (Ornstein, 1964; Davis, 1964). Dithiothreitol (5 mM) was included in all buffers. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 9% polyacrylamide gels as described by Neville (1971). Various standard proteins were used in the estimation of the molecular weight of the branching enzyme subunit. Protein bands were located by staining with Coomassie blue (Chrambach et al., 1967).

Location of Branching Enzyme Activity in Polyacrylamide Gels. Gels were quick frozen in powdered dry ice and sliced in 1-mm sections. Every 2 consecutive sections were combined and 0.5 mL of 50 mM glycylglycine buffer (pH 7.0) containing 5 mM dithiothreitol was added. The sections were macerated and incubated for several hours at 0 °C. Branching enzyme was measured as described above (assay A).

Ultracentrifugation in Sucrose Density Gradients. Sucrose density gradient centrifugation was carried out according to the methods described by 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 Tris-Cl buffer (pH 7.5) and 5 mM dithiothreitol. The gradients were layered with 100 μL of the above buffer solution containing 50 μg of rabbit heart lactate dehydrogenase, 50 μg of pig liver malate dehydrogenase, and 10 μg of either branching enzyme I or II. Centrifugation was carried out for 20 h and 7-drop fractions were collected. Lactate dehydrogenase activity was assayed by the method of Kornberg (1955) and malate dehydrogenase activity was assayed by the method of Ochoa (1955). Branching enzyme was determined as above (assay A).

Determination of Polyglucose in Branching Enzyme Fractions. Branching enzymes I and II, 200 μg, were diluted to 1 mL and dialyzed against 4 L of water for 12 h. The water was replaced 4 additional times with 12-h periods of dialysis. The dialyzed fractions were split, 60 μmol of glucose was added to one aliquot as internal standard, and both were made 2 N in HCl with reagent HCl and hydrolyzed 1 h in a boiling water bath. The samples were then frozen and lyophilized. The fractions were dissolved in 1.0 mL of water and centrifuged. The concentration of glucose was determined by measuring the amount of TPNH formed when aliquots were added to reaction mixtures containing yeast hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1974). The final amount was corrected for loss of the internal standard, usually 20%.

Formation of Branched Polysaccharides by Branching Enzyme. The specificity of *E. coli* B branching enzyme I was determined by assaying the decrease in absorbance of the glucan-I_2 complex of several α-glucans under the conditions described above (assay B). Reaction mixtures contained 5 units of branching enzyme per mL. The reactions were followed by the change in absorbance of the glucan-I_2 complex over the range of 400–700 nm and terminated after a constant absorbance was reached (usually 18–20 h). Polysaccharides were also formed by the coordinate action of branching enzyme I and phosphorylase *a* or glycogen synthase. Conditions described above (assay A) were used in the phosphorylase *a* branching enzyme system. When glycogen synthase was used, a reaction mixture contained per mL, 0.1 M Tris-acetate (pH 8.5), 800 nmol of ADPglucose, 2 mM glutathione, 100 μg of bovine plasma albumin, 0.5 mM $MgCl_2$, and 0.25 M citrate. No primer was added. Units of phosphorylase and glycogen synthase were kept constant at 1.5 IU per mL and the ratio of activity was varied by varying the branching enzyme added. Ratios of branching enzyme activity to chain elongation enzyme activity of 1 and 20 (based on enzyme units) were used. Reactions were followed by incorporation of radioactive glucose in the growing α-glucan and the appearance of I_2-complexing glucan. All reactions were stopped after 4 h by boiling for 5 min and polysaccharides were isolated by precipitation with 75% methanol containing 1% KCl.

Debranching of Polysaccharides and Separation of Linear α-1,4-Glucans. Branched polysaccharides (1–5 mg) were debranched at 30 °C with pullulanase (1 U/mL) in digests containing 50 mM sodium citrate buffer (pH 5.0) or at 37 °C with isoamylase (1 mg/mL) in digests containing 100 mM sodium acetate buffer (pH 5.5). After 20–24-h incubations the enzymes were inactivated by heating at 100 °C for 10 min. The

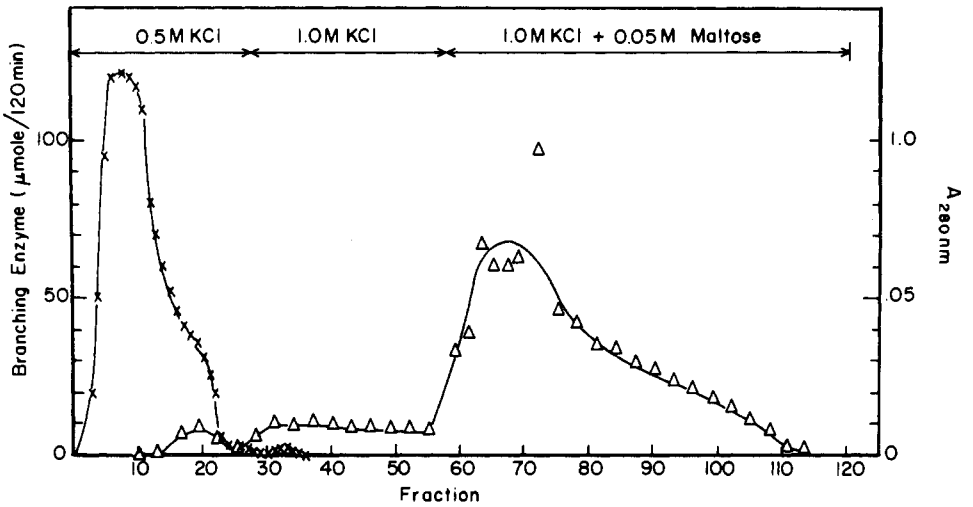

FIGURE 1: Chromatography of branching enzyme I on 4-aminobutyl-Sepharose. The procedure is described in the text. The fraction volumes were 30 mL: (×) absorbance at 280 nm; (△) enzyme activity.

average degree of polymerization (average dp) of the unit chains was determined by the ratio of total glucose equivalents vs. reducing glucose equivalents (Gunja-Smith et al., 1970). Total glucose equivalents were determined by the anthrone–sulfuric method (Ashwell, 1957) and reducing equivalents by a copper reducing method (Nelson, 1944).

Unit chains were fractionated on a Sephadex G-50 column (1.5 × 95 cm) equilibrated with 0.01 N NaOH containing 0.02% NaN_3. The column was calibrated with mixtures of oligosaccharides formed by complete debranching of amylopectin and the β-limit dextrin of amylopectin with pullulanase. A solution of linear chains (20–30 mg) was applied to the column and eluted with 0.01 N NaOH containing 0.02% NaN_3 at a flow rate of 16 mL/h and 4-mL fractions were collected. The average degree of polymerization of the fractions was determined as above. In routine separations 1–5 mg of carbohydrate was applied to the column and total carbohydrate determined in the fractions by the anthrone–sulfuric method.

Estimation of Molecular Weights of α-Glucans. Molecular weights of synthetic glycogens were estimated by gel filtration on a Sepharose-4B column (1.5 × 30 cm). Polysaccharides were eluted with a 0.01 N NaOH solution containing 0.02% NaN_3 and 1.6-mL fractions collected. The column was standardized with blue dextran (Pharmacia), rabbit liver glycogen, and dextran T-70 (average mol wt 70 000, Pharmacia). Total carbohydrate in fractions was determined by the anthrone–sulfuric method (Ashwell, 1957) or by liquid scintillation spectrometry.

Results

Purification of Branching Enzyme

(1) *Extraction of the Enzyme. E. coli* B strain AC70R1 cells, 605 g, were suspended in 2000 mL of 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 7000 psi pressure. The temperature was not allowed to increase above 18 °C. This constitutes the crude extract.

(2) *Isolation of the 70 000g Supernatant Fraction.* The crude extract was centrifuged at 16 000*g* for 10 min to remove unbroken cells. The supernatant that was obtained was centrifuged at 70 000*g* for 90 min. The supernatant fraction contained 85% of the recovered activity. This soluble activity is called branching enzyme I. The following steps, 3 through 6, describe the purification of this soluble activity.

(3) *Ammonium Sulfate Fractionation.* The 70 000*g* supernatant was made 30% saturated by the addition of crystalline ammonium sulfate and the mixture was stirred for at least 20 min and centrifuged at 16 000*g* for 20 min. The supernatant was collected and made 60% saturated by addition of solid ammonium sulfate, stirred, and centrifuged as above. The precipitate was dissolved in 50 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol and dialyzed against 2 changes of 5 vol of the same buffer.

(4) *Heat Treatment.* The dialyzed solution from ammonium sulfate fractionation was split into two equal volumes and heated to 55 °C in a water bath for 10 min with continuous swirling. After 10 min, the solution was cooled to 5 °C in an ice bath and centrifuged at 16 000*g* for 15 min.

(5) *DEAE-Cellulose Chromatography.* The supernatant solution from step four was absorbed onto a DEAE-cellulose column (5 × 48 cm, Whatman DE-52) which was equilibrated with 40 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol. The column was washed with 1 L of this buffer. The enzyme was eluted with a 10-L linear gradient of 0–0.6 M NaCl in the same buffer. Fractions were collected (280 mL), and the peak fractions, 11–23, were pooled and concentrated by ultrafiltration (PM-10 membrane, Amicon).

(6) *Chromatography on 4-Aminobutyl-Sepharose.* The concentrated DEAE fraction was absorbed onto a 4-aminobutyl-Sepharose column (2.5 × 21 cm) equilibrated with 50 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol. The column was washed successively with 400 mL of the Tris-dithiothreitol buffer, 900 mL of the buffer containing 0.5 M KCl, and 1000 mL of the buffer containing 1.0 M KCl. The enzyme was eluted with 1800 mL of the Tris–dithiothreitol buffer containing 1 M KCl and 0.05 M maltose (Figure 1). Fractions containing branching enzyme activity (56–105) were pooled and concentrated with an Amicon ultrafilter to 4.1 mL using a PM-10 membrane and dialyzed overnight against 2 L of 50 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol. This fraction constituted branching enzyme I.

(7) *Purification of Branching Enzyme II.* The 70 000*g* precipitate of step 2 was resuspended in potassium phosphate buffer (pH 6.8), containing 5 mM dithiothreitol, 10% sucrose, and 0.1 M NaCl, treated with α-amylase, and simultaneously purifed with glycogen synthase through 4-aminobutyl-Sepharose chromatography as described by Fox et al. (1976).

TABLE I: Purification of *E. coli* Branching Enzyme.

Step	Vol (mL)	Protein (mg)	Act. (units)	Sp act. (units/mg)	Glycogen synthase (units)
(1) Crude extract	2695	70 500	43 000	0.61	
(2) (a) 70 000*g* precipitate (branching enzyme II)	200	4 400	5 901	1.34	
(b) 70 000*g* supernatant (branching enzyme I)	2265	44 350	32 818	0.74	
(3) Branching enzyme I $(NH_4)_2SO_4$	480	23 940	24 900	1.04	
(4) 55 °C heat treatment	1060	16 730	32 286	1.99	
(5) DEAE-cellulose	290	6 620	5 695	0.86	
(6) 4-Aminobutyl-Sepharose	4.1	7.41	3 411	460	10.31
(7) Branching enzyme II	6.5	1.32	394	299	3.93

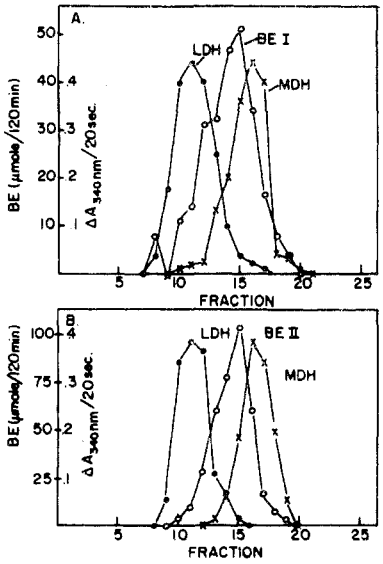

FIGURE 2: Sucrose density gradient centrifugation of branching enzymes I and II. BE, LDH, and MDH are the abbreviations for branching enzyme, lactate dehydrogenase, and malate dehydrogenase, respectively. The procedure is described in the text: (A) branching enzyme I; (B) branching enzyme II.

Fractions containing branching enzyme were pooled and concentrated to 6.5 mL by ultrafiltration as above and dialyzed overnight against 2 L of 50 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol. Table I summarizes the purification.

Both branching enzyme fractions contained very low glycogen synthase activities, but were free of α-amylase and R-enzyme activities. The final yield of branching activity summing both fractions was 9%. The enzymes are stable for months if stored at 4 or −85 °C, and stable to repeated freezing and thawing. Glucan was detected in both branching enzyme fractions. There was 1.66 mg of anhydroglucose units per mg of protein in branching enzyme I, but only 10 μg of anhydroglucose units per mg of protein in branching enzyme fraction II. Purified branching enzyme gave linear responses in both assays A and B. Assay A, the stimulation of phosphorylase *a* by branching enzyme, was 50 to 100 times more sensitive than assay B, the decrease in absorbance of the amylose-I_2 complex. Branching enzyme has a broad pH optimum from 6.5 to 8 when measured in assay B. Citrate appeared to be the best buffer over this range with inorganic phosphate and Tris-Cl giving 95 and 70% of the activity at pH 7.0, respectively.

Gel Electrophoresis of Branching Enzyme Fractions

The 4-aminobutyl-Sepharose fractions of branching enzymes I and II showed four protein staining bands after disc gel electrophoresis in the Davis Tris-glycine system (Davis, 1964). About 20 to 25 μg of protein was used for analysis. The two major bands corresponded to activity measured by the procedure indicated under Methods and had relative migration values to the tracking dye (R_m) of 0.5 and 0.62. No activity was detected with the two faint bands which migrated faster and slower than the major bands (R_m values of 0.20 and 0.80). Electrophoresis of 20 μg of protein of the branching enzyme fractions on sodium dodecyl sulfate gels according to the procedure of Neville (1971) showed one major band and zero to three minor bands. The electrophoretic mobilities of the major band in the sodium dodecyl sulfate gels were compared with standard proteins electrophoresed at the same time on separate gels. A plot of the distance of migration relative to the tracking dye (R_m) vs. the logarithm of the molecular weights of the standards (myoglobin, trypsin, fumarase, rabbit muscle pyruvate kinase, bovine serum albumin, and phosphorylase *a*) (obtained from Darnell and Klotz, 1975) gave a straight line and the R_m obtained for both branching enzyme fractions, 0.31, corresponded to a mol wt of 84 000. The average molecular weight of the branching enzyme subunit from 3 different purifications was 84 000 ± 2000.

Ultracentrifugation in Sucrose Density Gradients

Both branching enzyme fractions migrated faster than malate dehydrogenase (mol wt 72 000) and slower than lactate dehydrogenase (mol wt 140 000) in sucrose density gradient ultracentrifugation (Figure 2). Branching enzyme I, in addition, yielded a small second peak which migrated faster than both standards. The apparent molecular weights for branching enzyme I were 95 000 for the major peak and 178 000 for the minor peak. The apparent molecular weight of branching enzyme II was 87 000. Thus, the enzyme predominantly exists as a monomer, but has a low proportion of the activity as a dimer.

Action of Branching Enzyme

Action of Branching Enzyme on Linear and Branched α-Glucans. The branching action of purified branching enzyme I on various natural α-glucans was tested. Reactions were followed by the decrease in the absorption of the glucan-I_2 complex and terminated when a constant value was reached. Branching enzyme caused little decrease in absorbance and little increase in the number of branch points when allowed to react on two glycogens, from *E. coli* B and rabbit liver, for 24 h (Table II). In contrast, the action of branching enzyme on three amyloses (potato, potato (average dp = 300), and an amylose-like polymer isolated from SG-17, a branching enzyme negative mutant of *E. coli* (Fox et al., 1973)) was characterized by large decreases in absorbance of the glucan-I_2 complex (up to 95% at 660 nm), a shift in the maximum wavelength of the glucan-I_2 complex, and large increases in

TABLE II: Properties of Natural α-Glucans before and after Treatment with Branching Enzyme (BE) I and Synthetic α-Glucans Formed by the Combined Action of Branching Enzyme I and Phosphorylase or Glycogen Synthase.

Substrate enzyme system	λ_{max} (nm)		Unit chain av dp		α-1,6 bonds[a] (%)	
	−BE	+BE	−BE	+BE	−BE	+BE
E. coli B glycogen	440	440	11.2	10.2	8.9	9.7
Rabbit liver glycogen	480	440	14.3	13.2	7.0	7.6
Amylose	630	570	300	7.7		13.0
Amylopectin	540	470	25.6	10.4	3.9	9.6
β-Limit dextrin	530	450	10.1		9.9	
Be + phos a[b]		480		9.5		10.5
Be + GS[b]		480		10.9		9.1

[a] Percent release of glucose equivalents when treated with *Cytophaga* isoamylase or pullulanase. [b] Glycogen formed by combined enzymatic action, with a branching enzyme to chain elongation enzyme ratio of 20:1 (based on enzyme units).

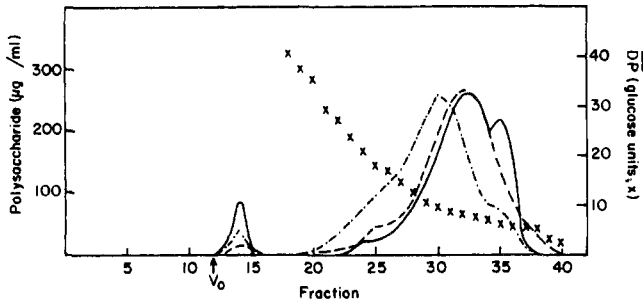

FIGURE 3: Fractionation of unit chains of debranched glycogens on Sephadex G-50: *E. coli* B glycogen (---), glycogen formed by branching amylose (—), and glycogen formed by branching amylopectin (---) with branching enzyme I. The average degrees of polymerization (DP) for fractions are included (×). The procedure is described in the text.

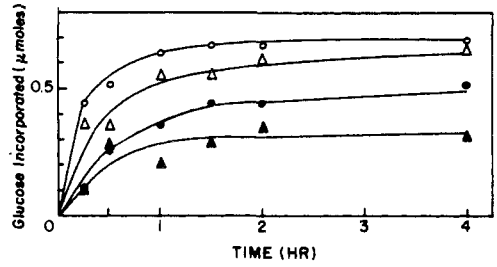

FIGURE 4: Formation of [^{14}C]glucan catalyzed by the simultaneous action of *E. coli* B branching enzyme I and glycogen synthase from ADP [^{14}C]glucose with ratios of branching enzyme activity to glycogen synthase activity of 1:1 and 20:1: (○) 20:1; (●) 20:1 branching enzyme heat killed; (△) 1:1; (▲) 1:1 branching enzyme heat killed. The procedure is described in the text.

the number of branched points (Table II). Similarly, branching enzyme introduced further branch points into amylopectin and decreased the wavelength maximum of the β-limit dextrin of amylopectin-I_2 complex (Table II). Although the products of branching of both amylose and amylopectin gave average unit chain lengths and percent of 1,6 bonds somewhat similar to glycogens, fractionation of the unit chains after enzymatic debranching with *Cytophaga* isoamylase showed these chains to be a more heterogeneous population than those of natural glycogens with more chains excluded and a large amount of chain lengths of 5–7 glucose units as determined by the ratio of glucose equivalents to reducing equivalents (Figure 3; untreated amylose eluted at fraction 14). In addition, the branched amylose was susceptible to debranching with pullulanase as measured by release of reducing power and the appearance of small unit chains when the pullulanase treated branched amylose was fractionated on Sephadex G-50 (not shown). Native glycogens are resistant to pullulanase (Lee et al., 1968).

Simultaneous Action of Branching Enzyme and Phosphorylase a or Glycogen Synthase. Reactions were allowed to proceed as described above with branching enzyme to phosphorylase *a* or glycogen synthase ratios of 1 and 20 (on a unit basis). Interestingly, reactions were similar when either phosphorylase *a* or glycogen synthase was used. Therefore, the data for only the glycogen synthase branching enzyme reactions will be presented with any minor differences noted for the phosphorylase branching enzyme reactions. No exogenous primer was added. However, branching enzyme I does contain endogenous glucan primer, but the amount of primer added with heat-killed branching enzyme failed to cause an increased glucose incorporation over water blanks in the phosphorylase *a* system. Endogenous glucan in the recovered product was less than 1% for the phosphorylase *a* branching enzyme reactions and 4 and 50% for the glycogen synthase-branching enzyme reactions at ratios of 1:1 and 1:20, respectively. No iodine positive material was observed at zero time for all reactions. Glucan synthesized in the presence of heat-killed branching enzyme had a wavelength maxima of 610–630 nm for the glucan-iodine complex. Typical time courses of the reactions and reactions containing heat-killed branching enzyme showed that at either ratio of activity, branching enzyme stimulated the incorporation of glucose into the glucan (Figure 4). In reactions where phosphorylase *a* was used, the theoretical 75% incorporation of the glucose was seen (based on phosphorylase equilibrium considerations), while reactions using glycogen synthase as the chain elongation enzyme were more quantitative and incorporated 88% of the glucose.

In reactions where the ratio of branching enzyme to chain elongating enzyme was 1:1 the product formed was not similar to native glycogen. As the reaction proceeded the wavelength maximum of 590 nm of the iodine stained glucan remained constant until glucose incorporation was essentially complete, suggesting that molecules of the same relative structure were being formed (Figure 5A). After incorporation was completed, the wavelength maximum did fall (from 60 to 120 min) indicating further branching did occur. However, the high-wavelength maximum of the glucan-I_2 complex is intermediate to that of amylopectin and amylose and thus the product is highly linear. Likewise, as the reaction progressed and the effective concentration of the glucan increased, the glucan precipitated from solution, again indicating a high degree of linearity.

Ratios of branching enzyme to either phosphorylase *a* or glycogen synthase of 20:1 gave products with wavelength

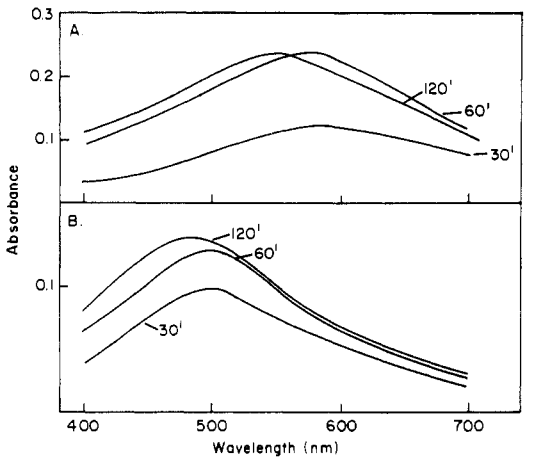

FIGURE 5: Absorption spectra of α-glucan-I_2 complexes of glucans formed by the simultaneous action of *E. coli* B branching enzyme I and glycogen synthase: (A) branching enzyme activity to glycogen synthase activity ratio of 1:1; spectra after 30, 60, and 120 min of incubation; (B) branching enzyme activity to glycogen synthase activity ratio of 20:1; spectra after 30, 60, and 120 min of incubation.

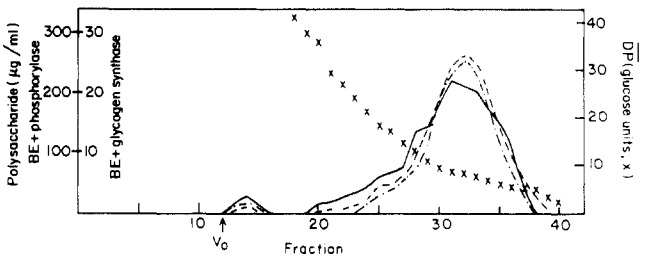

FIGURE 6: Fractionation of unit chains of debranched *E. coli* B glycogen (---), debranched glycogen (—) formed by the simultaneous action of *E. coli* B branching enzyme I and phosphorylase *a* (20:1 ratio of activity), and debranched glycogen (-·-) formed by the simultaneous action of branching enzyme and glycogen synthase (20:1 ratio of activity) by gel filtration on Sephadex G-50. Average degrees of polymerization (DP) for fractions determined by the ratio of total glucose equivalents to reducing equivalents are included (×).

maxima of the glucan-I_2 complexes and average unit chain lengths after enzymatic debranching with *Cytophaga* isoamylase similar to those of native glycogens (Table II). Following the iodine spectra during the time course of the reactions showed a final wavelength maximum of 480 nm (Figure 5B). The slight shift (from 500 to 480 nm) in the wavelength maximum with time may suggest a slight lag of branching action behind elongation. Estimation of molecular weights of the glucans formed during the time course of the reactions indicated that the glucan formed throughout the reaction was also of large molecular weight, greater than 10^7, as shown by exclusion on gel filtration on Sepharose 4B. Rabbit liver glycogen and blue dextran were also excluded on Sepharose 4B chromatography. Debranching of the synthetic glycogens and native *E. coli* B glycogen with *Cytophaga* isoamylase and fractionation of the unit chains on Sephadex G-50 showed similar profiles for the three glycogens (Figure 6). Both synthetic and native glycogens were resistant to hydrolysis by pullulanase.

Discussion

The use of 4-aminobutyl-Sepharose resin has enabled us to highly purify branching enzyme of *E. coli* B. DEAE-cellulose fractions were purified 100- to 200-fold with the aid of the 4-aminobutyl-Sepharose resin. Other glycogen biosynthetic enzymes, *E. coli* B glycogen synthase (Fox et al., 1976) and rabbit muscle glycogen synthase (Shaltiel and Er-El, 1973; Salsas and Larner, 1975), have previously been purified using 4-aminobutyl-Sepharose as an absorbant.

The recoveries of the branching enzyme from chromatography on 4-aminobutyl-Sepharose ranged from 65 to 85%. However, recoveries from DEAE-cellulose chromatography ranged from 15 to 60%. These low recoveries can partially be explained by the purification of nonspecific α-glucan primer, which eluted in the wash. In conditions of high glycogen (and glucan) content in the cells (i.e., cells grown on 0.4% glucose instead of 0.4% acetate), the high concentrations of primer inhibit the binding of branching enzyme to the DEAE-cellulose. Unpublished results showed that pretreatment with α-amylase in these conditions raised the recovery to 60%. The overall recovery of 9% was less than that of purified rabbit muscle branching enzyme which was 13 to 14% (Brown and Brown, 1966a). However, the specific activities of 299 and 460 μmol mg^{-1} are much higher than those reported for the same rabbit muscle branching enzyme fractions, 36 and 123 μmol mg^{-1}.

Both fractions I and II appeared to be similar and gave two major bands in disc gel electrophoresis which corresponded to branching activity. Electrophoresis in sodium dodecyl sulfate gels showed that both fractions had a subunit molecular weight of 84 000. Subunit molecular weights of animal branching enzymes have not been reported. However, the subunit molecular weight of potato Q-enzyme (EC 2.4.1.18, plant branching enzyme) has been reported as 86 000 (Borovsky et al., 1975) and thus the two enzymes are similar in this respect. The native molecular weight of rabbit muscle branching enzyme has been estimated at between 100 000 and 175 000. Sucrose density gradient centrifugation of *E. coli* branching enzymes I and II gave a predominant molecular form of monomers with a small association of branching enzyme I as a dimer (Figure 2). Interestingly, Q-enzymes of potato and spinach have native molecular weights similar to *E. coli* branching enzyme, 85 000 and 72 000 to 84 000, respectively (Borovsky et al., 1975; Hawker et al., 1974). Thus, both bacterial branching enzymes and Q-enzymes of plants have similar molecular weights of the subunits.

Branching enzymes from both animals (Krisman, 1962a; Larner, 1955) and bacteria (Walker and Builder, 1971; Sigal et al., 1965; Zevenhuizen, 1964) act on amylose and amylopectins. Purified branching enzyme of *E. coli* B exhibited the same broad specificity for α-1,4-glucans (Table II). Very few additional branch points, however, were introduced into *E. coli* B or rabbit liver glycogen when treated with the branching enzyme as shown by iodine staining and enzymatic debranching (Table II). The minimum length of the oligosaccharide chains transferred by branching enzyme can be estimated from the Sephadex G-50 elution profiles of branched amylose after enzymatic debranching with *Cytophage* isoamylase. The smallest chains detected are of an average length of 5 to 7 glucose units and in fact a peak of chains of these lengths occurred by incubation of potato amylose with branching enzyme (Figure 5). Other branching enzymes from liver, muscle, and potato (Verhue and Hers, 1966; Brown and Brown, 1966b; Drummond et al., 1972) have similar lower limits. However, the branching of the β-limit dextrin of amylopectin by the *E. coli* branching enzyme as indicated by a significant shift in the wavelength maximum and decrease of the absorption of the glucan-I_2 complex suggested that branching enzyme may branch internal chains and transfer branched oligosaccharides. Similarly, liver and potato branching enzymes can also transfer branched chains (Krisman, 1962a; Drummond et al., 1972).

Although the action of branching enzyme on amylose and amylopectin gave rise to products with some properties (i.e., wavelength maximum of the glucan-I_2 complex and average degree of polymerization of the unit chains after debranching) similar to native glycogen, fractionation on Sephadex G-50 of the unit chains after debranching with *Cytophaga* isoamylase yielded profiles indicating a more heterogeneous population than profiles of natural glycogen (Figure 3). In addition, unlike native glycogens, the branched glucans formed in this manner were subject to hydrolysis by pullulanase. The synthesis of glucan products most closely resembling natural products was achieved by the combined action of branching enzyme and phosphorylase *a* or glycogen synthase (Table II and Figures 5 and 6) in ratios of activity of 20:1. Similarly, Parodi et al. (1969) have observed that glycogen made by the coordinate action of liver glycogen synthase and branching enzyme resembled native glycogen in iodine stain and molecular size. However, glycogen from phosphorylase and branching enzyme had a lower molecular size. Doi (1969) observed that a product resembling amylopectin was synthesized by the coordinate action of potato branching enzyme and spinach starch synthase (ratios of activity from 15 to 75 on a unit basis). Amylopectins formed by the coordinate action of branching enzyme and phosphorylase, while having similar iodine staining properties and average unit chain lengths, do not have similar profiles of unit chains when fractionated by gel chromatography (Drummond et al., 1972). In the studies reported here, the structure of the glucan product was found to be more sensitive to the ratio of the activities of branching enzyme and the chain elongating enzyme than the chain elongating enzyme used.

Ratios of activity of branching enzyme to chain elongating enzyme of 20:1 and 1:1 were used. The ratio of branching enzyme to glycogen synthase in crude extracts of *E. coli* ranges from 4:1 to 8:1. Thus, ratios slightly above and below the in vivo ratio were selected in order to maximize differences. Both ratios stimulated the incorporation of glucose into glucan (Figure 4). This stimulation had previously been noted for bacterial branching enzymes (Walker and Builder, 1971; Fox et al., 1973). While the products formed in reactions with ratios of 1:1 were only slightly branched, the products of 20:1 ratio reactions resembled native glycogens in all respects studied. Iodine staining properties were similar (Table II and Figure 5). Fractionation of the products on Sepharose 4B showed that like native glycogens the synthetic glycogens have high molecular weights (greater than 10^7). Like native glycogens, the synthetic glycogens were resistant to hydrolysis by pullulanase. Finally, the profiles of unit chains after debranching with isoamylase on Sephadex G-50 chromatography were similar to the profiles of unit chains of native glycogens. The failure to make "glycogen" by the branching of amylose or amylopectin and the synthesis of glycogen by the two enzyme systems used suggests that glycogen is formed by the coordinate not successive action of branching enzyme and glycogen synthase.

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