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Immunological Characterization of *Escherichia coli* B Glycogen Synthase and Branching Enzyme and Comparison with Enzymes from Other Bacteria

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Escherichia coli B glycogen synthase and branching enzyme, although similar in amino acid composition, had no significant immunological cross-reactivity. The N-terminal sequences of the glycogen synthase were rich in hydrophobic residues, whereas branching enzyme had a higher content of acidic and basic residues. However, residues 21 to 28 of glycogen synthase and 7 to 14 of branching enzyme shared six of eight residues in common. Two fractions of branching enzyme, branching enzymes I and II, which can be isolated from *E. coli* B cell extracts, have been shown to be immunologically identical, suggesting that only one type of branching enzyme activity is present in *E. coli* B. Evidence has been obtained which indicates that *E. coli* B glycogen synthase and branching enzyme are antigenically very similar to glycogen synthases and branching enzymes from other enteric bacteria. No cross-reactivity with either enzyme was observed in cell extracts from photosynthetic bacteria.

Of the enzymes in the bacterial glycogen and starch biosynthetic pathway, ADPglucose synthetase (glucose-1-phosphate adenylyltransferase; EC 2.7.7.27) has provided the most interesting pattern of diversity in terms of its regulatory properties. Seven distinct classes of enzymes exist based on their activator specificity (27, 30). A preliminary immunological comparison of the ADPglucose synthetase from *Escherichia coli* B with the enzyme from some other sources has been conducted by Haugen et al. (12). Antiserum produced against the *E. coli* B ADPglucose synthetase cross-reacted with the enzyme from *Salmonella typhimurium*, with the formation of spurs. The same results were obtained in the reaction of anti-*S. typhimurium* ADPglucose synthetase against the *E. coli* B enzyme (20). These enzymes are both activated by D-fructose 1,6-bisphosphate, pyridoxal phosphate, and NADPH. No cross-reaction was observed with the spinach leaf enzyme, which is activated by 3-phosphoglycerate. Yung and Preiss (34) have shown that many of the bacterial ADPglucose synthetases can be at least partially inhibited by antibody prepared against *Rhodospirillum tenue* ADPglucose synthetase. A more rigorous immunological characterization of the ADPglucose synthetase enzyme among bacteria may provide much information on the divergence and relationship of bacterial classes and how the formation of different activator classes occurred during this process. Studies of this nature are still in progress. In addition, similar results for the remaining enzymes of the pathway, glycogen synthase and branching enzyme, may provide a more complete picture of divergence in the glycogen biosynthetic pathway.

Fredrick (10) has observed that starch phosphorylase enzymes and starch synthase enzymes have many characteristics in common and has proposed that the starch branching enzymes are also related to phosphorylase and starch synthase. Antibody produced against *Oscillatoria princeps* phosphorylase was shown to cross-react with *O. princeps* starch synthase as well as *O. princeps* starch branching enzymes, although less strongly. The relatedness of glycogen synthase and branching enzyme may also be true for *E. coli*.

Thus, this paper presents preliminary results on the immunological and structural characterization of similarities between glycogen synthase (EC 2.4.1.21) and branching enzyme (EC 2.4.1.18) from *E. coli* B. An immunological comparison of *E. coli* B glycogen synthase and branching enzyme with enzymes from a variety of bacterial sources is also reported.

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MATERIALS AND METHODS

[*U*-^{14}C]glucose 1-phosphate (255 mCi/mmol) and [*U*-^{14}C]glucose (327 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. [2-^{14}C]iodoacetic acid (3.9 mCi/mmol) was obtained from ICN Pharmaceuticals, Irvine, Calif. [2-^{3}H]iodoacetic acid (82 mCi/mmol) was obtained from Amersham. ADP[*U*-^{14}C]glucose was prepared from [*U*-^{14}C]glucose by the method of Preiss and Greenberg (29). Rabbit liver glycogen type III, rabbit muscle phosphorylase *a* (26 U/mg), and dithioerythritol were from Sigma Chemical Co., St. Louis, Mo. Sequenal grade 3-*N*-mercaptoethanesulfonic acid was obtained from Pierce, Rockford, Ill. Freund complete adjuvant was obtained from Difco Laboratories, Detroit, Mich. Ionagar no. 2 came from Colab Laboratories Inc., Chicago Heights, Ill. All other reagents were of the highest purity commercially available.

Homogeneous *E. coli* B glycogen synthase was prepared as previously described (9). The specific activity of the preparation used in these studies was 75 μmol/min per mg of protein in the primed assay, a total nitrogen assay (15) used as the basis for protein determination. Branching enzyme activity present in this preparation was less than 0.5% of the glycogen synthase activity, based on enzyme units in the assays described below.

Branching enzymes I and II from *E. coli* B were purified as previously described (3). The specific activities of branching enzymes I and II used in these experiments were 440 and 360 μmol/min per mg of protein, respectively, based on the total nitrogen assay. The branching enzyme I preparation used for antiserum production contained a 0.07% glycogen synthase contamination on an enzyme unit basis.

Maintenance and growth of organisms. Non-photosynthetic bacteria were maintained on nutrient broth agar slants; photosynthetic bacteria were maintained on stab cultures containing 0.3% yeast extract (Difco), 0.3% peptone (Difco), and 1.5% agar. They were grown in 1-liter cultures of either enriched medium containing 1.1% K_2HPO_4, 0.85% KH_2PO_4, and 0.6% yeast extract, with 0.6% glucose as the carbon source, or modified basal P medium containing 1.42% Na_2HPO_4, 0.68% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.0011% $CaCl_2$, 0.0246% $MgSO_4 \cdot 7H_2O$, 0.6% glucose, and trace elements (1 ml/liter), consisting of 0.05% $CaCl_2$, 1.67% $FeCl_2 \cdot 6H_2O$, 0.018% $ZnSO_4 \cdot 7H_2O$, 0.016% $CuSO_4 \cdot 5H_2O$, 0.018% $CoCl_2 \cdot 6H_2O$, and 2% EDTA. The pH was adjusted to 7.2 with NaOH. The cultures were grown on a rotary shaker.

Photosynthetic bacteria were grown anaerobically in 20-liter cultures of 550 medium as previously described (34). Cells were harvested by centrifugation and stored frozen at −20°C. The bacterial strains and growth conditions are summarized in Table 1.

Enzyme assays. (i) Glycogen synthase (primed synthesis). Incorporation of glucose into primer was performed as described previously (28). The reaction mixture contained 140 nmol of ADP[^{14}C]glucose (500 cpm/nmol), 10 μmol of bicine [*N*,*N*'-bis-(2-hydroxyethyl)glycine] buffer (pH 8.5), 5 μmol of potassium acetate, 2 μmol of GSH, 0.1 μmol of magnesium acetate, 100 μg of bovine serum albumin, 0.5 mg of rabbit liver glycogen, and enzyme in a total volume of 0.2 ml. The reaction was terminated after incubation for 15 min at 37°C by the addition of 2 ml of 75% methanol–1% KCl. Glycogen synthase enzyme fractions were diluted into a buffer containing 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)-NaOH buffer (pH 7.0), containing 5% sucrose and 5 mM dithioerythritol before assay. One unit of glycogen synthase activity was defined as 1 μmol of glucose transferred in 1 min under the conditions of the assay.

(ii) Branching enzyme. The basis of the assay was the stimulation by branching enzyme of the incorporation of glucose from glucose 1-phosphate into α-glucan catalyzed by rabbit muscle phosphorylase *a* (3–5). The reaction mixture contained, in a volume of 0.1 ml, 0.1 M sodium citrate (pH 7.0), 1 mM AMP, 50 mM [^{14}C]glucose 1-phosphate (50 cpm/nmol), 20 μg of crystalline rabbit muscle phosphorylase *a*, and branching enzyme. The reaction was initiated by the addition of glucose 1-phosphate, and incubation was carried out for either 90 or 120 min with no difference in result. The reaction was terminated by boiling for 1 min, 0.5 mg of carrier glycogen was added, and 2 ml of 75% methanol–1% KCl was added and cooled on ice for 5 min. The precipitate was collected by centrifugation, washed with 2 ml of methanol-KCl, and dissolved in 0.2 ml of water. The glycogen was precipitated by the addition of 2 ml of methanol-KCl and collected by centrifugation. The precipitate was then dissolved in 1 ml of water, and radioactivity present in a 0.5-ml portion was determined by liquid scintillation counting. Branching enzyme fractions were diluted into water before assay. One unit of branching enzyme activity was defined as 1 μmol of glucose incorporated into glucan per min under the conditions of the assay. Controls containing no branching enzyme or heat-

TABLE 1. Organisms and growth conditions

Organism	Growth medium[a]	Growth time	Temp (°C)[b]
E. coli B	1	16–18 h	37
E. coli B AC7OR1	1	16–18 h	37
E. aurescens ATCC 12814	2	16–18 h	37
S. typhimurium Lt-2	2	16–18 h	37
E. aerogenes ATCC 130485	1	16–18 h	37
K. pneumoniae ATCC 13883	2[c]	16–18 h	37
S. marcescens ATCC 274	1	16–18 h	30
A. hydrophila ATCC 7966	2[c]	16–18 h	30
R. sphaeroides ATCC 3224	3	12 days	RT
R. gelatinosa ATCC 17011	3	12 days	RT
R. molischianum ATCC 7137	3	9 days	RT

[a] Media: 1, enriched; 2, modified basal P; 3, 550.
[b] RT, Room temperature.
[c] Supplemented with 1% acid-hydrolyzed casein.

denatured branching enzyme (boiling for 1 min) 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.

Amino acid analysis. Protein samples were prepared for amino acid analysis by dialysis in 10% acetic acid and lyophilization. The lyophilized protein was dissolved in 88% formic acid, and norleucine was added in an amount equivalent to 5 to 10 nmol per subunit. Portions containing about 0.12 mg of protein were placed in hydrolysis tubes and dried in vacuo. Mercaptoethanesulfonic acid (0.2 ml of a 3 N solution) was added, and the solution was degassed and sealed in vacuo. Duplicate samples were hydrolyzed at 110°C for 24, 48, or 72 h. After hydrolysis, 30 μl of the hydrolysate was removed, neutralized with 15 to 18 μl of 10 N NaOH to about pH 2.2, and diluted with water to a total volume of 120 μl. Portions (25 μl each) were run on a Durrum D-500 amino acid analyzer. Recovery of amino acids was calculated from the total amount of nitrogen placed on the analyzer as determined by the micro Kjeldahl method (15).

Tryptophan was analyzed both from the amino acid hydrolysate by using an authentic tryptophan standard and spectrophotometrically by the method of Goodwin and Morton (11).

Cysteine and cystine were determined on protein samples that had been previously reduced and carboxymethylated with iodoacetic acid by the method of Crestfield et al. (8) modified for use with guanidine-hydrochloride (12).

N-terminal sequence determination. Automated Edman degradations were performed on a Beckman model 890C sequencer with dilute Quadrol protein program no. 030176. The protein (70 nmol of subunit) was placed into the cup and initially acid cleaved with heptafluorobutyric acid. The first residue was double coupled with phenylisothiocyanate before the normal cycles were begun. The phenylthiohydantoin derivatives of the amino acids were identified before or after trimethylsilylation by gas chromatography (17, 26) with a Packard model 419 chromatograph. Each residue was also identified, if possible, by two-dimensional thin-layer chromatography by the method of Kulbe (18). Histidine and arginine were identified as free amino acids after hydrolysis in 6 N HCl–0.1% $SnCl_2$ for 4 h at 150°C (21).

Protein samples were prepared for N-terminal sequence determination by the following procedures. Glycogen synthase enzyme concentrate (5 mg) was initially treated with 10 μg of α-amylase plus 2 μg of glucoamylase for 1 h at 37°C after carboxymethylation and suspension of the protein in 0.5 ml of 50 mM Tris-hydrochloride buffer (pH 7.5), followed by extensive dialysis against 10% acetic acid. This procedure was required to remove the bulk of the endogenous glucan present in highly purified glycogen synthase enzyme preparations which interferes with the degradation reactions.

Purified branching enzyme (100 nmol of subunit) contained little glucan and was carboxymethylated before sequence determination.

Preparation of antiserum. Preimmune serum was collected, and the rabbits were injected subcutaneously between the shoulder blades three times at 1-week intervals and then monthly for a total of 4 months with 1 mg of purified *E. coli* B glycogen synthase or 0.5 mg of purified *E. coli* B branching enzyme I. Protein samples (4 mg of glycogen synthase per ml and 1 mg of branching enzyme I per ml) were emulsified with equal volumes of Freund complete adjuvant before injection. Serum was collected 8 days after the last injection and stored frozen at −80°C. Serum from three rabbits injected with glycogen synthase was pooled and that from two rabbits injected with branching enzyme was pooled for use in these studies.

Preparation of enzymes for immunological comparison. Crude enzyme preparations from various bacteria were prepared for immunological studies by the following procedures. Cells (3 to 5 g) were sonicated in 25 ml of 0.1 M glycylglycine buffer (pH 7.0)–10 mM dithioerythritol and centrifuged for 10 min at 12,000 × *g*. Ammonium sulfate fractions of 0 to 30% and 30 to 60% saturation were obtained, suspended in a minimum volume of 50 mM HEPES-NaOH buffer (pH 7.0)–5% sucrose–5 mM dithioerythritol, and then dialyzed against 50 volumes of the same buffer. Ammonium sulfate fractions from bacteria that contained low amounts of enzyme activity were further purified on a DEAE-cellulose column equilibrated with the HEPES-NaOH buffer described above and eluted with a 0 to 0.6 M NaCl gradient. Enzyme-containing fractions were pooled, concentrated by 70% ammonium sulfate, and dialyzed against 50 volumes of HEPES-NaOH buffer. The fraction containing the highest enzyme activity was used in these experiments.

Double-diffusion plates. Ouchterlony double diffusion (24) was conducted on gels containing 1 mM potassium phosphate buffer (pH 7.0)–0.85% NaCl–1% Ionagar no. 2.

Neutralization of enzyme activity by antiserum. The effect of antisera on the activity of glycogen synthase and branching enzyme was determined by using the following reaction mixtures.

Glycogen synthase inactivation was monitored in reaction mixtures that contained 7.5 μmol of HEPES-NaOH buffer (pH 7.0), 7.5 mg of sucrose, 0.75 μmol of dithioerythritol, 100 μl of serum containing varying amounts of anti-glycogen synthase serum diluted into preimmune serum, and 0.02 U of purified or crude glycogen synthase enzyme preparation in a total volume of 0.25 ml. The mixture was incubated for 30 min at 30°C and then for 2 h on ice before assay. The reaction mixture was then centrifuged for 10 min at 10,000 × *g* before assay of the supernatant fraction for remaining primed activity.

Branching enzyme activity was neutralized by varying amounts of anti-branching enzyme serum in reaction mixtures containing 0.2 mg of bovine serum albumin, 10 μmol of glycylglycine buffer (pH 7.0), 1 μmol of dithioerythritol, antiserum, and 0.1 to 0.2 U of purified or crude branching enzyme preparation in a total volume of 0.21 ml. The reaction mixture was incubated and centrifuged as described above, and the supernatant fraction was assayed for remaining branching enzyme activity.

Purification of immunoglobulin fraction. Experiments to determine the effect of high ratios of antiserum on branching enzyme activity require the use of an immunoglobulin fraction that has been purified free from serum amylase activity. Serum amylase inhibits the branching enzyme assay by degrading the product as it is formed. Removal of serum amylase was accom-

plished in the following manner. Antiserum (5 ml) was diluted with an equal volume of 50 mM potassium phosphate buffer (pH 7.0)–0.85% NaCl, and applied to a 5-ml resin bed volume column of β-cyclodextrin–Sepharose 6B prepared as described by Vretblad (33), and equilibrated with the same buffer. The column was washed with 7.5 ml of the phosphate-NaCl buffer, with all eluates collected in the same receiver. The immunoglobulin fraction was then precipitated by the addition of saturated $(NH_4)_2SO_4$ solution to 40% of saturation. After collection by centrifugation, the precipitate was suspended in 1 ml of the phosphate-NaCl buffer containing 0.02% NaN_3 and dialyzed overnight against 1 liter of the same buffer. No detectable serum amylase activity remained in the immunoglobulin fraction after this procedure. Preimmune serum was treated in the same manner.

RESULTS

Amino acid composition of *E. coli* B glycogen synthase and branching enzyme. The amino acid composition of *E. coli* B glycogen synthase and branching enzyme is presented in Table 2. Homogeneous preparations of *E. coli* B glycogen synthase and branching enzyme are known to contain endogenous glucan (3, 9, 13, 16). This can affect amino acid analysis results. Hydrolysis of glycogen synthase and branching enzyme in 5.7 N HCl resulted in a 50% recovery of amino acid residues based on total nitrogen. To increase the recovery of free amino acid residues, hydrolysis was performed with mercaptoethanesulfonic acid, which has been reported (25) to improve recovery of amino acids in those samples containing carbohydrate. The data presented in Table 2 were obtained by using the procedures described in Materials and Methods and indicated recoveries of free amino acids of from 74 to 89% for the two enzymes.

TABLE 2. Amino acid composition of *E. coli* B AC7OR1 glycogen synthase and branching enzyme

Amino acid	Glycogen synthase (mol/50,000 g)	Branching enzyme (mol/84,000 g)
Aspartic acid	40.4	95.2
Threonine	20.2	37.0
Serine	37.0	38.3
Glutamic acid	46.4	76.2
Proline	20.5	37.5
Glycine	51.8	71.2
Alanine	46.6	54.1
Cysteine[a]	5.0	4.9
Valine	29.6	35.9
Methionine	8.7	19.6
Isoleucine	15.5	29.8
Leucine	49.0	70.6
Tyrosine	15.3	34.4
Phenylalanine	16.9	35.1
Histidine	14.1	35.2
Lysine	11.6	26.4
Tryptophan[b]	2.5	4.5
Tryptophan[c]	3.1	4.5
Arginine	25.7	54.1

[a] Determined as carboxymethyl cysteine.
[b] Determined from 3-*N*-mercaptoethanesulfonic acid hydrolysates.
[c] Determined spectrally (11).

N-terminal sequence of *E. coli* B glycogen synthase and branching enzyme. The N-terminal sequences of *E. coli* B glycogen synthase and branching enzyme are shown in Fig. 1. The repetitive yields for sequencing the glycogen synthase and branching enzyme were 92 and 96%, respectively. Initial yield values based on nanomoles of protein added to the sequencer were 69% for glycogen synthase and 98% for branching enzyme. The N-terminal sequences of these two enzymes are largely dissimilar. The N-terminus of glycogen synthase is rich in hydrophobic residues, whereas branching enzyme has a much higher content of acidic and basic residues. However, some degree of homology may exist in the region of residues 21 to 28 of the glycogen synthase enzyme and residues 7 to 14 of branching enzyme; among these eight residues, only two differences were noted. No regions of homology were observed at the N-terminus of either glycogen synthase or branching enzyme when compared with the ADPglucose synthetase enzyme (12).

Comparison of *E. coli* B branching enzymes I and II. Two fractions of branching enzyme activity have been purified from *E. coli* B (3). Branching enzyme I remains in the supernatant fraction after high-speed centrifugation; branching enzyme II forms pellets with glycogen. It has been suggested (3) that because the percent distribution of branching enzymes I and II varies with the glycogen content of the extract (high glycogen, high branching enzyme II), branching enzymes I and II are the same enzyme. An immunological comparison of these two activities supports this view. Double-diffusion experiments with branching enzymes I and II in adjacent wells gave precipitin lines of identity when antiserum directed against purified branching enzyme I was placed in the center well. Similarly, little difference was observed in the ability of anti-branching enzyme serum to neutralize the activity of branching enzyme I or II (Fig. 2).

Immunological comparison of *E. coli* B glycogen synthase and branching enzyme. The similarity of glycogen synthase and branching enzyme from *E. coli* B strain AC70R1 was tested by immunological methods. No precipitin bands were formed in double-diffusion experiments between anti-branching enzyme serum and purified glycogen synthase or between anti-glycogen synthase serum and purified branching enzyme. However, incubation of either glycogen synthase with high amounts of anti-branching en-

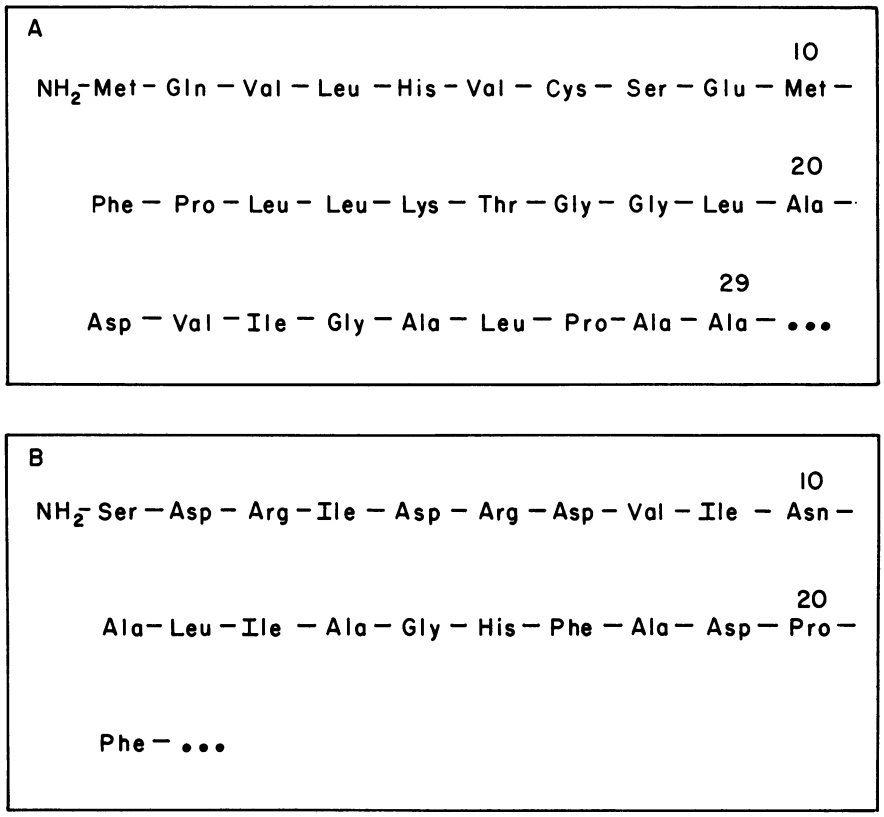

FIG. 1. (A) N-terminal sequence of *E. coli* B glycogen synthase. (B) N-terminal sequence of *E. coli* B branching enzyme.

zyme serum or branching enzyme with high amounts of anti-glycogen synthase serum resulted in neutralization of the enzymatic activities (Fig. 3). Neutralization of activity may be due to cross-reactivity between anti-glycogen synthase serum and branching enzyme or anti-branching enzyme serum and glycogen synthase. However, the possibility exists that this result is due to slight contamination of branching enzyme with glycogen synthase, or the reverse, in the highly purified antigens, thus producing a small quantity of contaminating antibodies. This was tested in the following manner. Neutralizations were performed after preincubation of the antisera with the enzymes. The preincubated antisera were centrifuged, and the supernatant fractions were used to test enzyme neutralization by standard procedures. The difference in neutralization between preincubation with enzyme and preincubation with buffer should then indicate the effectiveness of the enzyme to cross-react with a particular antiserum.

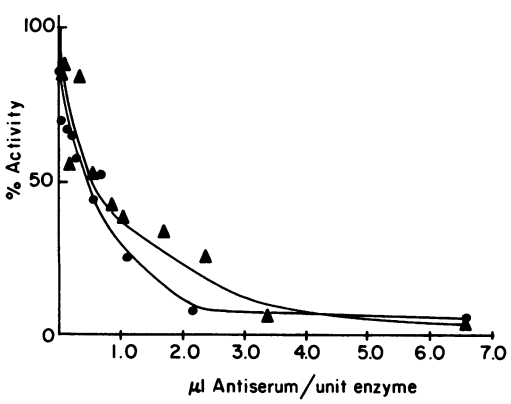

FIG. 2. Neutralization of *E. coli* B branching enzyme activity by anti-*E. coli* B branching enzyme serum. Neutralization of branching enzyme I (●) and branching enzyme II (▲). Neutralizations were performed as described in the text.

Figure 3 also shows the results of neutralization of glycogen synthase activity by anti-branching enzyme serum after pretreatment with 0.5 U of branching enzyme per μl of anti-branching enzyme serum. No significant difference was observed. The same pattern of results was observed when anti-branching enzyme serum was preincubated with 0.6 U of glycogen

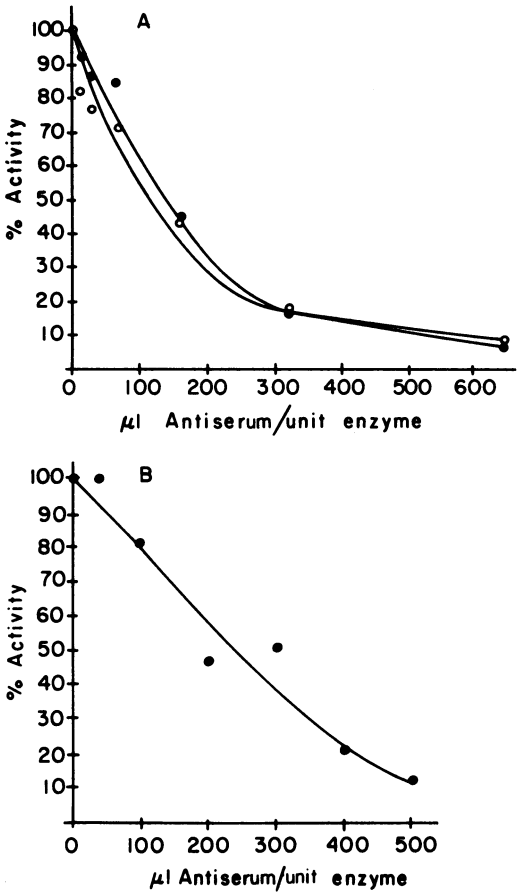

FIG. 3. (A) Neutralization of *E. coli* B glycogen synthase activity by anti-branching enzyme serum. ●, No branching enzyme pretreatment; ○, pretreatment with 0.5 U of branching enzyme per μl of antiserum. Antiserum pretreatment was conducted as follows. Anti-branching enzyme serum and branching enzyme (0.5 U of antiserum per μl) were combined and diluted 1:10 in 50 mM HEPES-NaOH buffer (pH 7.0)–5% sucrose–5 mM dithioerythritol. The solution was incubated on ice and then centrifuged for 10 min at 12,000 × *g*. The pellet was discarded, and the supernatant fraction was then used for neutralization of enzyme activity. Branching enzyme was left out of a second tube and used for the "no branching enzyme pretreatment" neutralization curve. (B) Neutralization of *E. coli* B branching enzyme activity by anti-glycogen synthase serum.

synthase enzyme or buffer and used to neutralize branching enzyme activity and when glycogen synthase activity was neutralized by anti-glycogen synthase serum pretreated with buffer or 0.5 U of branching enzyme per μl of antiserum (data not shown). These results indicate that the apparent cross-reactivity of anti-branching enzyme serum with glycogen synthase, or the reverse, is due to small quantities of contaminating antibodies and not to immunological similarity of glycogen synthase and branching enzyme.

Immunological relationship of *E. coli* B AC7OR1 glycogen synthase to other glycogen synthases. Antiserum produced against purified *E. coli* B AC7OR1 glycogen synthase yielded a single precipitin band when 10 to 50 μg of the purified enzyme or 0.2 U of crude *E. coli* B or AC7OR1 *E. coli* B enzyme prepared as described in Materials and Methods was used in an immuno–double-diffusion experiment (data not shown). The single band formed by the crude enzyme preparations was continuous with that formed by the purified enzyme. No spurs were observed. Partially purified enzymes from *S. typhimurium* and *Escherichia aurescens* are also cross-reactive with the anti-*E. coli* B glycogen synthase serum (data not shown). The single precipitin band formed by these enzymes is also continuous with that from the purified *E. coli* B glycogen synthase, indicating similarity of all enzymes tested. Again no spurs were observed. Preimmune serum was used as a control in these experiments and at no time showed cross-reactivity with any enzyme tested.

Figures 4 and 5 show the effect of antiserum on glycogen synthase activity in crude enzyme preparations from a variety of sources. Increasing amounts of antiserum inhibited purified *E. coli* B AC7OR1 glycogen synthase, crude *E. coli* B AC7OR1 glycogen synthase, and crude *E. coli* B enzyme in the same way (Fig. 4). The amount

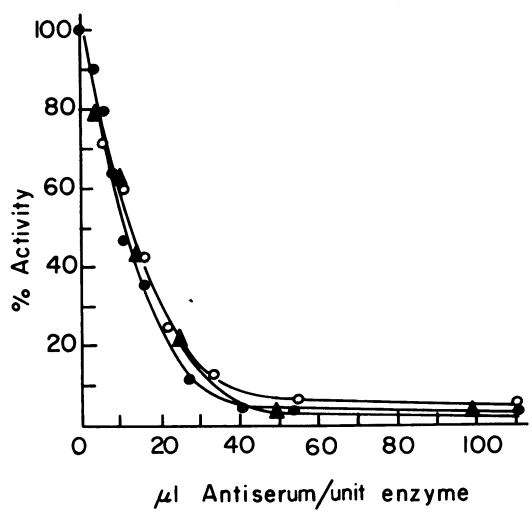

FIG. 4. Comparison of glycogen synthase enzymes from *E. coli* B and *E. coli* B AC7OR1 by neutralization of activity with anti-*E. coli* B AC7OR1 glycogen synthase serum. The procedures used were those described in the text. ●, Purified *E. coli* B AC7OR1 glycogen synthase; ▲, crude *E. coli* B AC7OR1 glycogen synthase; ○, crude *E. coli* B glycogen synthase.

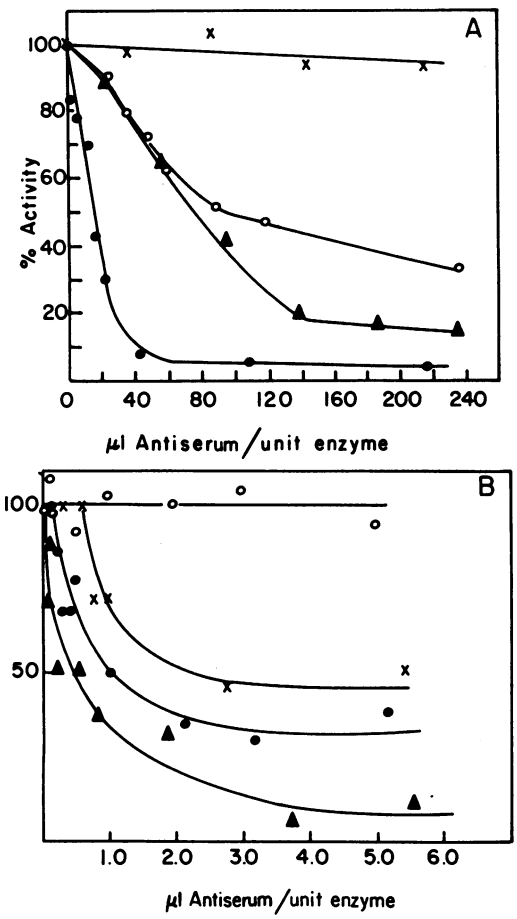

FIG. 5. (A) Comparison of glycogen synthase enzymes from several sources with anti-*E. coli* B AC7OR1 glycogen synthase serum. ●, *S. typhimurium*; ○, *K. pneumoniae*; ▲, *E. aerogenes*; ×, *S. marcescens*. Results from all sources are summarized in Table 3. (B) Comparison of branching enzymes from several sources with anti-*E. coli* B AC7OR1 branching enzyme serum. ▲, *E. aurescens*; ●, *E. aerogenes*; ×, *S. marcescens*; ○, *A. hydrophila*. Results from all sources are summarized in Table 3.

of antiserum required for 50% inhibition of activity was 11, 12, and 13 μl of antiserum per U of activity for the above enzymes, respectively. In addition, each enzyme was almost completely inhibited over the same range of antiserum concentration, indicating that a similar enzyme is present in all three preparations. Neutralization of enzyme activity from other sources is shown in Fig. 5 and Table 3. Cross-reactivity as a function of inhibition of activity was observed for *S. typhimurium*, *E. aurescens*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*, with 11, 13, 78, and 90 μl of antiserum per U of enzyme, respectively, required for 50% inhibition of activity. Little or no effect of antiserum was observed on enzyme activity from *Serratia marcescens*, *Aeromonas hydrophila*, *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas gelatinosa*, or *Rhodospirillum molischianum*. These enzymes did not cross-react with anti-*E. coli* B glycogen synthase serum in double-diffusion tests. No effect was seen on the activity of any enzyme tested when preimmune serum was substituted for antiserum.

Immunological relationship of *E. coli* AC7OR1 branching enzyme to other branching enzymes. Immuno–double-diffusion experiments performed with anti-*E. coli* B AC7OR1 branching enzyme serum gave reactions of identity when cross-reacted with *E. coli* B AC7OR1 branching enzymes I and II and crude *E. coli* B branching enzyme in the adjacent wells (data not shown). Partially purified enzyme preparations from *S. typhimurium*, *K. pneumoniae*, *E. aerogenes*, and *S. marcescens* also cross-reacted with anti-*E. coli* B branching enzyme serum and formed spurs when compared with the *E. coli* B branching enzyme antigen (data not shown). No cross-reaction in double-diffusion tests was observed with partially purified branching enzyme preparations from *R. sphaeroides*, *R. gelatinosa*, and *A. hydrophila*. Preimmune serum was used as a control and at no time showed cross-reactivity with any enzyme tested.

The effect of anti-*E. coli* B branching enzyme serum on the branching enzyme activity from a variety of sources is shown in Fig. 5 and Table 3. Increasing amounts of anti-*E. coli* B branching enzyme serum inhibited purified *E. coli* B AC7OR1 branching enzymes I and II, crude *E. coli* B, and crude *E. aurescens*. The amount of antiserum required for 50% inhibition was in each case about 0.5 μl of antiserum per U of enzyme. Each enzyme was almost completely inhibited at high antiserum/enzyme ratios. Neutralization of branching enzyme activity from crude enzyme preparations from *S. typhimurium*, *K. pneumoniae*, and *E. aerogenes* was also observed, but it was not as complete as with the *E. coli* strains. The amount of antiserum required for 50% inhibition was 1.5, 1.0, and 1.0 μl of antiserum per U of enzyme for the above enzymes, respectively. In addition, the maximal inhibition was also decreased to 65 to 70% (Table 3). The effect of antiserum on a crude enzyme preparation from *S. marcescens* was even less, with 3.0 μl of antiserum per U of enzyme required for 50% inhibition and about 56% maximal inhibition of activity at high antiserum/enzyme ratios. No effect on activity was observed when anti-*E. coli* B branching enzyme serum was incubated with branching enzymes from *R. sphaeroides*, *R. gelatinosa*, or *A. hydrophila* or when preimmune serum was substituted

for antiserum and incubated with any of the enzymes tested.

DISCUSSION

Various bacterial enzyme systems have been subjected to a careful immunological comparison among various bacterial sources (1, 2, 6, 7, 19, 22, 23, 31, 32, 35). One such system is tryptophan synthase (7, 23, 31). Results reported indicate that no antigenic determinants are shared by the two *trpA* and *trpB* gene products. However, comparisons within each gene product show a high degree of cross-reactivity in the enteric bacteria. In the α-chain, cross-reactivity is in agreement with sequence divergence.

Another sample of immunological comparison has been reported by Tronick et al. (32). Their results suggest that the glutamine synthetases of all gram-negative bacteria are covalently modified (possibly by adenylylation) and are also antigenically related. Recent results by Baumann and Baumann (2) and Baumann et al. (1) are in agreement with these findings. The presence of common features in the enzymes may indicate that the bacteria are derived from a common ancestor. The glutamine synthetases from all of the enteric bacteria display a high degree of antigenic homology in contrast to the enzymes from other sources.

Results presented in the case of glycogen synthase and branching enzyme from a wide distribution of bacteria indicate that cross-reactivity with *E. coli* B glycogen synthase and branching enzyme sera occurs only with enzymes from other closely related enteric bacteria. No cross-reactivity was observed between glycogen synthase from *S. marcescens* or *A. hydrophila* and anti-*E. coli* B serum. In addition, no cross-reaction was observed between branching enzyme from *A. hydrophila* and anti-*E. coli* B branching enzyme serum. However, branching enzyme from *S. marcescens* was partially neutralized by anti-branching enzyme serum. These two organisms are closely related to the enteric bacteria. No cross-reactivity with either anti-glycogen synthase or branching enzyme serum was observed when any enzyme from photosynthetic bacteria was tested.

Cross-reactivity of glycogen synthases and branching enzymes with anti-*E. coli* B sera is limited in each case to other enteric bacterial enzymes. This is particularly interesting with the glycogen synthase enzyme, where a reaction of identity is observed with *S. typhimurium* enzyme, indicating close similarity between the *E. coli* B and *S. typhimurium* glycogen synthase enzymes. This is in contrast to results observed for both the *S. typhimurium* branching enzyme and ADPglucose synthetase (12). In each case, cross-reactivity was observed between the *S. typhimurium* enzyme and anti-*E. coli* B serum, but without reactions of identity. The formation of spurs on double-diffusion plates was observed. Perhaps the divergence of these enzymes, particularly with ADPglucose synthetase, reflects the different selective pressures which have caused changes in the pattern of regulation of the enzyme.

Cross-reactivity between glycogen synthases and branching enzymes occurs only in bacteria which are in the same ADPglucose synthetase activator class (27, 30), indicating rather close similarity of these organisms and enzymes. A more extensive comparison of the glycogen biosynthetic enzymes among bacteria by similar immunological techniques with antisera directed against enzymes from other sources could provide information on the evolution of the glycogen biosynthetic pathway in bacteria. Such work is currently in progress.

The results presented here also indicate that *E. coli* B glycogen synthase and branching enzyme do not display a large degree of homology. N-terminal amino acid sequences for the two

TABLE 3. Summary of results from neutralization of glycogen synthase and branching enzyme activity from a variety of sources with anti-*E. coli* B glycogen synthase and branching enzyme sera

Enzyme source	Glycogen synthase		Branching enzyme	
	Antiserum (μl/U) for 50% inhibition	Maximum inhibition (%)	Antiserum (μl/U) for 50% inhibition	Maximum inhibition (%)
E. coli B AC7OR1	11	98	0.5	99
E. coli B	13	95	0.5	96
E. aurescens	13	95	0.5	98
S. typhimurium	11	98	1.5	66
E. aerogenes	78	95	1.0	65
K. pneumoniae	90	75	1.0	66
S. marcescens		5	3.0	56
A. hydrophila		5		0
R. sphaeroides		5		0
R. gelatinosa		20		0
R. molischianum		5		0

enzymes are dissimilar except for a region of eight amino acid residues, where only two differences are found. The possibility of limited homology in the inner amino acid residues of the enzyme cannot be ruled out at this time. The N-terminal sequence of either glycogen synthase or branching enzyme has little or no homology with this region of the *E. coli* B ADPglucose synthetase. In addition, the N-terminal sequence of *E. coli* B glycogen synthase is distinct from that of the rabbit skeletal muscle glycogen synthase (14).

The immunological similarity of *E. coli* B glycogen synthase and branching enzyme was also tested. The results indicate that branching enzyme may cross-react with anti-glycogen synthase serum, or the reverse. This view is rendered doubtful by experiments that use an enzyme pretreatment of the antisera to selectively remove the anti-branching enzyme or glycogen synthase antibodies, with no effect on the experimental result. The most likely conclusion is that small amounts of contamination in the highly purified antigens used for antiserum production were responsible for this result. Thus, in contrast to the results obtained by Fredrick (10) with *O. princeps* starch synthase and branching enzyme, where antigens were prepared from rather crude enzyme preparations obtained by preparative gel electrophoresis, little if any homology was observed between the *E. coli* glycogen synthase and branching enzyme.

Highly purified preparations of *E. coli* B glycogen synthase utilized in these studies were prepared from a derepressed strain (AC7OR1) which produces 6- to 10-fold higher specific activity in crude enzyme preparations than the wild type (9). The results presented here indicate that the enzyme produced in the derepressed strain is immunologically identical to the wild-type enzyme by two criteria, i.e., reactions of identity in double-diffusion tests and identical patterns of neutralization of activity by the anti-*E. coli* B AC7OR1 glycogen synthase serum. In addition, glycogen synthase activity present in crude preparations from *E. coli* B was totally neutralized by the anti-*E. coli* B AC7OR1 glycogen synthase serum, indicating that a single, identical enzyme is present in both the crude and purified enzyme preparations which catalyzed the reaction. Previous results have shown that the *E. coli* B glycogen synthase and the glycogen synthase from the derepressed *E. coli* B mutant have the same kinetic parameters (13).

The results presented here indicate a rather limited extent of immunological cross-reactivity among differing glycogen synthases and branching enzymes with anti-*E. coli* B sera as well as no apparent immunological homology between *E. coli* B glycogen synthase and branching enzyme. The limits of cross-reactivity might be widened based on reports by Zakin et al. (35) and Mouhli et al. (22). They report that fully denatured antigens produce antisera which have a wider specificity between aspartokinases-homoserine dehydrogenases I and II and aspartokinase II from *E. coli* K-12 compared with native antigens. Thus, this technique, applied to the glycogen biosynthetic enzymes, might provide a wider range of organisms that can cross-react with a given antiserum or allow identification of homologous regions among the enzymes of the glycogen biosynthetic pathway.

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