Isolation and Characterization of Glycogen Branching Enzyme from Rabbit Liver†

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ABSTRACT: Glycogen branching enzyme was isolated from rabbit liver. The highly purified enzyme shows a monomer molecular weight of 71 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and apparent molecular weights of 93 000 by sucrose density gradient sedimentation and 52 000 by gel-exclusion chromatography on Sephacryl S-300. No glucosamine, mannosamine, galactosamine, or sialic acid was detected in the protein. An amino acid analysis is reported. The spectrum of branching enzyme is that of a simple polypeptide, with $A^{1\%}_{280nm}$ = 24.6. Highly purified branching enzyme consists of several closely related active enzyme forms that can be resolved by isoelectric focusing in polyacrylamide gel. The major species of p*I* 5.7 is flanked by less abundant forms of p*I* 5.6 and 5.8. Seemingly identical enzyme forms are observed in crude extracts of rabbit liver, skeletal muscle, brain, and heart, although the absolute and relative concentrations vary among the tissues. Branching enzyme apparently does not exhibit tissue-specific isoenzymes.

Glycogen branching enzyme [1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase; EC 2.4.1.18] acts in concert with glycogen synthase (EC 2.4.1.11) during biosynthesis of glycogen. Although its deficiency is involved in a severe hereditary metabolic disease, type IV glycogenosis (Brown & Brown, 1966), it has been the least studied of the enzymes acting upon glycogen. Several earlier studies have resulted in purification and partial characterization of the branching enzyme from rabbit skeletal muscle (Gibson et al., 1971; Caudwell & Cohen, 1980); however, the small quantity of enzyme obtainable from this source has not permitted characterization of the molecular structure of the protein and detailed study of its catalytic mechanism.

Rabbit liver is a potentially abundant source of branching enzyme, since it contains 30 times more enzyme activity than muscle for a given weight of tissue. In the present work, rabbit liver branching enzyme has been purified and characterized with respect to its molecular weight, composition, and spectral properties. IEF-PAGE[1] and a method of staining for branching enzyme activity reported previously (Zimmerman & Gold, 1982) have been used to show that the enzymes from muscle and liver are probably identical and consist of several discrete molecular forms. Branching enzymes from rabbit brain and heart also appear to be identical with the liver enzyme.

Satoh & Sato (1982) have recently reported the isolation of branching enzyme from rat liver by means of affinity chromatography on glycogen–agarose adsorbents. The enzyme could be isolated as a ribonucleoprotein or as an nonconjugated protein, depending upon the type of affinity adsorbent used. Apparently, the RNA is not essential for activity, since the nonconjugated enzyme had ca. 40% the specific activity of the ribonucleoprotein, on the basis of protein content. This enzyme also exhibits microheterogeneity in electrophoretic separations.

Experimental Procedures

Materials. Frozen livers from 48-h-starved rabbits were obtained from Pel-Freez. Blue Sepharose Cl-6B, Sephacryl S-300, Polybuffer 74, and Polybuffer exchanger PBE 94 are products of Pharmacia Fine Chemicals. Ultrapure ammonium sulfate used in the enzyme isolation was obtained from Schwarz/Mann. Biolyte ampholytes and all electrophoretic reagents were products of Bio-Rad. Maltoheptaose was prepared as described previously (Hu & Gold, 1975). Phosphorylase *b* was prepared by the method of Fischer & Krebs (1962) and phosphorylase *a* by the method of Krebs & Fischer (1962).

Branching Enzyme Assays. Branching enzyme was assayed by a modification of method 1 of Gibson et al. (1971). Serial dilutions of purified enzyme or crude extracts were prepared in a buffer containing 5 mM Tris-HCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.5 (0.1 mg mL^{-1} bovine albumin was present when purified enzyme was diluted). Twenty microliters of diluted enzyme was incubated at 30 °C in a final volume of 120 μL containing 75 mM dipotassium glucose 1-phosphate, 2 mM AMP, and 6 EU mL^{-1} phosphorylase *a*, pH 6.8. Aliquots of 10 μL were taken at 15-min intervals and added to 50 μL of 1.5 M NH_3 to stop the reaction. Inorganic phosphate was determined as previously described (Hu & Gold, 1975). Activities were calculated from reactions in which the final rate of phosphate release was proportional to the concentration of enzyme; this is usually 0.02–0.05 μmol min^{-1} mL^{-1}. One enzyme unit (EU) is the amount of branching enzyme that causes an increase in the rate of production of inorganic phosphate of 1 μmol min^{-1}.

A convenient, semiquantitative assay was devised for rapid analysis of column fractions to determine which should be combined for further processing. The procedure was similar to that described above, but the sample volume was 10 μL and the final volume was 50 μL. After a 10-min incubation at 37 °C, the reaction mixture was treated with 1 mL of a solution containing 1.3 mM I_2, 2 mM KI, 40 mM sodium citrate, pH 5.6, and 3 mL of water. The color varies from yellow, in the absence of branching enzyme, through green, blue, violet, reddish brown, brown, and light tan, depending upon the amount of polyglucan synthesized and its degree of branching. The usefulness of the method lies in its speed and ability to

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[1] Abbreviations: IEF-PAGE, isoelectric focusing in a slab of polyacrylamide gel; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EU, enzyme unit; DEAE, diethylaminoethyl.

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accommodate a wide range of enzyme concentrations, so it is usually unnecessary to dilute the fractions or vary the incubation time.

Amylase Assay. The method of Bernfeld (1951) was modified to include 0.5% soluble starch, 6 mM NaCl, and 20 mM sodium phosphate, pH 6.9. Incubation was for 15 min at 25 °C, and reducing groups were determined by the method of Nelson (1944).

Protein Assay. Protein was determined by the dye-binding method of Bradford (1976) using a commercial reagent (Bio-Rad). Bovine γ-globulin (Cohn fraction II, Sigma) was used as a standard, assuming $A^{1\%}_{280nm} = 13.8$. Branching enzyme concentration was determined from A_{280} ($A^{1\%}_{280nm} = 24.6$) after the chromatofocusing step.

Amino Acid Composition. Pure branching enzyme was equilibrated with 10 mM Tris-HCl, pH 7.0, by gel filtration on Sephadex G-25 to remove mercaptoethanol and traces of UV absorbers; fractions preceding the protein were used as blanks. Portions were hydrolyzed in distilled 6 N HCl for 22 h at 112 °C and 0.1 torr and analyzed in duplicate for amino acids by standard methods (Spakman et al., 1958), while the remainder was used for determination of A_{280nm}, Coomassie blue binding (Bradford, 1976), and sulfhydryl groups. Total sulfhydryl groups were determined as described by Habeeb (1972) in the presence of 1% $NaDodSO_4$–70 mM Tris-HCl, pH 8.0. The ratio Trp/Tyr was determined by the method of Edelhoch (1967).

$NaDodSO_4$–PAGE. The discontinuous method of Laemmli (1970) was used with a Bio-Rad Protean slab cell. The sample buffer was modified to contain 20 mM sodium phosphate (pH 7), 2% $NaDodSO_4$, 1 mM EDTA, 1% 2-mercaptoethanol, 10% glycerol, and 0.03% bromphenol blue. The separating gel was 7.5% acrylamides, and the stacking gel was 4%. Gels were fixed in 50% ethanol–10% acetic acid and stained in the same solvent mixture containing 0.1% Coomassie blue R-250. Destaining was carried out in 25% ethanol–8% acetic acid.

Gel Exclusion Chromatography. A column of Sephacryl S-300 (90 cm × 1.6 cm) equilibrated with a buffer containing 0.20 M Tris-HCl and 0.20 M NaCl, pH 7.4, was run at 4 °C at a flow rate of ca. 15 mL h^{-1}. Samples were applied in 2 mL of buffer containing 20% sucrose, and fractions of 1.8 mL were collected. The void volume (V_0) was determined with rabbit liver glycogen and the total volume (V_t) with orthophosphate. Calibration curves are constructed as log M_r vs. K_{av}. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume at the maximum of each peak.

Extraction of Tissues for Analytical Electrofocusing (IEF-PAGE). Frozen tissues were homogenized at 0–4 °C in 2.5 volumes of 15 mM 2-mercaptoethanol containing 1 mM PMSF. Either a motor-driven Teflon pestle (liver and brain) or a Brinkmann Polytron (muscle and heart) was used. The homogenates were centrifuged at 160000*g* for 1 h at 5 °C and diluted with water; brain tissue produces artifacts in the area of application if high-speed centrifugation is omitted.

IEF-PAGE. IEF-PAGE was performed in the pH 4.5–7 range in a Bio-Rad 1405 horizontal cell. Gel dimensions were 11 cm × 20.5 cm × 0.15 cm; composition was 4.85% acrylamide, 0.15% *N,N'*-methylenebis(acrylamide), 2% ampholytes (three parts Biolyte 5/7 and one part Biolyte 6/8), 5% glycerol, and 0.1% maltoheptaose. Maltoheptaose is included as an initiator for glucan synthesis in the subsequent activity staining; although not necessary for development of the stain, the process appeared to be more reliable in its presence. Photopolymerization was catalyzed with 0.0005% FMN after degassing the solution. Electrode solutions were 1 M phosphoric acid and 1 M NaOH. A pH gradient was established by applying a current at constant power of 10 W (initial voltage 350 V) for 40 min across the 11-cm dimension. Samples of 10 μL were then applied to the gel surface near the pH 7 area, and the current was continued for 3.5 h (final voltage 1000 V) with cooling at 5 °C. The pH gradient was determined with a surface electrode.

Staining for branching enzyme activity was done by incubating the gel slab at 37 °C in 200 mL of a solution containing 6 EU mL^{-1} of phosphorylase *b*, 2 mM AMP, 10 mM dipotassium glucose 1-phosphate, and 0.125 M potassium maleate, pH 6.8 (Zimmerman & Gold, 1982). The gel was agitated occasionally and observed closely until cloudy bands of polysaccharide began to appear (ca. 1 h). Incubation time varies with enzyme concentration in a nonlinear fashion; careful observation of the bands is important because polysaccharide synthesis proceeds very rapidly after a variable lag time. The gel was then treated with a solution containing 1.3 mM I_2, 2 mM KI, and 40 mM sodium citrate, pH 5.6, to produce colorations between blue and reddish brown.

Isolation of Branching Enzyme. Tissue from 48-h-fasted rabbits was used to minimize potential interference from glycogen. Frozen liver (150 g) was chopped into cubes and homogenized for 60 s in a Waring Blendor with 375 mL of cold solution containing 15 mM mercaptoethanol, 10 mM EDTA, and 1 mM PMSF, pH 7.0. All the steps were carried out at 0–5 °C. The homogenate was centrifuged at 16000*g* for 30 min, and the supernatant was decanted through glass wool. It was then centrifuged at 100000*g* for 90 min, and the clear supernatant was recovered with a Pasteur pipet to avoid most of the incompletely sedimented material. Fat particles were removed by filtration through glass wool. The extract was adjusted to pH 5.7 with 1 N acetic acid and treated with 1 volume of cold distilled water and 1 volume of saturated ammonium sulfate (pH 5.7). The salt was added slowly with stirring and cooling so that the temperature did not rise above 7 °C. Another 150 g of liver was carried through the procedure up to this point, and the combined ammonium sulfate solutions were allowed to stand overnight.

After a centrifuging at 10000*g* for 30 min, the precipitate was dispersed in 160 mL of 38% saturated ammonium sulfate, pH 5.7. This was again centrifuged, and the precipitate was dissolved in 50 mL of 50 mM Tris-HCl–15 mM mercaptoethanol, pH 7.5, and dialyzed against the buffer. The sample was then applied to a short, wide column of DEAE-cellulose (Whatman DE-52) equilibrated with the same buffer. The column bed volume was 70 mL (g of protein)$^{-1}$. Elution was carried out with the equilibrating buffer at a fast flow rate (ca. 500 mL h^{-1}) until the protein content (A_{280}) of the eluate was negligible or had leveled off. Elution was then continued with the same buffer containing 0.10 M NaCl until a protein peak had emerged. Fractions containing the bulk of this protein were combined, treated with solid ammonium sulfate to give 60% saturation, and allowed to stand overnight.

The precipitate was centrifuged (16000*g* for 15 min), dissolved in ca. 50 mL of 25 mM histidine–HCl–15 mM mercaptoethanol, pH 6.2, and dialyzed against the same buffer. This solution was clarified by centrifugation and subjected to chromatofocusing (Söderberg et al., 1981) on a 1.3 cm × 36 cm column of Polybuffer exchanger PBE 94 equilibrated with the same histidine buffer. Elution was carried out with Polybuffer 74 diluted 1:10, pH 5.0, containing 15 mM mercaptoethanol. Fractions of 4 mL were collected, and the pH, A_{280}, and branching enzyme activity (semiquantitative method) of each fraction were determined. Enzyme activity peaked in

Table I: Purification of Rabbit Liver Branching Enzyme[a]

step	protein (mg)	activity (units)	sp act. (units/mg)	purification (x-fold)	yield (%)
supernatant from ultracentrifugation	30 300	(176 000)[b]	(5.8)[b]	(1)	(100)
first ammonium sulfate ppt dialyzed at pH 7.5	1 790	184 000	103	18	105
ammonium sulfate ppt following DEAE-cellulose	449	123 000	275	47	70
chromatofocused and treated with Blue Sepharose	12[c]	41 000	3 400	590	23

[a] Based on 300 g of frozen liver. [b] The activity was determined in a separate experiment with a freshly homogenized sample of frozen tissue centrifuged at 12000*g* for 10 min. [c] Corrected for the dye-binding capacity determined in this work.

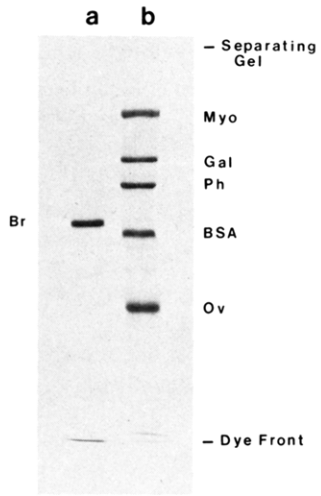

FIGURE 1: $NaDodSO_4$–PAGE of purified rabbit liver branching enzyme: (lane a) branching enzyme; (lane b) rabbit muscle myosin (205 000), *Escherichia coli* β-galactosidase (116 000), rabbit muscle phosphorylase *b* (97 400), bovine serum albumin (66 000), and chicken ovalbumin (45 000).

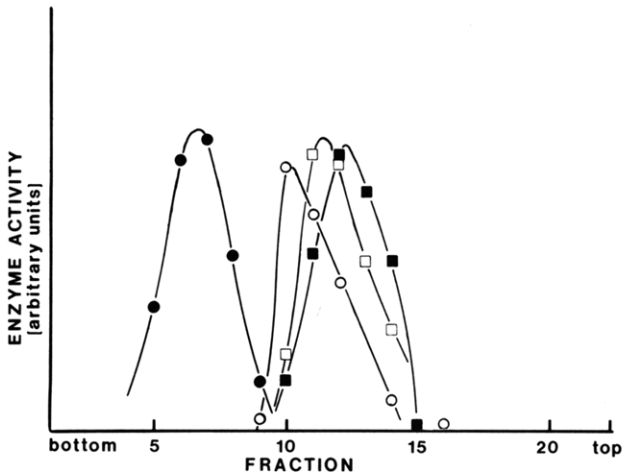

FIGURE 2: Sucrose density gradient sedimentation of purified rabbit liver branching enzyme: branching enzyme (○); rabbit muscle lactic dehydrogenase (144 000) (●); rabbit muscle glycerol-3-phosphate dehydrogenase (78 000) (□); rabbit muscle phosphoglucomutase (67 000) (■). Sedimentation was carried out in a Beckman SW 50.1 rotor for 15 h at 5 °C and 45 000 rpm. Gradients were 4.6 mL of 5–20% sucrose. Procedure was similar to that described by Gibson et al. (1971). Dehydrogenases were determined in continuous spectrophotometric assays (Bergmeyer, 1974). Branching enzyme was assayed by a variation of the quantitative method; all fractions were diluted equally, and only the 1-h time point was used.

the same fractions as protein, at ca. pH 5.6. Combined peak fractions were dialyzed against 3 volumes of saturated ammonium sulfate, pH 7, containing 15 mM mercaptoethanol. The precipitate was centrifuged, dissolved in 1–2 mL of 10 mM Tris-HCl–15 mM mercaptoethanol, pH 7.0, and dialyzed against the same buffer. The enzyme appears to be stable for months in this solution at 4 °C.

Occasionally, $NaDodSO_4$–PAGE revealed the presence of a significant amount of a 100 000-dalton impurity. This could be removed by passing the solution through a 1-mL column of Blue Sepharose Cl-6B equilibrated with the same Tris buffer. A representative purification is outlined in Table I.

Results

Purity. $NaDodSO_4$–PAGE shows the presence of a single major polypeptide and minor impurities in variable amounts (Figure 1). In addition to the M_r 100 000 impurity, which is removed by Blue Sepharose, there are traces of polypeptides having molecular weights of 46 000, 44 000, and 29 000. Amylase activity, which could affect the branching enzyme assay, was undectable; a maximum of 0.05 EU mg^{-1} could be present. The high concentration of branching enzyme present in the amylase assay (0.16 mg mL^{-1}) was shown to have little effect upon the assay. When a similar concentration of branching enzyme was added in the presence of swine pancreatic α-amylase, the activity did not change significantly. Glycogen debranching enzyme, which might also affect the branching enzyme assay, appears to be absent since its characteristic 160 000-dalton polypeptide is not observed in $NaDodSO_4$–PAGE. The preparation is estimated to be at least 95% pure. Impurities can be reduced further by gel exclusion chromatography on Sephacryl S-300, but with a considerable loss of yield.

Molecular Weight. The apparent molecular weight of the monomeric polypeptide chain of liver branching enzyme was estimated by $NaDodSO_4$–PAGE (Figure 1). A value of 71 000 ● 2000 was determined in experiments with several different preparations of enzyme.

The molecular weight of native branching enzyme was assessed by sedimentation in a sucrose gradient by using the method of Martin & Ames (1961) (Figure 2). A calculation of the molecular weight with reference to individual standard proteins gave 99 000 compared to lactic dehydrogenase, 91 500 compared to glycerol-3-phosphate dehydrogenase, and 87 900 compared to phosphoglucomutase. These values are remarkably similar to those reported by Gibson et al. (1971) for rabbit muscle branching enzyme, using the same method and standard proteins. A plot of sedimentation distance vs. $M_r^{2/3}$ of the standards gave a straight line from which a value of 93 000 could be estimated for the molecular weight of rabbit liver branching enzyme.

Gel exclusion chromatography on Sephacryl S-300 was also used to estimate the molecular weight of the native enzyme. A calibration curve constructed with ferritin (440 000), bovine

Table II: Amino Acid Analysis of Rabbit Liver Branching Enzyme

amino acid	residues/1000	residues/71 000 daltons
Asp	103.3	66
Thr	36.6	23
Ser	58.9	38
Glu	89.2	57
Pro	58.1	37
Gly	81.1	52
Ala	95.1	61
Cys[a]	10.6	7
Val	64.0	41
Met	15.2	10
Ile	41.8	27
Leu	97.0	62
Tyr	39.6	25
Phe	52.6	34
Trp[b]	21.1	14
Lys	54.0	35
His	32.2	21
Arg	49.6	32

[a] Determined as free sulfhydryl groups (Habeeb, 1972).
[b] Determined by method of Edelhoch (1967).

albumin (66 000), and ovalbumin (45 000) showed good linearity; branching enzyme has an apparent molecular weight of 52 000 in this system.

Composition. The amino acid composition of purified branching enzyme is given in Table II. An average residue weight of 111 can be calculated. The same protein solution used for amino acid analysis was used to determine the extinction coefficient at 280 nm and Coomassie blue binding in the protein assay of Bradford (1976) by calculating the concentration of protein from the concentrations and residue weights of the amino acids. The extinction coefficient $A^{1\%}_{280nm}$ = 24.6. Coomassie blue binding was found to be 2.0 times that of the standard, bovine γ-globulin, on the basis of the weight of protein.

Branching enzyme apparently contains no cystine, since none was observed in the amino acid analysis, but cysteine appears to be present because free sulfhydryl groups could be detected and estimated. Glucosamine, galactosamine, and mannosamine are all absent from the protein. These were examined by chromatographing a hydrolysate on the long column of the amino acid analyzer in the pH 5.28 buffer usually used for analysis of basic amino acids on the short column (Spakman et al., 1958). Hexosamine standards were eluted in positions where there was no interference from amino acids. No trace of any of the three hexosamines was observed under conditions where one residue in a 71 000-dalton polypeptide would have been readily detectable.

Ultraviolet Spectrum. The spectrum (Figure 3) shows no features suggesting the presence of components other than polypeptide. A_{280}/A_{260} = 1.56, and the ratio at the actual maximum and minimum is A_{278}/A_{248} = 2.02. A partial spectrum at a concentration 10 times that in Figure 3 gives no evidence for maxima between 300 and 400 nm. No color is apparent in concentrated solutions of branching enzyme.

Multiple Enzyme Forms. Although branching enzyme appears homogeneous in NaDodSO$_4$–PAGE, three to five closely spaced bands are revealed in IEF-PAGE. The same components appear whether the gel is stained for protein or for branching enzyme activity, indicating that the components are active enzymes differing slightly in isoelectric point. The bands are centered around pH 5.7, which agrees with the p*I* observed during chromatofocusing. The presence of multiple forms is not an artifact of isolation, since a crude rabbit liver

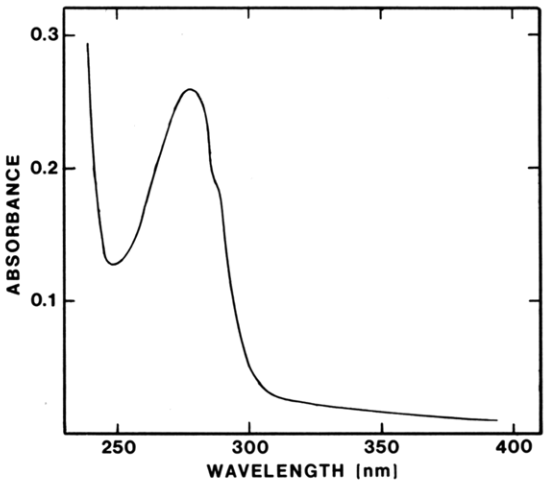

FIGURE 3: Ultraviolet spectrum of rabbit liver branching enzyme. The protein was dialyzed against 10 mM Tris-HCl, pH 7.0, and the dialysate was used as a blank.

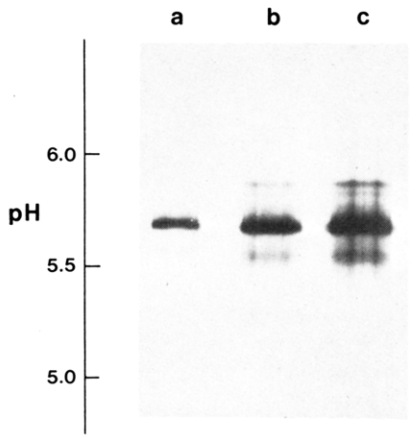

FIGURE 4: IEF-PAGE of a crude extract of rabbit liver. This procedure differs from the description under Experimental Procedures. An extract from a fed animal was centrifuged at low speed and subjected to phosphorolysis by endogenous phosphorylase and debranching enzyme; it was treated with 1 M potassium phosphate, 10 mM AMP, and 5 mM EDTA, pH 7.5, and dialyzed overnight against 5 mM Tris, 1 mM EDTA, and 5 mM mercaptoethanol, pH 7.5. (Similar experiments with 48-h-starved animals were done by omitting the phosphorolysis step.) The gel composition was 4% acrylamide, 0.2% *N,N'*-methylenebis(acrylamide), 2% ampholines (LKB) (three parts 5/7, one part 7/9), 0.1% maltoheptaose, 10% glycerol, and 0.02% ammonium persulfate. Extract was diluted 1:27 (lane a), 1:9 (lane b), and 1:3 (lane c). The gel was developed for branching enzyme activity.

extract shows similar components when stained for enzyme activity (Figure 4). Extracts from liver of 48-h-starved or fed animals gave similar patterns of active bands. In an experiment where purified enzyme and crude extract were subjected to IEF-PAGE side by side (Figure 5), only two of the major bands in each were visualized by activity staining, but the two bands corresponded closely in position.

Branching Enzyme in Other Rabbit Tissues. Enzyme activities vary widely among tissues. Supernatants from homogenates of frozen tissues were assayed, and the results are expressed as enzyme units per gram of wet tissue: liver, 660 EU g^{-1}; brain, 103 EU g^{-1}; skeletal muscle, 21 EU g^{-1}. Similar values were reported for the corresponding rat tissues by Satoh & Sato (1980). It is possible that amylase in the crude extracts is affecting the branching enzyme assay. This is unlikely because reaction rates were determined at concentrations of each extract where the rates were proportional to the added protein. We have observed in some cases that higher con-

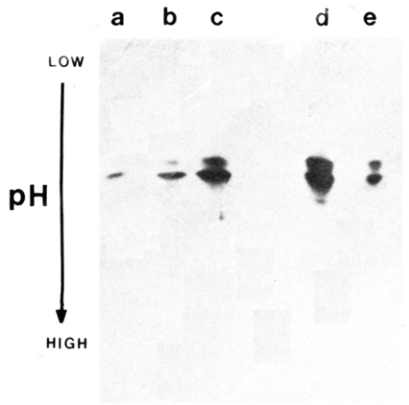

FIGURE 5: IEF-PAGE of crude extract of rabbit liver and purified branching enzyme. The procedure is described under Experimental Procedures. Lanes a–c are crude extracts diluted 1:27, 1:9, and 1:3, respectively. Lanes d and e are purified enzyme diluted 1:100 and 1:300, respectively. The gel was cut into two sections (lanes a–c and lanes d and e) to permit different development times and realigned for photographing.

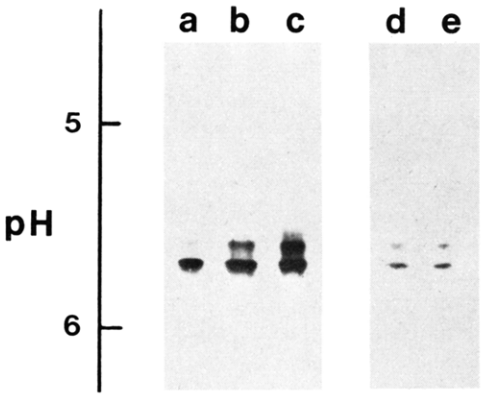

FIGURE 6: IEF-PAGE of crude extracts of rabbit liver, brain, skeletal muscle, and heart: skeletal muscle (undiluted) (lanes a and d); brain (1:2) (lane b), liver (1:9) (lane c), and heart (undiluted) (lane e).

centrations of a crude extract in the assay causes a precipitous fall in the activity; this is probably a result of amylase activity reaching a critical concentration where oligosaccharide primer is destroyed faster than it can be formed.

In order to compare the enzymes from different tissues in terms of their isoelectric points and distribution of enzyme forms, crude extracts were subjected to IEF-PAGE on the same gel and stained for enzyme activity. Because of the different levels of branching enzyme activity in the tissues, it was necessary to dilute the more active extracts so that roughly comparable enzyme activites (0.06–0.2 EU) could be applied to the gel; otherwise, it would be impossible to develop the activity stain simultaneously for all the extracts. Consequently, the amount of enzyme activity applied was limited by that in the least active extract, and the less abundant of the enzyme forms failed to develop. A comparison of extracts of liver (starved), brain, and skeletal muscle is shown in Figure 6a–c. The main enzyme band, focusing at pH 5.7, appears to be common to the three tissues; the band at pH 5.6, although common to the three tissues, is comparable in intensity to the main band in liver and brain but shows a significantly lower intensity in muscle. The minor components at pH 5.8 do not show in this gel. A direct comparison between skeletal and cardiac muscle is shown in Figure 6d,e; the patterns are identical. Although the relative amounts of the branching enzyme forms differ among the tissues, it appears that particular forms focus at the same pH, within the precision of the experiment, regardless of the tissue of origin.

Discussion

The molecular weight of rabbit liver branching enzyme cannot be decided unambiguously. $NaDodSO_4$–PAGE gave a value of 71 000, sucrose density gradient sedimentation gave a value of 93 000, and gel exclusion chromatography on Sephacryl S-300 resulted in a value of 52 000. The latter value is probably too low, and may be the result of adsorption of branching enzyme to the gel matrix. A similar result was obtained by Caudwell & Cohen (1980) with the rabbit muscle enzyme, although the numbers differ; $NaDodSO_4$–PAGE gave a subunit molecular weight of 77 000 while gel exclusion chromatography on Sephadex G-150 gave 60 000 daltons, the low value being ascribed to interactions with the gel. Gel exclusion chromatography of crude extracts of rabbit liver and muscle on Sephacryl S-300 (not reported above) gives well-defined peaks of branching enzyme in corresponding positions. Although this may not accurately reflect the molecular weight, it indicates that the enzymes are indistinguishable by this technique.

Our results with sucrose density gradient sedimentation compare well with those of Gibson et al. (1971), who reported a value between 92 000 and 103 000 for the muscle enzyme. It is not clear why this value is so high compared to the monomer size. Possibly it reflects a tendency of the protein to dimerize; a slight asymmetry in the sedimentation curve tends to support this idea. Rat liver branching enzyme (Satoh & Sato, 1982) gave a monomer molecular weight of 82 000 by $NaDodSO_4$–PAGE and a value of 98 000 by gel exclusion chromatography on Sephadex G-200. This reverses the situation observed with the rabbit enzymes and suggests that the rat branching enzyme has significantly different physical properties.

The specific activity of rabbit liver branching enzyme reported in this work is comparable to that found by Caudwell & Cohen (1980) for the rabbit muscle enzyme and is substantially greater than the activities reported by Gibson et al. (1971) for rabbit muscle enzyme and by Satoh & Sato (1982) for rat liver branching enzyme. It is not appropriate to draw conclusions from these results because the enzyme assay is difficult and can vary in different hands and the various investigators used different methods of estimating protein concentration.

Petrova & Filippova (1971) have reported the isolation of rabbit muscle branching enzyme in the form of a ribonucleoprotein containing a 2.5S RNA of unusual structure (Korneeva et al., 1979). Branching enzyme activity largely depended upon the presence of the RNA and could be reconstituted by addition of isolated RNA to a nonconjugated enzyme fraction. Other investigators have failed to confirm these results with the muscle enzyme (Gibson et al., 1971; Caudwell & Cohen, 1980). A recent report (Satoh & Sato, 1982) describes the isolation of rat liver branching enzyme as either a ribonucleoprotein or a nonconjugated protein, depending upon which of two glycogen-based affinity absorbents was used. In this case, the RNA is not essential for activity, but may activate by a factor of 2. We have looked for signs of an RNA conjugate in our work with the rabbit liver enzyme but have observed none; our purest branching enzyme is free of RNA, judging by its UV spectrum. IEF-PAGE of purified enzyme and crude tissue extract did not reveal any acidic ribonucleoprotein. Such a ribonucleoprotein might electrofocus at low pH where it is inactivated. Electrophoresis in polyacrylamide gel is reported to resolve the rat liver ribonucleoprotein into its components (Satoh & Sato, 1982).

Evidence described above indicates that the same family of branching enzyme forms is present in rabbit skeletal muscle, liver, brain, and heart. Our studies of crude tissue extracts with IEF-PAGE were made possible by a technique for activity staining that involves diffusing phosphorylase *b*, AMP, and glucose 1-phosphate into the gel and visualizing the resulting polyglucans with iodine (Zimmerman & Gold, 1982). This method is also useful for polyacrylamide gel electrophoresis, provided the concentration of acrylamide is no higher than 4.2%. Satoh & Sato (1980) have described a similar method in which the phosphorylase *b* is immobilized in the gel by addition prior to polymerization; this permits the use of higher concentrations of acrylamide but apparently requires longer development time and yields a product that cannot be stained with iodine. Both techniques have revealed the presence of multiple forms of branching enzyme in mammalian tissues. Rat skeletal and cardiac muscle, liver, and brain were shown by electrophoresis to contain ca. five microspecies that did not differ in position from tissue to tissue (Satoh & Sato, 1980). In the present work, the corresponding rabbit tissues were shown by IEF-PAGE to contain at least three microspecies that agreed closely in p*I* among the tissues.

Rabbit liver branching enzyme appears to contain no complex carbohydrate, since it is devoid of glucosamine, galactosamine, and mannosamine. Sialic acid also appears to be absent in the purified enzyme; treatment with neuraminidase resulted in no change in the distribution of enzyme forms in IEF-PAGE. The multiple enzyme forms are not the result of different numbers of sialic acid residues in a complex carbohydrate moiety. We cannot exclude the presence of carbohydrate containing only nonnitrogenous sugars.

Although no experiments were carried out with the rabbit enzyme, we have previously reported that the multiple forms of branching enzyme detected in extracts of human muscle and brain are not affected when the extracts are incubated with cyclic AMP dependent protein kinase, cyclic AMP, and ATP (Zimmerman & Gold, 1982). Since there is little difference between the branching enzyme patterns in extracts of liver from fed and starved rabbits, it seems unlikely that the enzyme forms differ in degree of phosphorylation or other regulatory modification.

A variety of other enzymes exist as families of proteins with slight differences in p*I*; sometimes the more acidic forms are elevated in tissues from aging individuals (Dreyfus et al., 1978). In several instances, this has been shown to be the result of progressive deamidation of glutamine or asparagine residues. Two specific asparagine residues of triosephosphate isomerase were identified as sites of nonenzymatic deamidation (Yuan et al., 1981); deamidation was postulated to be involved in the normal catabolism of the enzyme.

Apparently, the glycogen branching enzymes of both rabbit and rat do not consist of tissue-specific isoenzymes, at least in the four tissues of each species that have been investigated. The origin and role of the enzyme microspecies observed in all tissues remain to be elucidated.

Registry No. Glycogen branching enzyme, 9001-97-2.

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