Purification and Properties of Debranching Enzyme from Dogfish Muscle†

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ABSTRACT: Glycogen debranching enzyme (4-α-glucanotransferase amylo-1,6-glucosidase, EC 2.4.1.25 + 3.2.1.33) was purified 140-fold from dogfish muscle in a rapid, high-yield procedure that takes advantage of a strong binding of the enzyme to glycogen, and its quantitative adsorption to concanavalin A-Sepharose only when the polysaccharide is present. The final product was homogeneous according to the criteria of ultracentrifuge analysis and polyacrylamide gel electrophoresis in the presence and absence of dodecyl sulfate. A molecular weight of 162 000 ± 5000 was determined by sedimentation equilibrium analysis in good agreement with the value of 160 000 estimated by gel electrophoresis, but a low-sedimentation constant of 6.5 S suggests that the enzyme is asymmetric. The molecule appears to be made up of a single polypeptide chain with no evidence for multiple repeating sequences: it could not be dissociated into smaller fragments by dodecyl sulfate even after complete carboxymethylation; tryptic cleavage of the native protein yielded only two fragments of molecular weight 20 000 and 140 000 without loss of enzymatic activity. The amino acid composition of the enzyme is reported; no covalently bound phosphate or carbohydrate could be detected. All 32 sulfhydryl groups present were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions; eight reacted readily in the native enzyme without loss of catalytic activity, while substitution of eight additional ones lowered the activity by 50%. Inactivation was greatly reduced by glycogen; the polysaccharide also influenced markedly the electrophoretic behavior of the enzyme and large filamentous aggregates were formed when solutions of both were mixed. Purified debranching enzyme releases 3 μmol of glucose min^{-1} mg^{-1} at 19 °C, pH 6.0, from a glycogen limit dextrin and one-tenth this amount when the native polysaccharide is used as substrate; glycogen is quantitatively degraded in the presence of phosphorylase. None of the usual sugar phosphates or nucleotide effectors of glycolysis affected enzymatic activity. No phosphorylation by either dogfish or rabbit skeletal muscle protein kinase or phosphorylase kinase could be demonstrated, nor any direct interaction with phosphorylase as measured by SH-group reactivity, enzymatic activity, or rate of phosphorylase *b* to *a* conversion. Purification of the 160 000 molecular weight M-line protein of skeletal muscle resulted in the quantitative removal of debranching enzyme, indicating that the two proteins are different.

This investigation was undertaken as part of our continuing interest in the evolution of control mechanisms. Much of the work was carried out on the Pacific dogfish (*Squalus acanthias*), a primitive vertebrate possessing a well developed endocrine system and abundant in this geographic area. The isolation of several enzymes and structural proteins involved in the concerted regulation of glycogen metabolism and muscle contraction has already been described (see, for instance, Cohen et al., 1971; Malencik et al., 1975).

In the course of a study of dogfish glycogen synthase, a second high-molecular-weight protein was found to accompany the enzyme throughout its purification; separation was eventually achieved after digestion of endogenous glycogen by α-amylase and this second component was identified as a glycogen debranching enzyme by its ability to liberate glucose from glycogen phosphorylase limit dextrin. For this activity, two distinct reactions have to be catalyzed: first, transfer of a glucan segment of three residues from a side chain to another nonreducing end of the polysaccharide, leaving the glucose unit involved in the α-1,6 linkage exposed; then, hydrolysis of the remaining glucose stub by a specific α-1,6-glucosidase (Brown and Illingworth, 1962; Abdullah and Whelan, 1963; Brown et al., 1963; Brown and Illingworth, 1964; Brown and Illingworth-Brown, 1966).

Purification of the dogfish muscle debranching enzyme was undertaken because, as of now, only the rabbit muscle protein (Brown and Illingworth-Brown, 1966; Nelson et al., 1969; White and Nelson, 1974, 1975; Taylor et al. 1975), and that from baker's yeast (Lee et al., 1970) have been isolated and characterized in some detail. Furthermore, its strong affinity for glycogen and high concentration in muscle extract suggested that it might be involved in the structure of glycogen particles, or the regulation of enzymes associated with them. This manuscript describes the isolation in homogeneous form of the dogfish skeletal muscle enzyme and some of its physical, chemical, and enzymatic properties.

Materials and Methods

Con A[1]-Sepharose was obtained from Pharmacia; DEAE-cellulose from Whatman; uridinediphospho[U-^{14}C]glucose (UDPG) (200 mCi/nmol) from Amersham, England; trypsin

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[1] Abbreviations used are: UDPG, uridine diphosphoglucose; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Tris-glycerol buffer, 50 mM Tris, 10% glycerol, pH 7.4, containing 0.2 mM phenylmethanesulfonyl fluoride and 1 mM dithiothreitol; Nbs_2, 5,5′ dithiobis(2-nitrobenzoic acid); Con A, concanavalin A; DEAE, diethylaminoethyl; NADP, nicotinamide adenine dinucleotide phosphate; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetracetic acid; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates.

(TPCK-treated) and soybean trypsin inhibitor from Worthington. The buffer system commonly used throughout the purification consisted of 50 mM Tris, 10% glycerol, pH 7.4, containing 0.2 mM phenylmethanesulfonyl fluoride and 1 mM dithiothreitol (Tris-glycerol buffer). *Phosphorylase limit dextrin* was prepared by exhaustive phosphorolysis of shellfish glycogen with three-times crystallized rabbit muscle phosphorylase freed from traces of debranching enzyme by hydrophobic chromatography on aminobutylagarose as described by Taylor et al. (1975). The material obtained was precipitated with 66% ethanol, washed with acetone, redissolved in a small amount of water and dialyzed extensively before final lyophylization. Human salivary amylase, dogfish phosphorylase, and rabbit phosphorylase kinase were prepared according to Fischer and Stein (1961), Cohen et al. (1971), and Cohen (1973), respectively; α-glucosyl Schardinger dextrin was a generous gift from Dr. E. Y. C. Lee, University of Miami, Miami, Florida.

Enzyme Assays. Debranching enzyme was assayed by two procedures, both involving liberation of glucose from a phosphorylase limit dextrin. In crude fractions, assays were carried out as described by Nelson et al. (1969), but at 19 °C. On more purified fractions, e.g., after the Con A-Sepharose step, glucose production was measured at 340 nm by the reduction of NADP in a coupled assay involving hexokinase and glucose-6-phosphate dehydrogenase. The reaction, carried out in 0.5 ml of 50 mM imidazole-HCl, pH 7.0, 2 mM $MgCl_2$, 1 mM EDTA, 7 mM mercaptoethanol, 2.5 mM NADP, 5 mM ATP, 2.5 mg/ml of phosphorylase limit dextrin, 0.8 unit/ml of hexokinase, and 4 units/ml of glucose-6-phosphate dehydrogenase, was linear from 0.5 to 80 nmol of glucose produced min^{-1} ml^{-1}. The same buffer system was used for determination of phosphorylase activity in the direction of glycogen breakdown, except that, instead of limit dextrin and ATP, 10 μM glucose 1,6-diphosphate and 0.25% shellfish glycogen were added, and hexokinase was replaced by 1.5 units of phosphoglucomutase. The method of Hedrick and Fischer (1965) was followed for activity measurements in the direction of glycogen synthesis. Phosphorylase kinase was assayed according to Krebs (1966), but the Tris-glycerol buffer and glycogen synthase were according to Thomas et al. (1968). Glucose and glc-1P were determined enzymatically according to Slein (1963) and Bergmeyer and Klotzsch (1963), respectively.

Electrophoresis were carried out in the absence or presence of sodium dodecyl sulfate on 5% polyacrylamide gels according to Davis et al. (1967) and Weber and Osborn (1969), respectively; in the latter instance, half the phosphate concentration was used in order to shorten the running time. For molecular weight determination, the following marker proteins were used as standards: bovine serum albumin monomer and dimer (68 000 and 136 000, respectively), rabbit muscle phosphorylase (100 000; Cohen et al., 1971), and α and β subunits of dogfish muscle phosphorylase kinase (131 000 and 118 000, respectively; Pocinwong, 1975), *Escherichia coli* β-galactosidase (130 000), and the heavy chain of rabbit muscle myosin (200 000; Weber et al., 1975). Protein bands were stained with Coomassie blue and carbohydrates according to Fairbanks et al., 1971.

High-speed sedimentation equilibrium experiments (Yphantis, 1964) were performed at 4 °C in 50 mM Tris buffer, pH 7.4, containing 10% (w/w) sucrose, and 1 mM dithiothreitol. Number-, weight-, and z-average molecular weights were computed according to the program of Teller et al. (1969). Sedimentation velocity runs were carried out under the same conditions, or with 10% glycerol instead of sucrose. A partial specific volume of 0.73 was calculated from the amino acid composition of the enzyme (Cohn and Edsall, 1943).

The absorbancy coefficient was determined after extensive dialysis against Tris-glycerol buffer; a value $A_{280nm}^{1\%}$ of 17.8 was found, based on protein concentration determined by the refractometric method of Babul and Stellwagen (1969). *Amino acid analyses* were carried out on a Beckman 120C analyzer on samples hydrolyzed in 5.4 N HCl for 24, 48, 86, and 114 h at 110 °C in sealed tubes evacuated after several flushings with nitrogen. Tryptophan was measured spectrophotometrically (Edelhoch, 1967), and total cysteine after performic acid oxidation according to Hirs (1967).

Free sulfhydryl groups were titrated with NbS_2 following the procedure of Habeeb (1972). The native enzyme was carboxymethylated after dialysis against Tris-glycerol buffer containing 2 mM dithiothreitol in the presence of 100 mM neutralized iodoacetate. The reaction was allowed to proceed for 35 min at 19 °C in the dark and then stopped by addition of 250 mM β-mercaptoethanol.

Carbohydrate was determined by the phenol-sulfuric acid procedure of Ashwell (1966) and *phosphate* by a microassay (Shaw, 1971) on exhaustively dialyzed samples containing 2–3 mg of protein.

Results

Dogfish (*Squalus acanthias*) were netted in the waters of Puget Sound and kept for at least 3 days in a 4000-gal saltwater tank generously provided by the Department of Fisheries, University of Washington, before being sacrificed. They were killed by a blow on the head; dorsal and tail muscles were quickly excised, cooled in ice, and passed through a meat grinder; the muscle mince (usually 250–500 g) was suspended in 2 volumes of cold Tris-glycerol buffer and homogenized in a Waring blender for 1 min at high speed. The homogenate was centrifuged for 30 min at 23 000*g* in a Sorval RC 2B centrifuge and the supernatant was filtered through glasswool to remove a layer of fat. This crude extract was kept frozen at least overnight during which time a precipitate developed.

Ethanol Precipitation. The thawed extract was centrifuged, the supernatant was cooled to −2 °C, and ethanol (chilled to −60 °C) was added in 10-ml portions under good stirring to a final concentration of 23%. After centrifugation at 23 000*g* for 20 min, the sediment was homogenized in one-fifth the original volume of Tris-glycerol buffer and spun for 30 min at 48 000*g*; the pellet was washed with approximately 10 ml of the same buffer and recentrifuged. Both supernatants were combined and solid shellfish glycogen was added to a final concentration of 0.1%. This solution (100–200 ml) can be kept frozen for at least 1 week if needed.

Con A-Sepharose Chromatography. The above solution was centrifuged for 10 min at 48 000*g* and the supernatant was applied to a Con A-Sepharose column (usually 4 × 15 cm and containing an amount of adsorbant equal to at least one-fourth the volume of the original crude extract), previously equilibrated with 15 bed volumes of Tris-glycerol buffer containing 0.5 M NaCl, 1 mM each of $MgCl_2$, $MnCl_2$, $CaCl_2$, and 0.02% NaN_3 as a bacteriostatic agent. The column was washed with 3–4 bed volumes of the same buffer. In most instances, the 280-nm absorbancy of the effluent fell to less than 0.05 as contaminating proteins, including phosphorylase, phosphorylase kinase, and phosphatase, were removed. The debranching enzyme and glycogen synthase remained adsorbed and were eluted with the same buffer containing 0.1 M α-methyl mannoside (Figure 1). Rarely, some of the phospho-

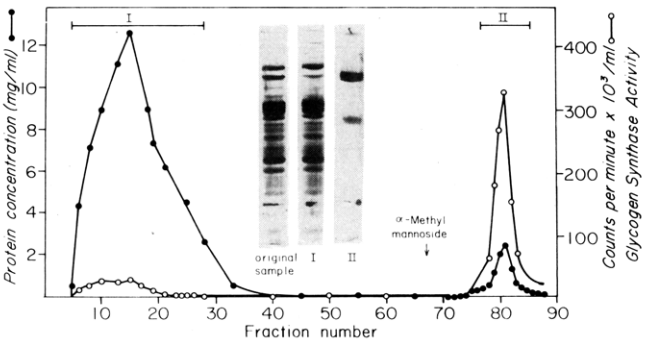

FIGURE 1: Purification of debranching enzyme and glycogen synthase by Con A-Sepharose chromatography. Extract from 250 g of dogfish muscle was precipitated with ethanol (see text) and a solution (50 ml) of the pellet was applied to a 2.5 × 20 cm Con A-Sepharose column. The standard Tris–glycerol buffer contained 2.5 mM glc-6P to protect glycogen synthase; 5-ml fractions were collected. Only glycogen synthase activity which coincides with debranching enzyme at this stage is illustrated. Approximately 100 μg of protein from the original sample and from pooled peak I, and 10 μg from pooled peak II were subjected to gel electrophoresis in the presence of dodecyl sulfate.

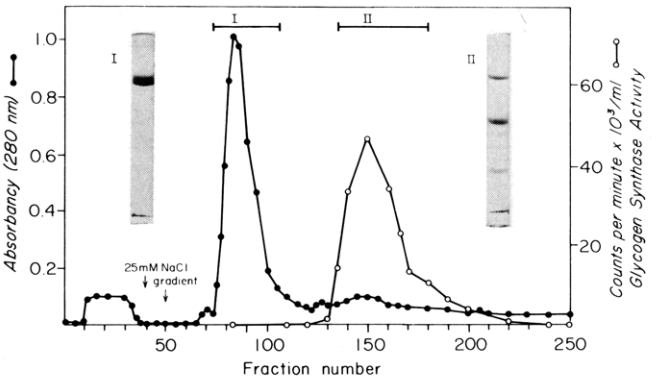

FIGURE 2: Separation of glycogen synthase and debranching enzyme. Conditions are described in the text, except that all solutions contained 2.5 mM glc-6P to protect glycogen synthase. Fraction size was 2.5 ml. Gel electrophoreses in the presence of dodecyl sulfate were carried out on ca. 10 μg of protein from pooled peak I and peak II (after concentration by lyophylization).

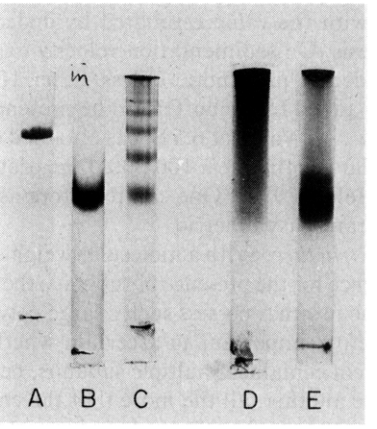

FIGURE 3: Disc gel electrophoresis of purified debranching enzyme. (A) In the presence and (B) in the absence of sodium dodecyl sulfate; (C) oligomeric forms. Partially purified enzyme fraction obtained after Con A-Sepharose chromatography; (D) before and (E) after amylase treatment. There is no dodecyl sulfate in C, D, and E.

rylase was also retained as visualized by the fact that the absorbancy of the effluent dropped very slowly. Elution was initiated, nevertheless, after 4 bed volumes, since, in any case, the contaminating phosphorylase could be readily separated in the next step. The opalescent fractions containing the debranching enzyme were pooled, 50 μg of crystalline human salivary amylase/100 ml of effluent was added and the digest was dialyzed for 24 h against several changes of Tris–glycerol buffer containing 0.02% NaN_3 upon which it became completely clear.

The Con A-Sepharose column can be regenerated by washing with 20 bed volumes of 10 mM Tris buffer, pH 7.4, containing 0.01% thimerosal (merthiolate), 1 M NaCl, 5 mM Na_2SO_3, and 1 mM each of $MgCl_2$, $CaCl_2$, and $MnCl_2$. The efficiency of the column diminishes upon successive recyclings (probably due to proteolysis of concanavalin A), and, usually, no more than 6–7 such operations were carried out.

DEAE-Cellulose Chromatography. The clear solution was applied to a 2 × 9 cm DEAE-cellulose column equilibrated with Tris–glycerol buffer, containing 0.02% NaN_3. The column was washed with the same buffer and then with buffer containing 25 mM NaCl to remove residual phosphorylase until the absorbancy of the effluent fell below 0.05; a 500-ml gradient from 25 to 350 mM NaCl was finally applied. This step readily separates amylase which is not retained, phosphorylase, and glycogen synthase from the debranching enzyme which emerges between 50 and 120 mM NaCl (Figure 2).

Fractions containing the bulk of the debranching enzyme (pool I, Figure 2) were concentrated in the Amicon Diaflo ultrafiltration cell using a XM50 membrane under occasional stirring (prolonged agitation denatures the enzyme) until the protein concentration increased to 3.5–3.8 mg/ml; beyond this point, an irreversible aggregation occurs. This enzyme solution can be stored frozen in Tris–glycerol buffer for at least 3 months without measurable loss of activity.

Table I summarizes the protocol of a purification. The fivefold increase in specific activity after DEAE-cellulose chromatography was due to removal of contaminating phosphorylase which was partially retained on the Con A-Sepharose. The overall purification was 140-fold. The purified enzyme was free of phosphorylase, phosphorylase kinase, and glycogen synthase activities. α-Amylase added in the course of purification was removed in the Con A-Sepharose step; total absence of this activity was confirmed by overnight incubation of the purified debranching enzyme preparation with glycogen in which an *increase* rather than decrease in iodine stain occurred. From the specific activity of the purified material, one can calculate that debranching enzyme accounts for approximately 0.7% of the soluble protein in dogfish muscle, a value comparable to that reported by Taylor et al. (1975) for the rabbit.

Molecular Weight. The enzyme displays a single band on gel electrophoresis carried out in the presence or absence of sodium dodecyl sulfate (Figure 3A,B). In the former case, a molecular weight of 160 000 was estimated by comparison with the protein markers listed under Methods.

Sedimentation equilibrium experiments gave results difficult to interpret because the native enzyme associates reversibly at a protein concentration of ca. 0.7 mg/ml, preventing extrapolation to zero concentration. Oligomerization could also be observed on gel electrophoresis (Figure 3C); a logarithmic plot of the mobility of the various bands was absolutely linear, indicating the formation of dimers, trimers, tetramers, etc. This association could be prevented by carboxymethylation of 8 out of 32 SH groups (see Methods), which could be carried out without loss of enzymatic activity. Under these conditions, a

TABLE I: Purification of Debranching Enzyme.

Step	Vol (ml)	Total Act.[b] (μmol of glc/min)	Total Protein[a] (mg)	Sp. Act.[b] (nmol min^{-1} mg^{-1})	Purification, Fold	Yield (%)
Crude extract	780	51.5	9830	5		
Crude extract, thawed, centrif	760	50.2	7900	6	1.2	97
Ethanol precip.	220	32.3	1230	26	5	63
Con A-Sepharose	110	25.3	165	150	30	49
DEAE-cellulose	120	24.6	34	730[c]	140	48

[a] Measured according to Lowry et al. (1951) in the first 4 steps, then spectrophotometrically using an adsorbancy index $A_{280nm}^{1\%}$ of 17.8. [b] For comparison purposes, all assays were carried out in the presence of 16 mM Tris-HCl, which corresponds to the amount carried over in the assay by the crude extract. Activities measured in the absence of Tris are approximately four times higher. [c] A specific activity of ca. 3000 ± 500 (3 μmol min^{-1} mg^{-1}) is obtained in 50 mM maleate buffer, pH 6.0, at 19 °C.

weight-average molecular weight M_w = 162 000 ± 5000 could be determined (M_n = 156 000 and M_z = 166 000) in good agreement with the value estimated by dodecyl sulfate gel electrophoresis. On sedimentation velocity experiments, the enzyme tended to precipitate unless either 10% glycerol or sucrose was added to the buffer; in the presence of these additives, a low $s_{20,w}$ value of 6.5 S was obtained corresponding to a molecular weight of ca. 100 000 if the protein were globular (Van Holde, 1975). One can therefore assume that the molecule is fairly asymmetric.

Subunit Structure. With a molecular weight of ca. 160 000 and no evidence for the presence of subunits, the enzyme would be among the few that possess such a large polypeptide chain. It was therefore important to ascertain whether or not the dogfish protein contained multiple subunits, perhaps strongly linked to one another, all the more that the enzyme exhibits two distinct catalytic functions (4-α-glucanotransferase and amylo-1,6-glucosidase activities; Brown and Illingworth, 1964; Brown and Illinworth-Brown, 1966). Neither reduction of the protein (1 mg/ml in Tris–glycerol buffer containing 1% dodecyl sulfate) with 140 mM mercaptoethanol for 1 h at 100 °C nor exhaustive carboxymethylation (after reduction with 2 mM dithiothreitol for 30 min at 19 °C) in the presence of 25 mM iodoacetate resulted in dissociation of the enzyme into smaller subunits.

Another alternative was that the single polypeptide chain was composed of multiple repeating sequences. If such segments contained a bond sensitive to proteolysis, one would expect the enzyme to be cleaved into small fragments. This was not the case: for instance, when 0.3 mg of the protein was digested with 5 μg of trypsin in Tris–glycerol buffer minus phenylmethanesulfonyl fluoride for up to 90 min, a peptide fragment of molecular weight approximately 20 000 was cleaved quantitatively after 10 min leaving material of molecular weight ca. 140 000. Surprisingly, there was no loss of catalytic function but, on the contrary, a 1.5-fold increase in activity. On prolonged digestion, a second split occurred leaving once more a rather large fragment of molecular weight ca. 120 000.

Amino Acid Composition and Sulfhydryl Groups. The amino acid composition of the enzyme is reported in Table II; it indicates a relatively high content of half-cysteine (32 residues/molecule) and 34 tryptophans responsible for a high-ultraviolet absorbancy $A_{280nm}^{1\%}$ of 17.8. All 32 SH groups reacted with Nbs_2 in the presence of sodium dodecyl sulfate, indicating the absence of S–S bonds (Figure 4). In the presence of glycogen, eight groups reacted readily without loss of enzymatic activity, while substitution of eight additional SH groups led to 50% inhibition. Omission of glycogen resulted in a much faster inactivation of the enzyme, implying either a direct interaction of the polysaccharide with some of the sulfhydryl groups at the active site or a change in conformation elicited by the substrate and resulting in a protection of some of the SH groups involved in enzymatic activity (Figure 4).

TABLE II: Amino Acid Composition of Dogfish Debranching Enzyme.

	Ca. No. of Residues/160 000 Daltons	
	From Dogfish ± SEM	From Rabbit Enzyme[a]
Lysin	78 ± 3	69
Histidine	53 ± 3	41
Arginine	70 ± 4	77
Aspartic acid	144 ± 4	127
Threonine	68 ± 3[b]	63
Serine	96 ± 5[b]	77
Glutamic acid	155 ± 6	177
Proline	70 ± 4	93
Glycine	95 ± 4	92
Alanine	78 ± 3	81
Valine	81 ± 3[c]	90
Isoleucine	61 ± 2[c]	80
Leucine	137 ± 3	140
Tyrosine	52 ± 3	61
Phenylalanine	58 ± 5	65
Half-cysteine	32[d]	42
Methionine	33 ± 3	28
Tryptophan	34	31
Total	1395	1434

[a] Values from Taylor et al., 1975. [b] Extrapolated to t = 0 h of hydrolysis. [c] Value for 84 h of hydrolysis. [d] A value of 32 was obtained by titration with Nbs_2 after denaturation.

No covalently bound phosphate (<0.2 mol/mol of protein) could be detected. Likewise, no firm evidence could be obtained for the presence of covalently bound carbohydrate. Staining of polyacrylamide gels by the periodate-Schiff procedure (see Methods) gave entirely negative results on samples that had not been treated with α-amylase; glycogen associated with the enzyme but which remained at the top of the gel gave, of course, a strong positive reaction. It should be mentioned, however, that because of the large size of the protein, this procedure would not have detected less than 5% carbohydrate

bound to the enzyme. The far more sensitive phenol-sulfuric acid test also gave a barely detectable reaction at the limit of sensitivity of the procedure, indicating that, at most, the enzyme contains 0.3% carbohydrate corresponding to less than three glycosyl residues per molecule.

Glycogen Binding. Dogfish debranching enzyme binds tightly to glycogen as already apparent by the Con A-Sepharose chromatography step: no binding to Con A occurred after α-amylase treatment. It does not penetrate polyacrylamide gels containing 0.1% of the polysaccharide; this exclusion did not appear to be due to a sieving effect, since the mobility of bovine serum albumin monomers and dimers (68 000 and 136 000, respectively) was unaffected under these conditions. Second, when an enzyme-glycogen mixture is subjected to electrophoresis, the protein is distributed throughout the gel from the origin to the leading edge (Figure 3D), while it migrates almost normally (Figure 3E) after α-amylase digestion of the polysaccharide. Third, addition of a concentrated solution of debranching enzyme (>2 mg/ml) to a 1% solution of glycogen produces a filamentous precipitate within a few minutes which cannot be redissolved by dilution. These filaments were not due to the rapid formation of amylose because they did not stain dark blue with iodine and were readily soluble in 0.1% dodecyl sulfate.

Enzymatic Activity. Dogfish debranching enzyme is inactive below pH 4.6 and above pH 9 with an optimum between 5.5 and 6.3 (phosphate-citrate buffer), barely lower than that reported for the rabbit enzyme (pH 6.6; Nelson et al., 1969) but similar to that of the yeast protein (Lee et al., 1970). Dogfish debranching enzyme is distinctly more active on glycogen limit dextrin than on the intact polysaccride. Relative to the phosphorylase limit dextrin (taken as 1) rates of 0.6, 0.1, and 0.1 were observed for α-glycosyl Schardinger dextrin, liver, and shellfish glycogen, respectively. A specific activity of 3.0 $\pm$ 0.5 μmol of glucose released min^{-1} mg^{-1} of protein was obtained in the presence of a saturating concentration of phosphorylase limit dextrin (10 mg/ml) in 50 mM maleate buffer, pH 6.0, at 19 °C. No measurement could be carried out at 30 °C because of rapid inactivation at this temperature. In the presence of phosphorylase *b*, the enzyme brought about a quantitative degradation of glycogen. Crystalline phosphorylase *b* (11 μg) and debranching enzyme (8 μg) were added to purified shellfish glycogen (ca. 1 mg/ml in 0.1 M phosphate buffer, pH 6.5, containing 1 mM AMP and 0.02% NaN_3). After overnight incubation at 19 °C, 0.36 μmol/ml of glucose and 4.40 μmol/ml of glc-1P were produced, for a total of 4.76 μmol/ml of glc + glc-1P. Complete hydrolysis with α-1,6-, α-1,4-amyloglucosidase (Keppler and Decker, 1970) yielded 4.77 μmol/ml of glucose. Phosphorylase alone yielded 1.36 μmol/ml of glc-1P and negligible amounts (<0.1%) of glucose. The dogfish protein can also incorporate glucose into glycogen, but this reaction proceeds at measurable rates only at very high concentrations of glucose (60 mM). Tris is an inhibitor of the enzyme (75% inhibition at 15 mM) as reported earlier for the rabbit protein (Brown and Illingworth-Brown, 1966; Nelson et al., 1969).

Regulation of Enzyme Activity. The activity of debranching enzyme was not altered by a variety of effectors involved in the control of glycogen metabolism, such as 1 mM each of AMP, ADP, ATP, UDPG, glc-1P, glc-6P and P_i. It was not phosphorylated by rabbit or dogfish phosphorylase kinase or protein kinase with or without cyclic 3',5'-AMP. Incubation of 100 μg of debranching enzyme for 30 min at 19 °C with 0.5 mM [γ-^{32}P]ATP (3.5×10^8 cpm/μmol of ATP) and 2 mM Mg in the presence of 10 μg of either kinase resulted in no incorporation of radioactivity. Introduction of one phosphate group/molecule would have been accompanied by the incorporation of ca. 200 000 cpm; i.e., the procedure would have been easily sensitive enough to detect the incorporation of $^1/_{100}$ of a phosphoryl residue/mole. Under the above conditions, therefore, no obvious regulation of the enzyme could be found.

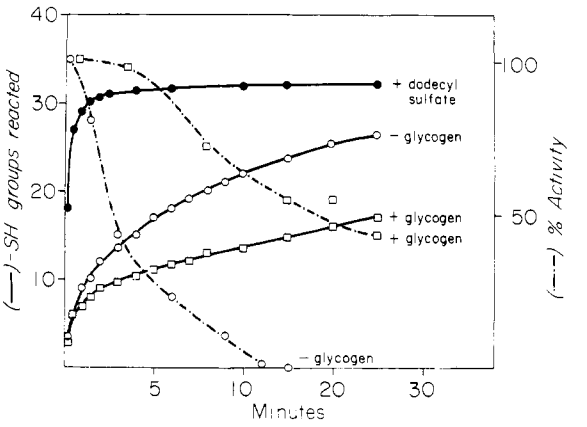

FIGURE 4: Titration of SH groups in dogfish debranching enzyme. The enzyme, stored in Tris-glycerol buffer containing 1 mM dithiotheritol, was dialyzed for 16 h against an excess of Tris-glycerol buffer without the sulfhydryl reagent. The protein (0.3 mg in 0.2 ml) was added to 1.0 ml of phosphate buffer, pH 8.0, containing 1 mM EDTA with or without glycogen (25 mg/ml final concentration). The mixtures were titrated with 10 mM Nbs_2, and the reaction was stopped by diluting 50 μl of citrate buffer, pH 6.5, containing 140 mM β-mercaptoethanol. The reaction was followed by changes in absorbancy at 410 nm; enzymatic activity was determined by the coupled assay described under Methods. For total SH group determination, the phosphate buffer contained 2% sodium dodecyl sulfate.

Absence of Interaction with Phosphorylase. Because of the strong binding of debranching enzyme to glycogen and, indeed, its presence in muscle glycogen particles, the possibility occurred that it might interact with phosphorylase or some of the enzymes involved in the control of glycogen metabolism. As yet, no obvious interaction could be detected, even though, occasionally, some phosphorylase remained adsorbed on the Con A-Sepharose column. Addition of increasing amounts of debranching enzyme up to a twofold molar excess did not affect either phosphorylase *b* (measured in the presence of 1 mM AMP) or *a* activities, when these were tested both in the direction of glycogen synthesis or breakdown. To check its possible involvement in the phosphorylase *b* to *a* conversion, 2.2 mg/ml of phosphorylase *b* was preincubated overnight with or without 2.2 mg/ml of debranching enzyme in the presence and absence of 2% glycogen, and conversion was initiated by addition of 80 μg/ml of Ca^{2+}-activated rabbit muscle phosphorylase kinase (see Methods). In no instance was the phosphorylase *b* to *a* reaction affected, though glycogen increased the rate of interconversion approximately fourfold. Likewise, addition of a twofold molar excess of dogfish muscle phosphorylase *b* to the debranching enzyme did not affect its reaction with Nbs_2 as followed both by loss of enzymatic activity or the rate of substitution of the SH groups.

Debranching Enzyme and the M-Line Protein. The M line of striated muscle contains a protein of molecular weight 160 000 (Masaki and Takaiti, 1974). Gel electrophoresis of the purified material showed the presence of a second component of molecular weight 90 000, which was identified as phosphorylase. This finding led to the obvious suspicion that the 160 000-dalton protein might be the debranching enzyme.

Dogfish skeletal muscle was first extracted and washed several times with 0.1 M KCl and the remaining structural proteins were dissolved in 0.6 M KCl–pyrophosphate buffer (modified Hasselbach–Schneider solution; Huxley and Hanson, 1957). While ca. 80% of the debranching enzyme was removed at low ionic strength, 20% remained with the contractile proteins. Ammonium sulfate precipitations, according to the procedure of Masaki and Takaita (1974), entirely separated the debranching enzyme from even crude fractions of M-line protein. This result was not too surprising, since the amino acid compositions of both proteins differ markedly.

Discussion

Dogfish muscle debranching enzyme was obtained in homogeneous form in ca. 50% yield by a three-step purification procedure. The initial ethanol precipitation does not appear to damage the enzyme: a control purification in which this step was replaced by adsorption on DEAE-cellulose yielded material of identical specific activity that displayed the same aggregation behavior in the ultracentrifuge. The specific activity of the purified enzyme (3.0 ± 0.5 units/mg at 19 °C) is of the same order of magnitude as that obtained for the rabbit enzyme (8–10 units/mg at 30 °C; Nelson et al., 1969; Brown et al., 1973), considering the difference in temperature at which the assays were carried out. Since dogfish normally live at 4 to 7 °C, it is not surprising that this enzyme, like most others isolated from this organism, is very unstable at temperatures above 20 °C.

The determination of the molecular weight by ultracentrifugation analysis posed a number of problems, due to the fact that the enzyme aggregates readily under certain conditions and appears to be rather asymmetric, complicating the interpretation of ultracentrifugation data. Indeed, the calculated molecular weight of the dogfish enzyme from sedimentation velocity experiments ($s_{20,w} = 6.5$) is 140 000 using the empirical formula of Halsal (1967) and as low as 100 000 according to the equation of Van Holde (1975) for globular proteins.

Sedimentation equilibrium analysis of the partially carboxymethylated but still enzymatically active protein yielded a molecular weight of 162 000, in good agreement with the value estimated from dodecyl sulfate gel electrophoresis. This molecular weight agrees closely with those recently reported for the rabbit enzyme, although the latter protein displays a higher sedimentation coefficient with $s_{20,w}$ values of 7.68 S, Nelson et al. (1969); 7.66 S, Brown et al. (1973); and 8.1 S, Taylor et al. (1975). A mixture of both enzymes yielded a single band on dodecyl sulfate gel electrophoresis. This similarity extends to their amino acid composition (see Table II), as indicated by a low divergence factor (Harris and Teller, 1973) of 0.035. This result is not totally unexpected: a previous comparative study of dogfish and rabbit phosphorylase indicated a degree of isology of the order of 85–90% between the two enzymes on the basis of their amino acid compositions (divergence factor of 0.026), and partial amino acid sequences, corresponding to no more than 1.5 mutations per 100 residues per 100 million years of divergence of the two species (Cohen et al., 1973). This remarkably low rate of evolution was attributed to the fact that phosphorylase is involved in a highly complex system of regulation and that much of its surface had to be devoted to either control or catalysis and hence had to be conserved. A similar degree of conservation for the debranching enzymes might imply that it is subjected to similar constraints and, therefore, that it might also play an important role in the structure of glycogen or the control of its metabolism. The debranching enzyme displays both 4-α-glucanotransferase and amylo-1,6-glucosidase activities (Brown and Illingworth, 1964; Brown and Illingworth-Brown, 1966; Nelson et al., 1969), which might also be responsible for its large size. The protein could not be dissociated under various denaturing conditions, including total substitution of its sulfhydryl groups. The fact that trypsin cleaves only a small fragment makes it unlikely that the enzyme is composed of multiple identical subunits. But it does not discount the possibility that the enzyme might be the product of the fusion of two genes each coding for a specific function, a situation resembling the aspartokinase–homoserine dehydrogenase system of Cohen (1969).

The enzyme did not appear to be affected by the usual effectors of glycolysis; no phosphorylation could be demonstrated. One must, therefore, assume that the enzyme exists exclusively in an active state, and, hence, that phosphorylase is always provided with α-1,4-polyglucan chains of sufficient length to carry out its enzymatic function. Dogfish contains 1.25 g of phosphorylase/kg of muscle (Cohen et al., 1971) with a specific activity of ca. 10 μmol of glc-1P produced min^{-1} mg^{-1} of protein at pH 7.0, 19 °C; this would allow for the release of 12 500 μmol of glc-1P min^{-1} kg^{-1} of muscle. The concentration of debranching enzyme is 185 mg/kg with a specific activity of 2.5 μmol of glucose released min^{-1} mg^{-1} at pH 7.0, 19 °C, which enables the transfer of 460 oligosaccharide fragments min^{-1} kg^{-1} of muscle. Hence, each transfer (coupled with the disappearance of an α-1 $\rightarrow$ 6 branch point) generates a new segment with a mean chain length of ca. ten α-1,4-glycosyl residues susceptible to being attacked by phosphorylase. Therefore, the potential activity of the two enzymes ($12\,500/4600 = 2.7$) is of the same order of magnitude, implying that even if the debranching enzyme were rate limiting, no large slow down of glycogen degradation would result. Furthermore, from the maximum amount of lactic acid produced (ca. 30 μmol min^{-1} ml^{-1} of intracellular water), it has been concluded that only a fraction (ca. 10%) of the total phosphorylase activity is expressed even under conditions of strong contraction (for review, see Fischer et al., 1971).

Besides its catalytic activity, the debranching enzyme might also serve a structural function. Its tight binding to glycogen and the filamentatous precipitate that results when solutions of both are mixed indicate a multivalent type of interaction, reminiscent perhaps of the action of lectins. The debranching enzyme may thus play a distinct role in the structure of glycogen particles (Meyer et al., 1970) in which it is present as a major component (Nelson et al., 1972; Taylor et al., 1975). It has been previously reported (Heilmeyer et al., 1970, Haschke et al., 1970; Nelson et al., 1972) that the enzymes included in such particles react differently than those in solution. While no direct interaction of debranching enzyme with phosphorylase could be demonstrated here, it might, nevertheless, influence the activity of other enzymes in the protein–glycogen complex and, therefore, should be included in attempts to study the regulation of glycogen metabolism in reconstituted systems.

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References

Abdullah, M., and Whelan, W. J. (1963), *Nature (London) 197,* 979.

Ashwell, G. (1966), *Methods Enzymol. 8,* 85.

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.

Bergmeyer, H. U., and Klotzsch, H. (1963), Methods of Enzymatic Analysis, Bergmeyer, H. U. Ed., New York, N.Y., Academic Press, p 131.

Brown, D. H., Gordon, R. B., and Illingworth-Brown, B. (1973), *Ann. N.Y. Acad. Sci. 210,* 238.

Brown, D. H., and Illingworth, B. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1783.

Brown, D. H., and Illingworth, B. (1964), in Control of Glycogen Metabolism, Whelan, W. J., and Cameron, M. P., Ed., London, Churchill, p 139.

Brown, D. H., Illingworth, B., and Cori, F. C. (1963), *Nature (London) 197,* 980.

Brown, D. H., and Illingworth-Brown, B. (1966), *Methods Enzymol. 8,* 515.

Cohen, G. N. (1969), *Curr. Top. Cell. Regul. 1,* 184.

Cohn, E. J., and Edsall, J. T. (1943) in Proteins, Amino Acids and Peptides, Van Nostrand-Reinhold, Princeton, N.J.

Cohen, P. (1973), *Eur. J. Biochem. 34,* 1.

Cohen, P., Duewer, T., and Fischer, E. H. (1971), *Biochemistry 10,* 2683.

Cohen, P., Saari, J. C., and Fischer, E. H. (1973), *Biochemistry 12,* 5233.

Davis, C. H., Schliselfeld, L. H., Wolf, D. P., Leavitt, C. A., and Krebs, E. G. (1967), *J. Biol. Chem. 242,* 4824.

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Fairbanks, G., Steck, T., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Fischer, E. H., Heilmeyer, L. M. G., and Haschke, R. H. (1971), *Curr. Top. Cell. Regul. 4,* 211.

Fischer, E. H., and Stein, E. A. (1961), *Biochem. Prep. 8,* 27.

Habeeb, A. F. S. A. (1972), *Methods Enzymol. 25,* 457.

Halsal, H. B. (1967), *Nature (London) 215,* 880.

Harris, C. E., and Teller, D. C. (1973), *J. Theor. Biol. 38,* 347.

Haschke, R. H., Heilmeyer, L. M. G., Meyer, F., and Fischer, E. H. (1970), *J. Biol. Chem. 245,* 6657.

Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 4,* 1337.

Heilmeyer, L. M. G., Meyer, F., Haschke, R., and Fischer, E. H. (1970), *J. Biol. Chem. 245,* 6649.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.

Huxley, H. E., and Hanson, J. (1957), *Biochim. Biophys. Acta 23,* 229.

Keppler, D., and Decker, K. (1970), in Methoden der Enzymatischen Analyse, Vol. 2, Bergmeyer, H. U., Ed., Weinheim/Bergstr., Germany, Verlag Chemie, p 1089.

Krebs, E. G. (1966), *Methods Enzymol. 8,* 543.

Lee, E. Y. C., Carter, J. H., Nielsen, L. D., and Fischer, E. H. (1970), *Biochemistry 9,* 2347.

Lee, E. Y. C., and Whelan, W. J. (1971), *Enzymes, 3rd Ed. 5,* 191.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Malencik, D. A., Heizmann, C. W., and Fischer, E. H. (1975), *Biochemistry 14,* 715.

Masaki, T., and Takaiti, O. (1974), *J. Biochem. (Tokyo) 75,* 367.

Meyer, F., Heilmeyer, L. M. G., Haschke, R. H., and Fischer, E. H. (1970), *J. Biol. Chem. 245,* 6642.

Nelson, T. E., Kolb, E., and Larner, J. (1969), *Biochemistry 8,* 1419.

Nelson, T. E., White, R. C., and Watts, T. E. (1972), *Biochem. Biophys. Res. Commun. 47,* 254.

Pocinwong, S. (1975), Ph.D. Thesis, University of Washington, Seattle, Washington.

Shaw, M. C. (1971), *Anal. Biochem. 44,* 288.

Slein, M. W. (1963), in Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N.Y., Academic Press, p 117.

Taylor, C., Cox, A. J., Kernohan, J. C., and Cohen, P. (1975), *Eur. J. Biochem. 51,* 105.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164,* 66.

Thomas, J. A., Schlender, K. K., and Larner, J. (1968), *Anal. Biochem. 25,* 486.

Van Holde, K. E. (1975), *Proteins 3rd Ed., 1,* 225.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Weber, K., and Osborn, M. (1975), *Proteins 3rd Ed., 1,* 180.

White, R. C., and Nelson, T. E. (1974), *Biochim. Biophys. Acta 365,* 274.

White, R. C., and Nelson, T. E. (1975), *Biochim. Biophys. Acta 400,* 154.

Yphantis, D. A. (1964), *Biochemistry 3,* 297.