DEBRANCHING ENZYME FROM RABBIT SKELETAL MUSCLE; EVIDENCE FOR THE LOCATION OF TWO ACTIVE CENTRES ON A SINGLE POLYPEPTIDE CHAIN

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1. Introduction

Glycogen degradation in mammalian skeletal muscle is carried out by two enzymes. The sequential phosphorolysis of the outer α 1,4 linked glucosyl units to yield glucose 1-phosphate is catalysed by glycogen phosphorylase (EC 2.4.1.1) until four glucosyl units remain before an α 1,6 branch point. The continued action of phosphorylase then requires the elimination of the α 1,6 branch point, a reaction that occurs in two distinct stages. An oligo 1,4-1,4 glucan transferase (EC 2.4.1.25) first transfers a maltotriosyl unit from a donor to an acceptor chain, and the α 1,6 glycosidic bond which is exposed is then hydrolysed by an amylo 1,6 glucosidase (EC 3.2.1.33). The oligo 1,4-1,4 glucan transferase (termed transferase) and amylo 1,6 glucosidase (termed glucosidase) were found to copurify until an essentially homogenous protein species was obtained [1,2], suggesting that the two activities of the debranching system form an enzyme complex. We recently showed that debranching enzyme (mol. wt 164 000) had no subunit structure, suggesting it might be one of the few enzymes in which the two active centres are present on the same polypeptide chain [3]. In this paper, we demonstrate that by limited proteolysis of debranching enzyme, it is possible to almost completely inactivate the transferase component, while leaving the glucosidase component hardly unaffected, providing further evidence that there are two active centres on the single polypeptide chain.

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2. Materials and methods

Debranching enzyme was purified from rabbit skeletal muscle and stored at O°C in 50 mM glycerophosphate–2 mM EDTA–1 mM dithiothreitol, pH 7.0 (buffer A) at 15 mg/ml [3]. Glycogen phosphorylase *b* was prepared by the method of Fischer and Krebs [4] and free from traces of debranching enzyme activity by hydrophobic chromatography [3]. It was used both in Assay One described below, and also to make phosphorylase limit dextrin. Worthington Trypsin (TPCK treated) and chymotrypsin were obtained from Cambrian Chemicals Ltd., London, U.K. Electrophoresis in the presence of sodium dodecyl sulphate [5] was done using standard marker proteins as described previously [3,6]. High speed sedimentation equilibrium of debranching enzyme was performed with a Spinco Model E Analytical Ultracentrifuge [3].

2.1. *Measurement of debranching enzyme activity*

Assay one: The debranching of a phosphorylase limit dextrin was measured by coupling the reaction to phosphorylase, the latter activity being assayed by exploiting the difference is p*K* between the substrate inorganic phosphate and the product glucose 1-phosphate [3]. The incubation mixture comprised 3.0 ml of a solution containing 10 mM potassium phosphate, 100 mM KCl, 1.0 mM AMP, 0.1% limit dextrin and 0.2 mg/ml phosphorylase *b*, pH 6.80, thermostatted at 25.0°C. The reaction was initiated with rate limiting quantities of debranching enzyme (0.01–0.02 ml). After an initial lag period, the rate of decrease in pH becomes linear with time, and the rate of reaction was measured from this section of the recorder trace [3].

Assay two: This method makes use of the slight reversibility of the glucosidase reaction. 0.1 ml of a solution containing glycogen 48 mg/ml and [^{14}C]-glucose 12 mM in glycyl–glycine 50 mM, pH 7.2 was warmed at 30°C for two min. The reaction was initiated with debranching enzyme (0.02 ml) diluted in ice-cold glycyl–glycine pH 7.2 containing 15 mM mercaptoethanol, and the [^{14}C]glucose incorporated into glycogen was analysed after 15 min [3,7]. The answers were subtracted from reaction blanks carried through the procedure in an identical manner except for the substitution of enzyme by dilution buffer.

3. Results

3.1. *Effect of limited proteolysis on the structure and activity of debranching enzyme*

The activity of debranching enzyme was found to be very stable, despite the extremely long polypeptide chain of 164 000 daltons. It lost no activity upon storage for two months at 0°C, and was completely stable for 20 h at 25°C in buffer A, when measured by either Assay One or Assay Two. The activity was also rather resistant to proteolytic attack, but after prolonged incubation with trypsin or chymotrypsin (1% by weight), some inactivation was observed in Assay One, though very little in Assay Two (table 1). However, when the protease concentrations were raised ten fold, a clear distinction appeared between Assay One and Assay Two. Whereas 5% or less of the original activity was now measured by Assay One, there was a retention of 36% or 65% of activity in Assay Two with chymotrypsin and trypsin respectively (table 1).

Table 1
Effect of protease digestion on the activity of debranching enzyme

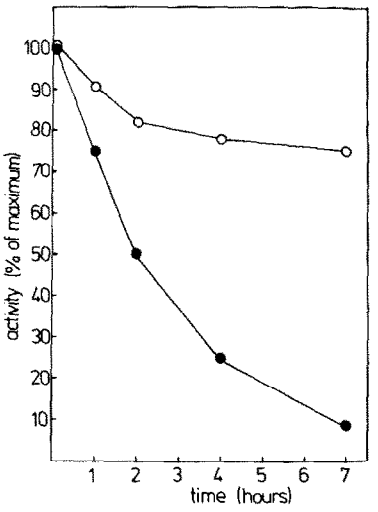
Protease	Debranching enzyme activity (%)	
	Assay One	Assay Two
None	100	100
Chymotrypsin (1%)	40	77
Trypsin (1%)	37	88
Chymotrypsin (10%)	5	36
Trypsin (10%)	4	65

Debranching enzyme, 5 mg/ml ($A^{1\%}_{280}$ = 17.5 [3]), was incubated with 0.05 mg/ml (1%) or 0.5 mg/ml (10%) protease for 20 h at 25°C. Activities of portions were then measured by Assay One and Assay Two. Control reactions done in the absence of protease showed no significant decrease in activity over this time, by either method. The traces of protease unavoidably carried over into Assay One, had no effect on the activity of the phosphorylase *b* present in this coupled assay.

Since the differential effect of proteolysis appeared to be greatest with trypsin, the time course of this reaction was investigated more carefully (figure 1A). As expected, activity declined much more rapidly in Assay One than in Assay Two, and after seven hours digestion 75% of activity remained in Assay Two, but only 10% in Assay One.

Dodecyl sulphate gel electrophoresis was done in order to see whether loss of activity in Assay One correlated with the cleavage of specific peptide bonds. After a one hour digestion with trypsin, although little activity had disappeared in either assay, extensive cleavage of the protein had nevertheless occurred, and protein staining material corresponding to the position of the native enzyme was no longer visible. Six major subfragments were observed, the largest of which possessed a mol. wt of 82 000 (fig.1B). Although most of the activity measured by Assay One disappeared between 1 and 7 h, no new subfragments appeared and all that was observed was a gradual disappearance of the six major protein staining bands obtained after one hour. This suggests that the loss of activity is associated with further extensive cleavage of the polypeptide chain to yield fragments that are too small to be detected by gel electrophoresis, after disaggregation in dodecyl sulphate.

The effect of tryptic digestion on the size of debranching enzyme in the absence of denaturing solvents was also investigated. Debranching enzyme emerged from a Sephadex G-200 column as a single symmetrical peak at a V/V_0 of 1.5 (not illustrated). The peak tube analysed by high speed sedimentation equilibrium centrifugation showed a homogeneous distribution and a mol. wt of 157 000 (fig.2), in close agreement to that determined previously [3]. Debranching enzyme digested with 10% trypsin for 7 h was also subjected to gel filtration. Most of the 280 nm absorbing material and all the activity measured by either Assay One or Assay Two co-eluted as a single symmetrical peak at a V/V_0 of 1.6, i.e. little different from the untreated enzyme (not illustrated). The peak tube, which possessed 70% of



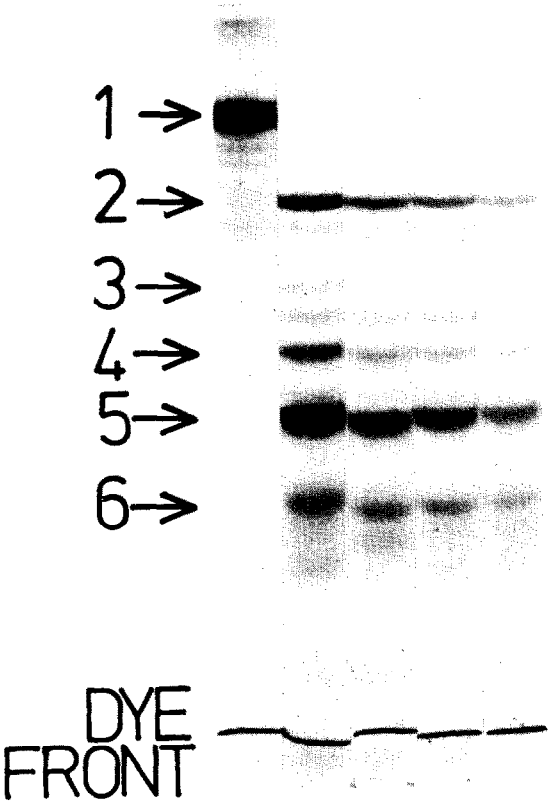


Fig.1. Effect of trypsin on the structure and activity of debranching enzyme. (A)Debranching enzyme, 5 mg/ml, was incubated with trypsin, 0.5 mg/ml, at 25° C. At various time intervals, portions were removed and tested for activity by Assay One (■) and Assay Two (□). (B)Dodecyl sulphate gel electrophoresis of debranching enzyme on 7.5% acrylamide gels, done at each time point after addition of trypsin, 5 μg of protein was applied to each gel. Component 1 is native debranching enzyme (160 000 daltons), and components 2 to 6 are the major subfragments derived by limited proteolysis. The estimated mol. wts of the bands are: band 2, 82 000; band 3, 60 000; band 4, 51 000; band 5, 41 000; band 6, 32 000. The faint minor components at time = 0 which amount to less than 3% of the total protein are trace degradation products of debranching enzyme, and appear upon prolonged storage, presumably as a result of the action of traces amounts of endogenous proteases. Migration is from top to bottom.

the specific activity of the untreated enzyme in Assay Two, and 15% of the specific activity in Assay One, again showed a homogeneous distribution when analysed by high speed sedimentation equilibrium centrifugation. However the mol. wt had now decreased to 122 000 (fig.2). Since this fraction still showed all the six major subfragments upon dodecyl sulphate electrophoresis, the 20% decrease in molecular size presumably results from the removal of small peptides too small to be observed by gel electrophoresis. The results also demonstrate that the six major fragments obtained by tryptic digestion still interact with one another at pH 7.0 in the absence of denaturing conditions.

4. Discussion

The results given in table 1 and fig.1 indicate that Assay One and Assay Two measure different catalytic parameters of debranching enzyme. Assay One must certainly measure transferase activity, and in all probability measures a combination of both transferase and glucosidase activities. The inactivation of

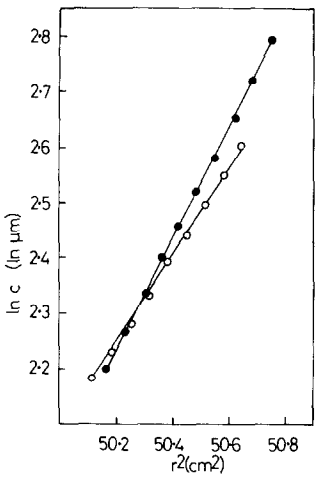


Fig. 2. High speed sedimentation equilibrium centrifugation of debranching enzyme. In sodium glycerophosphate buffer, pH 7.0, containing 2.0 mM EDTA and 7 mM mercaptoethanol. (●—●)Native enzyme mol. wt, 157 000. (○—○Enzyme after 7 h digestion with 10% trypsin and gel filtration on Sephadex G-200, mol wt, = 122 000. The plots of log fringe displacement against the square of distance from the centre of rotation were obtained from interference patterns photographed after 24 h at 16 000 rev/min. The initial protein concentrations were 0.25 mg/ml.

debranching enzyme measured in Assay One can therefore not by itself be ascribed to the loss of just one of the two activities. However, under the same conditions the activity measured by Assay Two only decreased slightly. Since the glucosidase component must be functional for Assay Two to work, the results indicate that inactivation in Assay One must have resulted from a specific loss of transferase activity. The data then further suggest that Assay Two is indeed a highly specific assay for glucosidase, and that contrary to previous suggestions [8], this reaction does *not* require transferase action to cover the added [^{14}C]-glucose units so that they are retained in the glycogen. The specific loss of one of the two activities of debranching enzyme provides strong evidence that there are two active centres on the single polypeptide chain. If it is assumed that the structures of human and rabbit muscle debranching enzymes are analogous, then six cases of glycogen storage disease type IIIB, in which glucosidase was normal but transferase was absent [9] lends further credence to this theory.

It is well established in aspartokinase-homoserine dehydrogenase of *E. coli* K 12, that the aspartokinase activity and the site for feedback inhibition by L-threonine are located in the N-terminal portion, and the homoserine dehydrogenase activity in the C-terminal portion of a single polypeptide chain of 86 000 daltons [10]. Similarly, in DNA polymerase of *E. coli* B, the 5′-3′ exonuclease is located in the N-terminal 30% of the chain, while the polymerase and 3′-5′ exonuclease are located in the C-terminal 70% of the 109 000 dalton chain [11]. In these examples, the conclusions were based on chemical analysis of both chain termination mutations in which only the N-terminal portion and consequently just one of the activities was synthesized, and also by limited proteolysis of the enzymes. In the former example, chymotryptic attack destroyed aspartokinase activity, and generated a C-terminal fragment of 55 000 daltons which contained just the homoserine dehydrogenase activity. In the latter example, subtilisin cleaved the molecule into two fragments, each with their distinctive activities, which were readily separable from one another by gel filtration or ion exchange chromatography. In these classical systems, the chain appears to fold into two distinct globular domains from the N-terminus of the protein, each retaining its own independent activity when cleaved from the other. It would be interesting to know whether the situation is analogous in debranching enzyme, since this appears to be the first example of a mammalian polypeptide chain that is 'double headed'. However, the assignment of glucosidase or transferase to a specific region on the linear polypeptide chain has not yet proved possible. Although an 85–90% loss of transferase activity was associated with a 20% decrease in the size of debranching enzyme, the remaining 10–15% of transferase activity still emerged with the glucosidase activity from Sephadex G-200, in the 122 000 dalton component. No physical separation of the two activities has therefore yet been achieved.

Human debranching enzyme deficiencies have been classified into two major subgroups, type IIIA and IIIB, based on measurements carried out with muscle and liver extracts prepared after tissue biopsy [9]. Van Hoof and Hers [9] found that 34 of 45 patients were of type IIIA, characterised by a total absence of both transferase and glucosidase activity

in either muscle or liver. However, a further six patients were of type IIIB, in which although both transferase and glucosidase were totally absent in liver, only transferase was absent in muscle, glucosidase apparently being normal. If it is assumed that cases of type IIIB are single gene mutations (which is likely as debranching enzyme deficiency shows autosomal recessive inheritance) then the structural information on the muscle enzyme described in this paper allows at least one possible explanation for the molecular basis of the type IIIB deficiency. If glucosidase were located in the N-terminal and transferase in the C-terminal protion of the chain, then particular chain termination mutations might lead to the production of just glucosidase and not transferase activity. If such a fragment were stable in muscle but rapidly inactivated in liver, this would be consistent with the clinical measurements. The type IIIA subgroup might either be a series of amino acid substitutions which each simultaneously destroy both transferase and glucosidase, or alternatively a defect in a control gene required for the synthesis of this enzyme.

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