ATTACHMENT OF CARBOHYDRATE TO ENZYMES INCREASES THEIR CIRCULATORY LIFETIMES

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1. Introduction

In previous reports [1–6] we have described the coupling of a number of enzymes to cyanogen bromide-activated dextran to produce soluble enzyme–dextran conjugates. Enzymes modified in this way have stability properties that are superior to those of the corresponding native enzymes. Thus conjugation of catalase, α- and β-amylases, and trypsin with dextran decreased their susceptibility to heat inactivation [1–5]. Following conjugation, the stability of trypsin in the presence of protein denaturants such as urea and sodium dodecyl sulfate was markedly increased, and the enzyme became resistant to inhibition by naturally-occurring protease inhibitors [2,3]. Enzyme–dextran conjugates were also shown to be more resistant than the corresponding native enzymes to proteolytic inactivation, both autolytic [2,3] and by added exogenous proteases [5]. We have now investigated the behavior of two synthetic enzyme–dextran conjugates in experimental animals with a view to determining whether enzymes modified by attachment of carbohydrate are likely to have properties making them more useful than unmodified enzymes for the therapy of metabolic disorders that are responsive to administration of exogenous enzymes. In this report the effect of attachment of dextran on the in vivo behavior of *Bacillus amyloliquefaciens* α-amylase and bovine liver catalase is described. Our findings suggest a new approach to increasing the duration of therapeutic usefulness of certain enzymes used for treatment of metabolic disorders. A preliminary account of this work has been published [7].

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2. Materials and methods

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1.) was a 4-times crystallized preparation from *Bacillus amyloliquefaciens* supplied by Sigma Chemical Company, St. Louis, MO (sold as *Bacillus subtilis* α-amylase). Beef liver catalase (hydrogen peroxide : hydrogen peroxide oxidoreductase, EC 1.11.1.6) was also from Sigma. Dextrans (av. mol. wt approx. 10^6 and 60 000–90 000) were from Fisons Ltd. (Loughborough, England) and Sigma, respectively.

α-Amylase activities were determined by measuring the rate of release of reducing sugars from soluble starch at 37°C and pH 5.5, using a copper reducing power method [8]. During the determination of α-amylase activities in blood samples, deproteinization of enzyme digests using barium hydroxide and zinc sulfate [9] was necessary prior to measurement of reducing sugars released by enzyme action. One unit (U) of α-amylase activity is the amount that releases 1 μmol reducing sugars/min from soluble starch under the above conditions. Specific activities are expressed as U/mg protein.

Catalase activity was measured spectrophotometrically [10]. Digests (3.0 ml) contained hydrogen peroxide (2.9 ml 11.8 mM hydrogen peroxide solution in 0.05 M phosphate buffer, pH 7.0) and 0.1 ml suit-

ably diluted enzyme solution. The time taken for the absorbance of the mixture to decrease from 0.45–0.40 (i.e., from a hydrogen peroxide concentration of 10.3–9.2 mM) was measured and used to calculate enzyme activity. One unit (U) of catalase brings about the decomposition of 1 μmol hydrogen peroxide/min at 25°C. Specific activities are expressed as U/mg protein.

Soluble conjugates of α-amylase and catalase with dextran (mol. wt 10^6 and 60 000–90 000, respectively) were prepared by treatment of the polysaccharide under carefully controlled conditions with cyanogen bromide, followed by interaction of the enzyme with the activated dextran under appropriate conditions (α-amylase, pH 9.0, 4°C, 24 h; catalase, pH 8.0, 23°C, 20 h). After conjugation, traces of residual unconjugated enzyme were removed from the products by gel chromatography on Sephadex G-100 (α-amylase) or Ultrogel AcA 34 (catalase), or by using concanavalin A–Sepharose [11]. The relative amounts of carbohydrate and protein in both conjugate preparations, on a weight basis, were nominally 10 : 1. The conjugated enzyme preparations had specific activities of 170 U/mg (α-amylase) and 12 000 U/mg (catalase) compared with the specific activities of the corresponding native enzymes which were 400 U/mg and 17 000 U/mg, respectively.

3. Results and discussion

To investigate the circulatory lifetime of α-amylase the native and conjugated forms of the enzyme were administered to rats; in these animals the level of circulatory α-amylase is low and it has a different pH optimum than does the bacterial enzyme, allowing specific determination of the administered enzyme without difficulty. Catalase could not conveniently be tested in rats because of the high endogenous blood catalase levels; resort was therefore made to using acatalasemic mice in which blood catalase levels are very low [12].

Samples of native and conjugated *Bacillus amyloliquefaciens* α-amylase (approx. 400 U α-amylase activity; 1 mg protein in the case of native enzyme and 2.5 mg protein in the case of the conjugated enzyme) were administered to rats (av. wt approx. 200 g) via their tail veins. Blood samples were removed from the tail veins of the animals at intervals over the period of 2 h and assayed for bacterial α-amylase activity. A similar procedure was followed in the case of catalase in the acalasemic mice; samples of native and conjugated enzyme (approx. 12 000 U catalase; 0.7 mg protein in the case of native enzyme and 1 mg protein in the case of conjugated enzyme were administered to the animals (av. wt approx. 30 g) via their tail veins and catalase levels in blood samples removed from the animals over the period of 2 h were measured. In addition, the catalase–dextran conjugate and native catalase (approx. 50 000 U catalase; 3 mg or 4 mg enzyme protein) were administered intraperitoneally to acatalasemic mice, followed by measurement of blood catalase levels over the period of 24 h.

Injection of both native enzymes and their dextran conjugates directly into the circulation of the experimental animals resulted in initially high blood enzyme levels, which decreased with time. However, the conjugated enzymes were, in both cases, removed at markedly lower rates than were the native enzymes. Thus 2 h after injection into rats, 75% of the conjugated α-amylase remained in the blood, but only 16% of the native enzyme was still present in the circulation (fig.1). Conjugated catalase also had a much

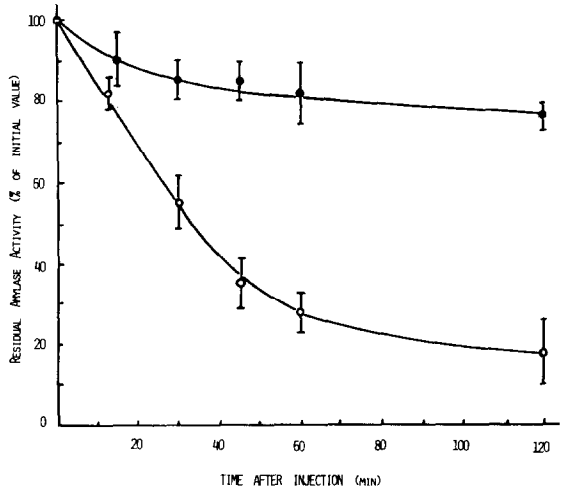

Fig.1. Removal of *Bacillus amyloliquefaciens* α-amylase from the circulation of rats after intravenous administration of native enzyme (o) or α-amylase–dextran conjugate (•). In both cases 400 U amylase activity (1 mg protein in the case of native amylase and 2.5 mg protein in the case of conjugated amylase) were injected. The average values and ranges for groups of three rats are shown.

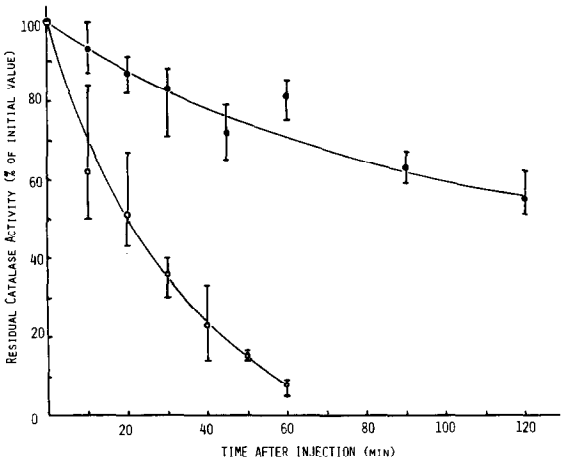

Fig.2. Removal of bovine liver catalase from the circulation of acatalasemic mice after intravenous administration of native enzyme (○), or catalase–dextran conjugate (●). In both cases 12 000 U catalase activity (approx. 1 mg protein) were injected. The average values and ranges for groups of four mice are shown.

greater circulatory lifetime than did the native enzyme. 60 min after intravenous administration to acatalasemic mice, 93% of the native enzyme had been removed, whereas the level of conjugated enzyme activity fell by less than 30% in the same time (fig.2). The half-lives of native and conjugated catalase were 17 min and 140 min. When injected intraperitoneally into acatalasemic mice, native catalase appeared in the blood stream rapidly, maximum activity levels being reached 2 h after injection, compared with the 4.5 h required to attain maximum blood catalase levels following intraperitoneal injection of the catalase–dextran conjugate. Following delivery by the intraperitoneal route, the maximum blood catalase level obtained with the conjugated enzyme was three times higher than that reached with native enzyme; the blood catalase level returned to normal within 4.5 h of injection of native enzyme but 22 h after administration of conjugated catalase the enzyme was still present at a level of 33% of the maximum level attained.

We have shown previously that conjugation of enzymes with dextran produces extremely high molecular weight aggregates containing many enzyme molecules and many dextran molecules, linked both intermolecularly and intramolecularly [3]. Thus, the decreased rate of passage of catalase from the peritoneal cavity into the circulation following conjugation with dextran would be expected simply on a molecular weight basis. What could not have been predicted with certainty was the finding that carbohydrate attachment extends the lifetimes of the enzymes in the circulation. Removal of proteins and glycoproteins from the circulation can take place by a number of mechanisms, including excretion in the urine, specific uptake from the blood by interaction of structural features in the macromolecules with cell-surface receptors, and non-specific uptake by the reticuloendothelial system, particularly the spleen and the Kupffer cells of the liver. The susceptibility of a protein to clearance in these different ways is likely to depend on its molecular properties such as structure, charge and molecular weight [13]. In the case of α-amylase there is substantial excretion of the native enzyme in the urine, suggesting that the increased circulatory lifetime is the result of decreased susceptibility of the conjugated enzyme to clearance by urinary excretion consequent to the increase in molecular weight caused by conjugation. In the case of catalase, however, which in its native form has a molecular weight outside the range of susceptibility to glomerular filtration, this effect is unlikely to be involved and some alternative explanation must be invoked to explain the increased circulatory lifetime. On the basis of molecular weight alone, a faster rate of clearance of the conjugated enzyme from the circulation might have been anticipated, because of increased susceptibility of the conjugate to be uptaken by the reticuloendothelial system [13]. However, previous studies [14] have indicated a relationship between the plasma half-life of a protein and its isoelectric point, the half-life increasing as the isoelectric point decreases, in accordance with other indications that uptake by cells of macromolecules is favored by their having cationic properties [13]. The same phenomenon may be involved in the present case, since conjugation of enzymes with cyanogen bromide-activated dextran results in a modification of surface lysine residues, presumably accompanied by a decrease in isoelectric point. An additional factor that may be involved in prolonging the circulatory lifetime of the modified enzymes is the effect of the attached carbohydrate chains serving

to 'disguise' the enzymes making them more compatible with the vascular system, less susceptible to the action of proteolytic enzymes, and preventing their recognition and uptake by the reticuloendothelial system. Further studies are in progress to investigate in detail the relative importance of molecular weight and charge properties of conjugated enzymes and the structure of the carbohydrate component in determining the circulatory lifetimes of enzyme-carbohydrate conjugates.

Extension of the circulatory lifetimes of proteins by coupling of dextran to them may have important practical applications, particularly from the point of view of increasing the therapeutic value of enzyme and non-enzymic proteins when their effectiveness depends on their retention in the circulation. For example, the efficacy of L-asparaginase as an anti-leukemia agent, and the antihemophilic action of Factor VIII both depend on their presence in the bloodstream [15,16] and both of these substances are rapidly cleared following administration to subjects under treatment. Attempts to increase the duration of therapeutic usefulness by using these, and other, proteins in the form of dextran conjugates would appear worthy of investigation.

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