COMPARISON OF THE ACTIVE SITES OF ALPHA AND BETA AMYLASES

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One attempt to catagorize enzymes according to mechanistic classes was made by Koshland (1953) who suggested that enzyme catalyzed reactions were essentially of two types which were designated as single and double displacement reactions. By analogy with known organic reactions it was proposed that enzymes in the two classes might alter the stereochemical course of a reaction by employing divergent reaction mechanisms. On this basis, it was suggested that the stereochemistry of an enzyme reaction might serve as a guide for placing enzymes in the proper reaction class.

Because there are so few enzymic reactions catalyzed at optically active centers, the relationship between substrate-product stereochemistry and reaction classes does not appear to have been investigated. In an attempt to make such a comparative study, we have incepted a program of research on two amylases.

The guiding assumption for the interpretation of the results of these studies proposes that enzymes operating by an identical mechanism at a common reaction center but with different stereochemistry would have identical catalytic amino acids. On the other hand, enzymes operating by different mechanisms would probably, but not necessarily, possess different catalytic amino acids (Koshland et al., 1958).

The subject enzymes, alpha amylase (hog pancreas) (Caldwell et al., 1952) and beta amylase (sweet potatoes) (Balls et al., 1948), have been reported to hydrolyze the alpha-1 → 4-glucosyl bonds of starch with retention and inversion of configuration, respectively (Freeman and Hopkins, 1936). It has also been demonstrated using ^{18}O isotopically enriched water that both of these enzymes rupture the C_1-O bond of starch during catalysis (Halpren and Liebowitz, 1959; Mayer and Larner, 1959).

In an attempt to establish the reaction class in which alpha amylase presided, the stereochemistry involved during hydrolysis was quantitatively investigated. Beta amylase is probably a single (back-side) displacement enzyme since it quantitatively produces products with inverted configuration (Thoma and Koshland, 1960a).

Experimental

Amylodextrin of average chain length 69 from acid hydrolysis of superlose (Stein and Hall) was isolated by acetone precipitation and was kindly supplied by Dr. J. A. Effenberger. The enzyme substrate was prepared by saturating a solution with the dextrin by boiling for 30 minutes in distilled water, cooling to room temperature, centrifuging and concentrating. The average chain length of the fractionated amylodextrin was 36 glucose units (Dimler et al., 1952; Nelson, 1944).

Optical rotation experiments to examine the stereochemical course of hydrolysis were initiated by adding 1 ml of alpha amylase (5 mg/ml) to 3 ml of substrate (1.68%) at pH 6.9 in 0.06 M phosphate and 0.08 M NaCl. The optical rotation of a 1 ml aliquot of the mixture to which 0.2 ml of water was added was followed as a function of time using a Rudolph precision polarimeter at the sodium D line. The observed rotation was extrapolated to "zero" time to assess the optical rotation immediately subsequent to hydrolysis but before the onset of mutarotation. To a second aliquot, 0.2 ml of concentrated ammonium hydroxide was added and the rotation of the completely mutarotated sample measured. The measured rotations were corrected for the presence of enzyme and ammonium hydroxide to give the rotation of the carbohydrates. Glucose and maltose, the sole reaction products, were quantitatively fractionated on a charcoal column (Miller, 1960) and assayed by the anthrone method (Dimler et al., 1952).

To test for the possibility that tyrosine is involved in alpha amylase activity, difference spectra of the enzyme in 0.05 M NaCl between pH 6.0 and 10.0 (0.06 M phosphate or carbonate buffers) were measured using alpha amylase at pH 6.0 in 0.06 M phosphate and in 0.05 M NaCl in the reference cell. The measurements were performed with a Cary Model 14 automatic recording spectrophotometer between 230 and 350 mμ. The total tyrosine content was estimated by the procedure of Hermans (1962). To investigate the possibility that alpha amylase contained phosphate which might be involved in catalysis, the phosphate content of the enzyme was determined by the method of Chen et al. (1956). A sample of protein of 5.6 mg was exhaustively dialyzed and employed for the assay.

Chloride free enzyme was prepared by three passages through a

42 X 1 cm column of G 25 Sephadex (Pharmacia Laboratories) in 0.01 M calcium acetate buffer at pH 6.9. A micro modification of the method of Bergman et al. (1957) indicated the level of chloride in these preparations was less than 10^{-8} M. The hydrolytic activity of the enzyme was measured by Nelson's (1944) modification of Somogyi's method (1938) using appropriate buffer blanks. V_m and K_m between pH 3.5 and 10.0 were evaluated by the method of Lineweaver-Burk (1934) in the presence and absence of 0.05 M NaCl. As a further probe of the binding site(s) of alpha amylase, inhibition by 0.8 X 10^{-2} M cyclohexaamylose was attempted.

Results and Discussion

The report that the hydrolysis products of alpha amylolysis of starch mutarotated downwards (Freeman and Hopkins, 1936) is consistent with any hydrolytic mechanisms which will produce either complete or partial retention of configuration. For example, if solvent reacts from above and below the ring of a free carbonium ion (one not bound to the enzyme) then a mixture of anomers would result. If this mixture contained 50% of the alpha species, the solution would mutarotate downward as the system approached equilibrium. However, partial retention of configuration was ruled out by following the course of mutarotation of the products of hydrolysis of amylodextrin. Assuming quantitative retention of configuration upon hydrolysis, the change on mutarotation was calculated to be 0.423° on the basis of the glucose and maltose formed which compares favorably to the observed change of 0.421° $\pm$ 0.013°. The agreement between the measured and calculated result assures that the stereochemistry is not altered upon hydrolysis. An enzyme promoting quantitative retention of configuration upon hydrolysis may operate either by a double or front-side displacement reaction. Consequently, the mutarotation data for alpha amylolysis of starch does not allow a definitive classification of the reaction mechanism. Using the same approach for beta amylase, it was established that this enzyme gave quantitative inversion of configuration upon hydrolysis of the glucosyl bond (Thoma and Koshland, 1960a). The observation that these enzymes either completely retain and invert configuration upon hydrolysis seems to convincingly preclude the possibility that the products are formed without the intervention of the enzyme after it has produced a carbonium ion or other reactive intermediate.

The amino acids involved in the activity of alpha amylase were assessed in part by measuring the influence of pH on the enzyme action. Plots of log V_m vs. pH for the amylase were typical triphasic curves

(Dixon and Webb, 1958) indicating enzyme action depended on groups with ionization constants of pK 4.6 and 6.7 for the chloride free and 5.3 and 7.8 for the chloride activated enzyme. Similar experiments with beta amylase indicated pK's of 3.75 and 7.0 (Thoma and Koshland, 1960b). Additional experiments on the beta enzyme pointed to carboxyl and imidazolium side chains as the catalytic amino acids (Thoma and Koshland, 1960b).

The pH difference spectra of alpha amylase in 0.05 M NaCl indicated no measurable ionization of tyrosine below pH 9.0 while phosphate determination revealed the molar ratio of enzyme to phosphate was 50 or greater. These experiments exclude both tyrosine and phosphate as the group with the alkaline pK responsible for alpha amylase activity. Since the enzyme is not inactivated by sulfhydryl reagents (Caldwell et al., 1945) and since the N-terminal can be altered without affecting activity (McGeachin, 1963), these groups also appear to be excluded from playing a catalytic role. The possibility that chloride is mandatory for hydrolysis can also be discarded because the chloride free preparation has 5% of the original activity at pH 6.9.

A reasonable hypothesis consistent with the data above is that imidazolium and carboxyl groups (Dixon and Webb, 1958) are responsible for catalysis of alpha amylase. Similar assignments have been made for other carbohydrases (Larner and Gillespie, 1955; Myrbäck, 1957; Neely, 1958; 1959; Ono et al., 1958). Although other possibilities for groups which have pK's near neutrality have been proposed (Bernhard, 1959; Erlanger, 1960; Porter et al., 1958), there does not appear to be good precedence for their occurrence in proteins.

Since the same type of catalytic groups promote the catalysis of alpha- and beta amylase (Thoma and Koshland, 1960b), it is tempting to conclude that the two enzymes operate by a common mechanism. If this conclusion is correct, the different stereochemistry of the products is then simply a consequence of the specific "guidance" of the water molecule involved in hydrolysis to the reaction center by the enzyme (Mayer and Larner, 1959). Therefore, alpha- and beta amylase are probably both single displacement enzymes, the former operating by a front-side and the latter by a back-side displacement reaction.

Although the catalytic side chains of the enzymes appear to be identical, experimental evidence indicates that the binding sites are distinctly different. The K_m of beta amylase shows very slight pH sensitivity but no dependence on dissociable groups between pH 3.5 and 9.0 (Thoma and Koshland, 1961) while the K_m of alpha amylase shows pH

variation with dependence on groups with ionization constants of pK 5.7 and 8.7. Although beta amylase is inhibited by cyclohexaamylose (K_i = 5 X 10^{-4} M) and by the internal segments of a starch chain (Thoma and Koshland, 1960c), alpha amylase is not detectably inhibited by 0.8 X 10^{-2} M cyclohexaamylose and hydrolyses the internal segments of starch chains. Assuming a precision of ± 5% for the enzymic assay, a ratio of the binding affinities of beta amylase to alpha amylase for cyclohexaamylose can be calculated to be at least 10^4.

The interpretations of the experimental results reported above harmonize well with Koshland's (1958) suggestion that the catalytic centers of enzymes promoting similar reactions might be the same. However, substrate specificity and the action pattern of these two amylases is independent of the catalytic site and probably dictated by the structure of the binding sites. A similarity in reaction mechanism between two enzymes promoting different stereochemistry is encouraging since such parsimony in nature will surely simplify the biochemists task of understanding enzyme action.

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