THE NUMBER OF BINDING SITES OF SWEET POTATO BETA AMYLASE

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Received June 7, 1963

The number of binding sites per molecule of enzyme is becoming of increasing interest because of its relation to the understanding of the biosynthesis of proteins, the efficiency of catalysis and the correlation of protein structure to function. Recently it was found (Thoma and Koshland, 1960a) that cyclohexaamylose competitively inhibits beta amylase and hence it seemed possible to use this compound for an equilibrium dialysis study on this high molecular weight enzyme.

To obtain highly pure sweet potato beta amylase, 50 mg of two times crystallized enzyme prepared by the procedures of Balls et al. (1948) were chromatographed on a 2 x 25 cm ECTEOLA cellulose (0.64 meq/g) column after purification on a dextran gel column (Thoma and Koshland, 1960b). The protein was placed on the cellulose column in a 0.0125 molar sodium phosphate buffer at pH 7.05 and eluted with a concave salt gradient of sodium chloride using the procedure of Peterson and Sober (1959). The first and second reservoirs contained 150 ml of eluting buffer and the third contained 150 ml of 0.75 molar sodium chloride in the phosphate buffer.

For the equilibrium dialysis experiments, purified beta amylase in 0.05 molar acetate buffer at pH 4.8 was dialyzed against radioactive cyclohexaamylose (0.056 μc/mg) at various concentrations in the acetate buffer. The cyclohexaamylose was prepared by the procedure of Andersen et al. (1962), and the dialysis procedure followed that of M. E. Koshland et al. (1962). To establish that the radioactive dextrin had equilibrated in the 44 hour interval of the experiments, a control was run in which acetate buffer replaced the enzyme solution. On measuring the radioactivity of the two compartments the counts were found to be the same within experimental error (ca. 2-4%) showing the establishment of equilibrium.

Radioactivity was measured by adding very slowly 50 μl aliquots to 1 ml of p-(diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide

(hydroxide of Hyamine 10-X purchased from Packard Instruments) and stirring vigorously by swirling while the aliquots were added to the vials. The contents of the vials were then mixed with 14 ml of a phosphor solution prepared by mixing one part of dioxane to 14 parts of a 0.3% solution of 2-phenyl-5-(4-biphenyl) 1,3,4-oxadiazole (PBD) in xylene. The samples were counted at -8 to -10° C in a liquid scintillation spectrometer for 10 minutes within 4-6 hours after preparation. The presence of beta amylase at a concentration of 90-100% of that used in the equilibrium dialysis experiments did not alter significantly the efficiency of counting (± 2-4%). Since a 1% error in quenching will be reflected by a 5-7% increase in the number of binding sites, it is possible that the actual value could be 10-28% greater than indicated from the Scatchard plots (cf. below). During dialysis the enzyme lost no activity.

Assurance that the enzyme had not hydrolyzed cyclohexaamylose during the period of equilibration was obtained in two ways. First, on chromatography of solutions after equilibrium dialysis, the only radioactive spot corresponded to cyclohexaamylose. Second, during the 72 hours of incubation at 25° C no significant increase in the amount of reducing materials was detected indicating that the dextrin was not hydrolyzed.

When the data are plotted on Scatchard plots (Scatchard, 1949), the results shown in Figure 1 are obtained. Since there appears to be some doubt about the molecular weight of native beta amylase*, the data are plotted in terms of the monomer weight of 50,000. The intercepts on the ordinate of Figure 1 give the number of binding sites in moles per 50,000 g of beta amylase. This is seen to be 1.0 within experimental error. Thus using the published value for the molecular weight of 152,000 (Englard and Singer, 1950), there are 3 sites per molecule of enzyme.

This weight per binding site is of considerable interest since the molecular weights of some alpha amylases are reported as 50,000 g (Fischer and Stein, 1960). Beta amylase apparently can be added to a growing list of proteins which are composed of subunits (Deal et al., 1963; Madsen and Cori, 1956; Frieden, 1958; Burk and Greenberg, 1930; Townend et al., 1961; Kagi and Vallee, 1960; Saddi et al., 1961).

* A personal communication from M. Burr and D. Yphantis indicates a molecular weight of 215,000 for the native enzyme and 50,000 for the depolymerized enzyme using equilibrium ultracentrifugation in contrast to a reported figure of 152,000 for the native molecule (Englard and Singer, 1950).

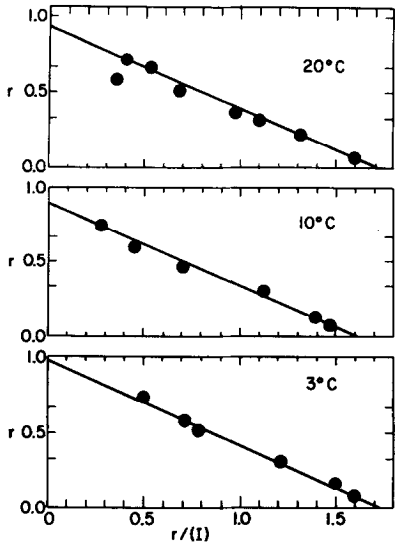

Figure 1. Scatchard plot of Equilibrium Dialysis Data for C^{14}-Labeled Cyclohexaamylose-Beta Amylase System. 0.05 M acetate buffer, pH 4.8, 8 x 10^{-5} M beta amylase, 5-50 x 10^{-5} M cyclohexaamylose (0.56 μc/mg), temperature as indicated. Symbol r represents ratio of moles of inhibitor bound per 50,000 g of enzyme and (I) represents molar concentration of free inhibitor.

From the slope of the Scatchard plots, the dissociation constant of the inhibitor-amylase complex was calculated to be $1.7 \pm 0.1 \times 10^{-4}$ M and is considered to be equal to this value within experimental error at the three temperatures investigated. The dissociation constant determined from the dialysis experiment is in reasonable agreement with the value, $2.15 \pm 0.15 \times 10^{-4}$ M determined kinetically (Thoma and Wellner, 1962) at 2.5°, 10.5°, and 20° C, respectively, using as substrate, sodium chlorite-oxidized amylodextrin of 23 residues average length. The agreement between the inhibitor constant from dialysis and the Michaelis constant (Thoma and Koshland, 1960a) suggests that the Michaelis constant is approximately equal to the binding constant for substrate (a non-reducing end of a starch chain).

A significant feature of the Scatchard plots is their linearity which is testimony that the binding sites are acting independently at least in the concentration region of inhibitor studied. The homogeneity and independence of the binding sites is also indicated by the linearity of the Lineweaver-Burk and Dixon plots both for a starch substrate and for the amylodextrin substrate (Thoma and Koshland, 1960a; Bailey and French, 1957; Thoma and Koshland, 1960b).

The observation that there is one inhibitor site per monomer molecule of protein makes it tempting to postulate that the native enzyme possesses the same number of catalytic centers although this finding per se does not constitute a proof of this postulation. The possibility of three binding sites per 150,000 g of enzyme all of which are not catalytic seems improbable but the possibility cannot be positively discarded. Normal Michaelis-Menten kinetics would be followed as long as the binding sites are acting independently (Reiner, 1959). It has been reported that RNAase has two homogeneous and independent binding sites for 5'-cytidylic acid (Schellman, 1963) but only one catalytic site (Gundlach et al., 1959; Barnard and Stein, 1959; Hirs et al., 1960). An analogous situation for the beta amylase and cyclohexaamylose system, however, does not seem warranted for the following reasons: (a) The cyclic dextrin is a large molecule, larger than the smallest rapidly hydrolyzed substrate, maltotetrose, while the nucleotides tested with RNAase are analogs of the split products. (b) Cyclohexaamylose is bound as tightly as the substrate (assuming $K_m \cong K_s$) whereas this is not true for the nucleotide system (Schellman, 1963). (c) Because of the rigidity of the cyclic dextrin (French, 1957), steric obstructions would seem to hinder a tandem arrangement.

To add further support to this contention, inhibition studies of beta amylase with the cycloamyloses were performed. According to Johnson et al., (1942), a plot of log $(\frac{V_o - V_i}{V_i})$ vs. log [I] where V_i and V_o are, respectively, velocities in the presence and absence of inhibitor, I, will lead to linear plots of slope n. The value of n corresponds to the number of moles of inhibitor bound per active site. Since the measured values of n were found to be 0.93 and 0.98 for cyclohexa- and cycloheptaamylose, respectively, one molecule of inhibitor is effective in blocking one catalytic site. For these reasons, it appears likely that there is one catalytic site per 50,000 g of beta amylase.

Acknowledgement

Part of this research was conducted at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission. It was also supported in part by grant RG 8500 of the National Institutes of Health and by a grant from Corn Industries Research Foundation.

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