# Mechanism of Maltal Hydration Catalyzed by β-Amylase: Role of Protein Structure in Controlling the Steric Outcome of Reactions Catalyzed by a Glycosylase†

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*Received February 5, 1991; Revised Manuscript Received April 17, 1991*

ABSTRACT: Crystalline (monomeric) soybean and (tetrameric) sweet potato β-amylase were shown to catalyze the cis hydration of maltal (α-D-glucopyranosyl-2-deoxy-D-*arabino*-hex-1-enitol) to form β-2-deoxymaltose. As reported earlier with the sweet potato enzyme, maltal hydration in $D_2O$ by soybean β-amylase was found to exhibit an unusually large solvent deuterium kinetic isotope effect ($V_H/V_D = 6.5$), a reaction rate linearly dependent on the mole fraction of deuterium, and 2-deoxy-[2(a)-$^2$H]maltose as product. These results indicate (for each β-amylase) that protonation is the rate-limiting step in a reaction involving a nearly symmetric one-proton transition state and that maltal is specifically protonated from above the double bond. This is a different stereochemistry than reported for starch hydrolysis. With the hydration catalyzed in $H_2O$ and analyzed by gas–liquid chromatography, both sweet potato and soybean β-amylase were found to convert maltal to the β-anomer of 2-deoxymaltose. That maltal undergoes cis hydration provides evidence in support of a general-acid-catalyzed, carbonium ion mediated reaction. Of fundamental significance is that β-amylase protonates maltal from a direction opposite that assumed for protonating starch, yet creates products of the same anomeric configuration from both. Such stereochemical dichotomy argues for the overriding role of protein structures in dictating the steric outcome of reactions catalyzed by a glycosylase, by limiting the approach and orientation of water or other acceptors to the reaction center.

Reported findings, showing that different glycosylases convert prochiral substrates such as glycals or exocyclic enitols to products of the same anomeric configuration as formed from their glycosidic substrates, recently led us to propose (Chiba et al., 1988; Weiser et al., 1988; Hehre, 1989) that the steric outcome of glycosylase-catalyzed reactions is determined by protein structure and is not linked to the preexisting substrate configuration. This concept provides new insight into the mode of control of product configuration by glycosyl-mobilizing enzymes that have been described as "retaining" or "inverting" enzymes (Koshland, 1953; Thoma et al., 1963; Reese et al., 1968; Capon, 1969; Sinnott, 1990), with β-amylase generally cited as exemplifying the latter. The reported findings encompass reactions with glycals catalyzed by α- and β-glucosidases (Hehre et al., 1977; Chiba et al., 1988), β-galactosidase (Lehmann & Zieger, 1977), exocellulases (Kanda et al., 1986; Claeyssens et al., 1990), and glycogen phosphorylases (Klein et al., 1982; Klein & Helmreich, 1985), as well as reactions with exocyclic enitols catalyzed by β-galactosidase (Brockhaus & Lehmann, 1977; Schlesselmann, 1981), α- and β-glucosidases (Hehre et al., 1980; Schlesselmann et al., 1982; Weiser et al., 1988), and glycogen phosphorylases (Klein & Helmreich, 1985; Klein et al., 1986; Johnson et al., 1990). Comparable findings also have been described for enzymes that act on glycosidic substrates with inversion of configuration although, for these, the coverage is limited. Glucodextranase was shown to catalyze the hydration of D-*gluco*-heptenitol[1] (Hehre et al., 1980), and trehalase, the hydration of D-*gluco*-octenitol[1] (Weiser et al., 1988)—in each case forming a product of the same (β) configuration as formed on hydrolyzing dextran or α,α-trehalose. However, no study providing such comparison has been reported for an enzyme of this type reacting with a glycal, the endocyclic double bond of which would likely be oriented differently at an active site than the exocyclic double bond of a hept- or octenitol.

In a previous study (Hehre et al., 1986), the reactivity of sweet potato β-amylase in catalyzing the hydration of maltal[1] was described, but the anomeric configuration of the hydration product was not determined. Elucidation of the full stereochemistry of this reaction would provide a significant test of the concept of primarily topological control of product configuration, as well as an unusual opportunity to gain new basic understanding of how β-amylase functions as a catalyst. This enzyme, prototype of the various glycosylases that convert glycosidic substrates to products of inverted configuration, has

† This study was supported by Senior International Research Fellowship I-F06-TWO-I330 (to E.J.H.) from the National Institutes of Health; by Research Grants DMB 89-04332 from the National Science Foundation (to E.J.H.) and CA-16054 and Core Grant P30 CA-13330 from the National Cancer Institute, National Institutes of Health (to C.F.B.); and by NMR Instrumentation Grants I-S10-RR02309 from the National Institutes of Health and DMB-8413723 from the National Science Foundation.

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[1] Abbreviations: maltal, α-D-glucopyranosyl-(1→4)-2-deoxy-D-*arabino*-hex-1-enitol; GLC, gas–liquid chromatography; D-*gluco*-heptenitol, 2,5-anhydro-1,2-dideoxy-D-*gluco*-hept-1-enitol; D-*gluco*-octenitol, (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol; TMS, trimethylsilyl derivative.

0006-2960/91/0430-6769$02.50/0 

long been considered to be a single-displacement enzyme. It has been pictured as acting by direct displacement (Koshland, 1953, 1959; Sinnott, 1990), or by a carbonium ion mediated mechanism with a directed "backside" approach of water postulated to provide configurational inversion (Mayer & Larner, 1959; Koshland et al., 1962; Thoma et al., 1963, 1968, 1971). Evidence has, in fact, been lacking regarding what role substrate configuration and enzyme have in determining the steric outcome of reactions catalyzed by β-amylase.

The present investigation of the stereochemistry and mechanism of the maltal hydration reaction was undertaken with crystalline preparations of both tetrameric (sweet potato) and monomeric (soybean) β-amylase. As the three-dimensional X-ray crystallographic structure of the latter enzyme (complexed with α-cyclodextrin) has recently been resolved to 3 Å (Dr. Bunzo Mikami, Kyoto University, personal communication), we envisioned the possibility that maltal might provide a useful small substrate for investigating the enzyme's catalytic site structure and possible constraints on the approach of water to the reaction center. Present results are discussed with respect to defining the catalytic mechanism of the maltal hydration reaction, including the factors envisioned as determinants of the stereochemistry and their bearing on reactions catalyzed with other substrates by β-amylase. The findings are also discussed in connection with related results which have indicated that the process of glycosylase catalysis is separable into a "plastic" protonation phase and a "conserved" phase concerned with steric outcome.

## Materials and Methods

*β-Amylase.* Crystalline sweet potato β-amylase [955 μmol of maltose liberated from starch/(min·mg) at pH 4.8 and 20 °C] was purchased from Sigma as a suspension in ammonium sulfate. Dialysis against 0.05 M acetate buffer of pH 5.0 gave a stock solution of 16.8 mg (15 500 units)/mL. Crystalline soybean β-amylase (isozyme 2), 860 μmol of maltose liberated from amylopectin/(min·mg) at 37 °C and pH 5.4 (Morita et al., 1975; Mikami & Morita, 1988), was kindly provided by Professor Y. Morita (Kyoto University) as a solution in pH 5.4 buffer/glycerol 3:2 v/v). Before use, this was dialyzed against several changes of 0.05 M acetate buffer at pH 4.4 containing 5 mM mercaptoethanol. Concentrations (mg/mL protein) were estimated from the optical density at 280 nm of suitably diluted solutions at pH 4.4, assuming $E^{1\%}_{280} = 17$ (Mikami & Morita, 1988).

*Maltal.* Hexa-*O*-acetylmaltal, prepared as previously described (Hehre et al., 1986), was deacetylated with 0.03 M sodium methoxide in dry methanol (25 °C, 3 h). Pure maltal was recovered by chromatography on dry silica gel 60 with ethanol/ethyl acetate, 2:5, as solvent. Fractions showing a single spot on TLC were pooled and dried under vacuum. Maltal was kept (at −20 °C) as a 100 mM solution in dry methanol.

*2-Deoxymaltose Standards.* A sample of pure, anomerically equilibrated 4-*O*-α-D-glucosyl-2-deoxy-D-glucose was obtained as a product of the action of cyclodextrin glycosyltransferase on soluble starch plus 2-deoxy-D-glucose (Kitahata et al., 1974; Hehre et al., 1986). Its use as a control in connection with GLC and $^1$H NMR spectroscopic analyses of the products formed by the action of β-amylase on maltal are described under Results. A second 2-deoxymaltose sample, recovered in crystalline form from a digest of buckwheat α-glucosidase with maltose as donor and 2-deoxy-D-glucose as acceptor (Chiba et al., 1975), provided the basis for assigning the relative retention times of the trimethylsilylated derivatives of α- and β-2-deoxymaltose on GLC. Proof of the α-anomeric configuration of the crystalline compound was obtained by Chiba and Kimura (1990) as follows. A freshly prepared solution of the crystals on trimethylsilylation and GLC (with arabitol as internal standard) showed a predominant peak of $R_{arabitol}$ 1.93 plus a second (minor) peak with a slightly longer retention time, $R_{arabitol}$ 2.00. After anomerization (25 °C, 24 h) these two TMS-2-deoxymaltose peaks appeared nearly equal in size. To identify the anomer responsible for the predominant peak, 0.6 mg of the crystalline 2-deoxymaltose was treated briefly (1.7 min, 25 °C) with a dilute (1.5 μg/mL) solution of crystalline *Aspergillus niger* α-glucosidase (Chiba, 1988). A freeze-dried aliquot was trimethylsilylated and, on GLC, showed major product peaks with retention times corresponding to TMS-α-2-deoxy-D-glucose and TMS-α-D-glucose standards. Residual TMS-2-deoxymaltose appeared as a dominant early peak (α-anomer) and minor later peak (β-anomer) in roughly the same proportions as the untreated crystals.

*Gas–Liquid Chromatography.* Test samples (1 mg of carbohydrate) were lyophilized, then heated (70 °C, 15 min) in 100 μL of trimethylsilylating reagent (hexamethyldisilazane/trimethylchlorosilane/pyridine, Gasukuro Kogyo, Ltd., Tokyo), and examined (1-μL sample) with a Shimadzu Chromatopak C-R3A gas chromatograph and integrating recorder. The runs were isothermal at 245 °C.

*$^1$H NMR Spectra.* Spectra were recorded in $D_2O$ (99.75 atom % D) at 25 °C with a Varian 500-MHz spectrometer. Chemical shifts (ppm) refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt.

*Primary Deuterium Isotope Effect.* Two 0.45-mL samples of a dialyzed solution of crystalline soybean β-amylase (10.7 mg/mL) in 0.05 M acetate buffer of pH 4.4 were used. One was further dialyzed at 10 °C against two successive changes of the pH 4.4 buffer—each time for 24 h. The second sample was similarly dialyzed against two changes of 0.05 M acetate-$d_3$/NaOD buffered $D_2O$ at pD 4.4 to exchange the enzymes's $^1$H for $^2$H atoms. Following measurement of the absorbance at 280 nm with an aliquot of each dialyzed sample (diluted 1:30 with the pH 4.4 buffer) 50 μL of the pD 4.4 buffer was added to the enzyme in $D_2O$ to equalize the protein concentration (at 9.6 mg/mL) in both samples. For use, the enzyme in water was diluted to 1.6 mg/mL with the pH 4.4 buffer.

*Proton Inventory.* Two 0.50-mL samples of a solution of soybean β-amylase (12 mg/mL) at pH 4.4 were dialyzed (10 °C, 48 h)—one against three changes of $D_2O$ buffered at pD 4.4, the other against the pH 4.4 buffer. The enzyme in $D_2O$ (400 μL treated with 2.8 μL of 2-mercaptoethanol) had $OD_{280}$ = 18.67. The enzyme in $H_2O$ (150 μL) was treated with 3 μL of 2-mercaptoethanol and 305 μL of pH 4.4 buffer to give a solution containing one-third the protein concentration, $OD_{280}$ = 6.22.

## Results

In preliminary experiments, an isothermic (245 °C) GLC program was found to give clear separations of the trimethylsilylated derivatives of maltal and the anomers of 2-deoxymaltose from each other, and also to allow quantitative determination of each component in known mixtures of maltal plus low proportions of 2-deoxymaltose. Under these conditions, TMS-α-2-deoxymaltose emerged at $R_{maltal}$ 1.20; that of TMS-β-2-deoxymaltose, at $R_{maltal}$ 1.45. Reaction conditions were also found in which digests of maltal with either sweet potato or soybean β-amylase in aqueous buffers yielded hydration products in measurable amounts (by GLC) after very brief incubation. Hydration catalyzed by the soybean enzyme

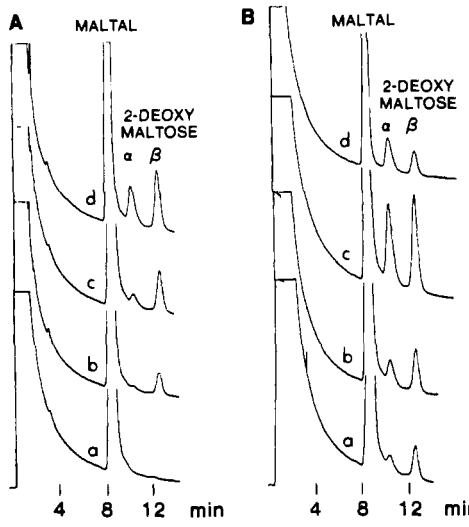



FIGURE 1: (A) GLC records of trimethylsilyl derivatives of (a) 60 mM maltal in pH 5.0 buffer, 40 °C, 10 min, and (b–d) components of reaction mixtures of 60 mM maltal with 16.8 mg (15 500 units)/mL crystalline sweet potato β-amylase, incubated at pH 5.0 and 40 °C for 1, 3, and 10 min, respectively. (B) GLC records of trimethylsilylated derivatives of (a–c) components of reaction mixtures of 60 mM maltal with 9.05 mg (7800 units)/mL crystalline soybean β-amylase incubated at pH 4.4 and 40 °C for 1, 5, and 15 min, respectively, and (d) a mixture of 20 mM maltal plus 3 mM anomerically equilibrated 2-deoxymaltose standards at pH 4.4.

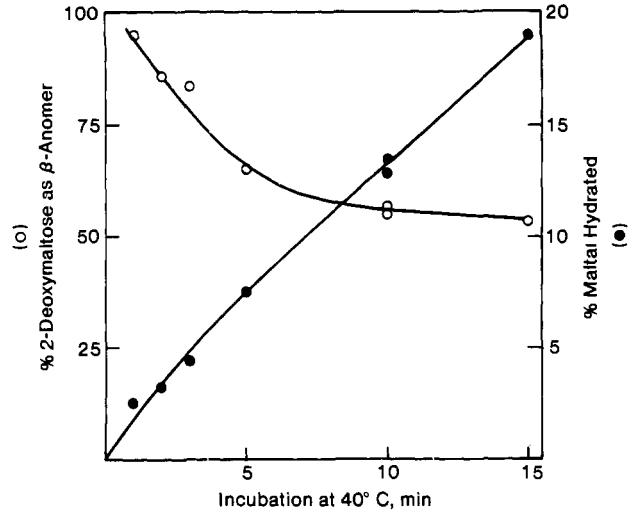



FIGURE 2: Time course of hydration of 60 mM maltal by 16.8 mg (15 500 units)/mL crystalline sweet potato β-amylase at pH 5.0 and 40 °C. (●) % (mM) of maltal hydrated; (○) % of the 2-deoxymaltose hydration product present as the β-anomer ($R_{maltal}$ 1.455). Data points from integration of peak areas in GLC records.

was noted to proceed much more slowly in $D_2O$ than in $H_2O$, as reported earlier (Hehre et al., 1986) for the reaction with sweet potato β-amylase.

In order to determine the steric outcome of the maltal hydration reaction, replicate 0.21-mL samples of 100 mM maltal in methanol were dried under vacuum in small test tubes and then warmed to 40 °C. To examine the sweet potato β-amylase, each of a series of such samples was treated with 0.35 mL of enzyme [16.8 mg (15 500 units)/mL in 0.05 M acetate buffer of pH 5], also prewarmed to 40 °C. A sample of maltal in buffer alone was included as a control. Individual test mixtures were held at 40 °C for 1, 2, 3, 5, 10, or 15 min, and then a 50-μL sample was quick-frozen in a bath of solid $CO_2$/acetone and stored at −20 °C until the series was assembled. All samples were then dried from the frozen state in a high vacuum lyophilization apparatus, subjected to trimethylsilylation, and examined by GLC. A comparable experiment was carried out with crystalline soybean β-amylase [9.05 mg (7800 units)/mL] in 0.05 M acetate buffer at pH 4.4 containing 5 mM mercaptoethanol.

Figure 1A illustrates the GLC patterns for the TMS derivatives formed: (a) with the maltal/buffer control (40 °C, 10 min); (b–d) with maltal/sweet potato β-amylase digests incubated at 40 °C for 1, 3, and 10 min, respectively. In (a) the peak of TMS-maltal alone is present; in test mixtures (b–d), two new peaks at $R_{maltal}$ 1.20 and 1.45 are found that correspond, respectively, to derivatized α- and β-2-deoxymaltose. At 1 min (b), the $R_{maltal}$ 1.45 peak of the β-anomer is predominant with only a trace of the α-anomer present. On longer incubation (c and d; 3 and 10 min) the signals of both TMS-2-deoxymaltose anomers are increased, with that at $R_{maltal}$ 1.45 representing the primary enzymic product, β-2-deoxymaltose, remaining dominant.

A similar pattern was found (Figure 1B) for the hydration of maltal by soybean β-amylase. Scan a, of a digest incubated for 1 min at 40 °C, shows the predominance of TMS-β-2-deoxymaltose over TMS-α-2-deoxymaltose; scans b and c show its continued dominance during 15 min at 40 °C. Integration of the signals recorded (scan c) for the 15-min digest shows the β-anomer to represent 55% of all derivatized 2-deoxymaltose. This proportion is still clearly above the 46% of β-anomer found present in a concurrent control of 3 mM anomerized 2-deoxymaltose plus 20 mM maltal in buffer (Figure 1B-d).

Figure 2 illustrates the time course of the hydration of 60 mM maltal catalyzed by 16.8 mg/mL sweet potato β-amylase at 40 °C and pH 5.0. The percent of substrate hydrated and the percent of 2-deoxymaltose present as the β-anomer were calculated from the integrated areas of peaks in the individual GLC records. Under the test conditions, which provided for nearly 20% hydration of maltal in 15 min, the proportion of 2-deoxymaltose present as the β-anomer declined from 95% at 1 min to 54% at 15 min, reflecting substantial but incomplete nonenzymic anomerization of the primary enzymic product.

*Studies of Maltal Hydration in $D_2O$ Catalyzed by Soybean β-Amylase.* Experiments were carried out to learn the extent to which the previously reported behavior of sweet potato β-amylase in hydrating maltal in $D_2O$ (Hehre et al., 1986) would apply qualitatively and quantitatively to the reaction catalyzed by soybean β-amylase.

To determine the direction of protonation of maltal, a test mixture was prepared comprising 100 μmol of maltal freshly dried from solution in methanol plus 1.0 mL (10.7 mg) of soybean β-amylase that had been exhaustively dialyzed against 0.05 M acetate-$d_4$ buffer of pD 4.4 to exchange its $^1H$ for $^2H$ atoms. The test mixture was incubated at 40 °C for 6 h and then subjected to preparative paper chromatography (Hehre et al., 1986) along with a 2-deoxymaltose standard. The hydration product (6.9 mg) was recovered as a white amorphous powder. For analysis by NMR spectroscopy, it was dissolved in a small volume of $D_2O$, dried under vacuum, and finally redissolved in $D_2O$ to give a 30 mM solution.

$^1H$ NMR spectra at 500 MHz of the recovered product and control compounds are presented in Figure 3. Spectrum T is of the 2-deoxymaltose-$d_1$ product from the test digest of maltal in $D_2O$ by soybean β-amylase. It is best compared with spectrum C (both recorded at 25 °C) of anomerically equilibrated protio 2-deoxymaltose synthesized by cyclodextrin glycosyltransferase from starch and 2-deoxy-D-glucose. Both T and C show a signal at 5.36 ppm, representing the H′-1

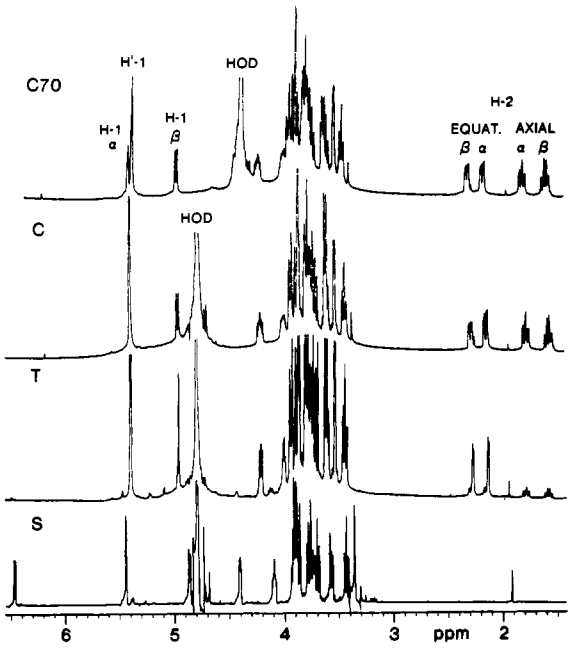



FIGURE 3: $^1$H NMR spectra recorded in $D_2O$ at 500 MHz (at 25 °C unless otherwise noted). S, 30 mM maltal; T, 30 mM anomerized 2-deoxymaltose-$d_1$ isolated from a digest of maltal by soybean β-amylase in $D_2O$; C, 25 mM anomerically equilibated 2-deoxymaltose synthesized by cyclodextrin glycosyltransferase acting on starch and 2-deoxy-D-glucose; C70, same compound as in C, but spectrum recorded at 70 °C.

resonance of 2-deoxymaltose plus H-1 of the α-anomer (these resonances are resolved in spectrum C70 recorded at 70 °C). Test and control spectra also show a resonance at 4.92 ppm assignable to the H-1 of β-2-deoxymaltose. In the protio standard (spectra C and C70) this is a doublet with a coupling constant of 10 Hz, whereas in spectrum T it appears as a singlet. The absence of coupling at 4.92 ppm in T is evidence for the presence of a deuteron in place of a proton at the axial position at C-2 in the product of maltal hydration catalyzed in $D_2O$ by the soybean β-amylase.

In the upfield region, spectra T and C show multiplets at 1.52 and 1.75 ppm representing the H-2 axial resonances, respectively, of β- and α-2-deoxymaltose, as well as multiplets at 2.09 and 2.24 ppm representing the H-2 equatorial resonances, respectively, of α- and β-2-deoxymaltose (Hehre et al., 1986). These assignments of the H-2 resonances of the reducing residues were confirmed by homonuclear decoupling experiments. Especially to be noted in spectrum T is that the H-2 equatorial resonances have lost the germinal coupling and (by integration) are 4 times larger than the H-2 axial resonances, whereas, in spectrum C the H-2 equatorial and axial resonance signals are of equal magnitude. This is further clear evidence that a deuteron is present at the axial position at C-2 of the 2-deoxymaltose-$d_1$ produced from maltal by the soybean enzyme. Integration of the H-2 resonances in spectrum C (and of the H-1 resonances in spectrum C70) indicates that the β-anomer represents 51% of the 2-deoxymaltose equilibrated in $D_2O$.

*Primary Deuterium Isotope Effect.* Two sets of test mixtures were prepared, each containing 37.5, 50, 75, or 120 mM maltal plus either 9.6 mg/mL soybean β-amylase in $D_2O$ at pD 4.4, or 1.6 mg/mL of the enzyme in aqueous buffer at pH 4.4. The mixtures were set up at 2-min intervals and incubated in small sealed tubes at 40 °C for 120 min. Each was then quick-frozen in a bath of solid $CO_2$/acetone, kept at −20 °C until all were collected, and finally lyophilized. The dry residues (originally 2.4–3.6 μmol of maltal) were silylated (70 °C, 15 min) and then subjected to GLC analysis.

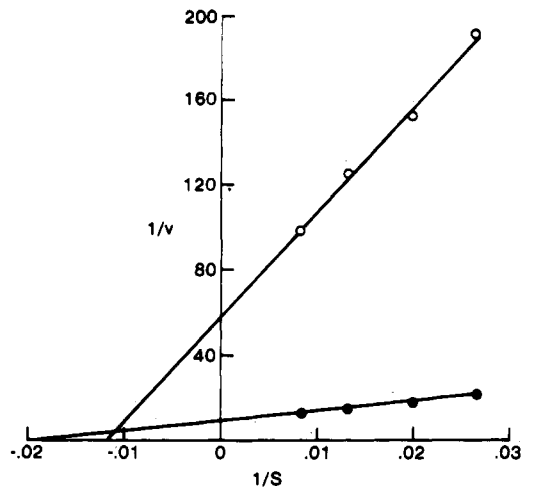



FIGURE 4: Primary solvent deuterium kinetic isotope effects for maltal hydration catalyzed by crystalline soybean β-amylase at pH (pD) 4.4 and 40 °C. Reciprocal plots of velocity $[[\mu mol/(min \cdot mg)]^{-1}]$ versus maltal concentration $[(mM)^{-1}]$. (●) Data points from integrated GLC peaks of components of reactions at pH 4.4; (○) data points from integrated GLC peaks in reactions at pD 4.4; linear curves in each case are computed by the iterative method of Wilkinson (1961).

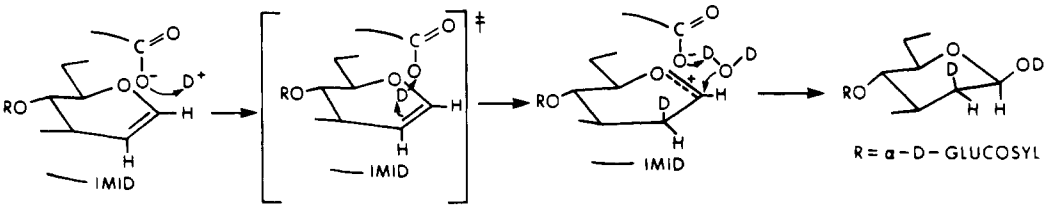
From the recorded areas of the peaks for residual maltal (8.5 min), α-2-deoxymaltose (10.3 min), and β-2-deoxymaltose (12.45 min), the percent hydration in each mixture was calculated as well as the velocity of hydration [$v$ = μmol/(min·mg)] at each concentration of maltal ($S$ = mM). All reactions were found to be initial. Figure 4 illustrates plots of $1/v$ versus $1/S$ for the digests at 40 °C with soybean β-amylase in $D_2O$ at pD 4.4 and for those with the enzyme in $H_2O$ at pH 4.4. Large differences in the $V_{max}$ values and in the slopes distinguish the enzymic activity in $D_2O$ and $H_2O$. When computed according to the procedure of Wilkinson (1961), the hydration catalyzed in $H_2O$ showed $V = 0.11 \pm 0.004$ μmol/(min·mg), $K = 54 \pm 3.8$ mM, and $V/K = 0.002$; that catalyzed in $D_2O$ gave $V = 0.017 \pm 0.0006$ μmol/(min·mg), $K = 87 \pm 6.2$ mM, and $V/K = 0.0002$. Thus, $^DV = 6.5$ and $^DV/K = 10$. The effect was not due to inactivation of the enzyme in the pD 4.4 buffer during the assay procedure. Enzymes at pD 4.4 and at pH 4.4 were preincubated (40 °C, 120 min) prior to assay, diluted 5-fold (and adjusted to contain 20% pD 4.4 buffer and 80% pH 4.4 buffer by volume), and then assayed for the maltal hydration rate on incubation at 40 °C for 120 min. The rates were indistinguishable.

*Proton Inventory.* Reaction mixtures, in duplicate, comprised 50 μmol of maltal plus either 50 μL of soybean β-amylase, $OD_{280}$ = 18.67, in $D_2O$ at pD 4.4 (0.992 atom % D); 50 μL of the enzyme, $OD_{280}$ = 6.22, in $H_2O$ at pH 4.4 (0 atom % D); or 25 μL of each of the pH 4.4 and pD 4.4 enzyme solutions (0.496 atom % D). Incubation was at 40 °C for 50 min, after which each digest was quick-frozen and lyophilized, trimethylsilylated, and analyzed by GLC to obtain the μmol of 2-deoxymaltose formed/mg of enzyme. As shown in Figure 5, a strictly linear relationship was found between the rate of maltal hydration and the mole fraction of solvent deuterium, indicating the presence of a single proton in the transition state.

## Discussion

In our initial study of maltal hydration catalyzed by the tetrameric sweet potato β-amylase (Hehre et al., 1986), the reaction was found to differ from starch hydrolysis in several fundamental respects. The conversion of this prochiral (enolic) glycosyl substrate to an analogue of maltose exhibited an

Scheme I



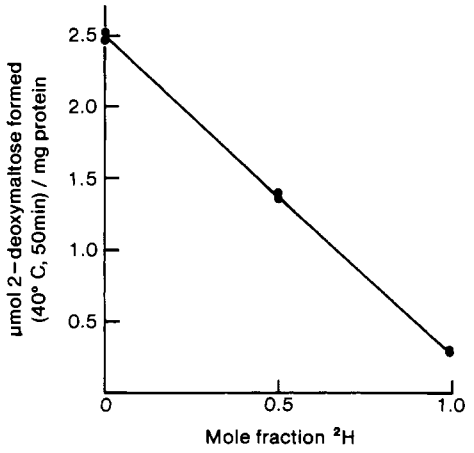




FIGURE 5: Proton inventory for the reaction of hydration of 100 mM maltal catalyzed by soybean β-amylase at pH (pD) 4.4. Observed reaction rates (μmol of 2-deoxymaltose formed/mg) in duplicate mixtures incubated at 40 °C for 50 min are plotted as a function of mole fraction of deuterium.

unusually large solvent deuterium kinetic isotope effect, $V_H/V_D$ ca. 8, 6 times the value of 1.25 recorded (Koshland et al., 1962) for starch hydrolysis, and a linear relation between reaction rate and mole fraction of deuterium. Moreover, it produced 2-deoxy-[2(a)-$^2$H]maltose on deuteriohydration, indicating that the β-amylase protonated the double bond of maltal from a direction opposite that assumed for protonating the glycosidic oxygen atoms of starch. Present findings demonstrate that the hydration promoted by the monomeric soybean β-amylase shows these same unusual features. In addition, the important parameter of steric outcome (unresolved in our earlier study) has been elucidated. Data obtained by GLC analysis of reactions catalyzed in water (Figures 1 and 2) show conclusively that both sweet potato and soybean β-amylase convert maltal specifically to the β-anomer of 2-deoxymaltose. This result, coupled with the finding that both forms protonate maltal from above the plane of the D-glucal ring, shows that β-amylase catalyzes a cis hydration of the double bond. The combined stereochemical finding and kinetic isotope effects observed for hydration by both tetrameric and monomeric β-amylase provide independent sets of data that agree and together furnish a strong basis for assigning a catalytic mechanism of the maltal hydration reaction.

Thus, the data support a well-defined transition-state structure for the reaction (Scheme I). The observed stereospecific protonation of the double bond of maltal by both β-amylases shows that each has a functional group (possibly a carboxyl group) close to and above the double bond of bound maltal. The exceptionally large solvent (i.e., primary) deuterium kinetic isotope effect found for the catalyzed hydration reaction indicates that the rate-determining step must involve proton transfer. Finally, the observed linear dependence of the rate of maltal hydration on the mole fraction of deuterium suggests the presence of a single proton undergoing change in the transition state, with an approximately symmetrical position of the hydrogen suggested by the large magnitude of the primary deuterium isotope effect (Klinman, 1978).

Present data provide new evidence consistent with a mechanism involving a general-acid-catalyzed protonation step with formation of a transient 2-deoxymaltosyl carbonium ion/enzyme intermediate (Scheme I) that is captured by a water molecule specifically directed to the reaction center to yield the observed β-anomeric product. The observed cis hydration supports this mechanism although it does not rule out the possibility of a concerted addition of water to the double bond. The latter appears less likely as it would require substantially more energy than the mechanism of Scheme I, and as it also has not been observed in model studies with vinyl ethers (Salomaa et al., 1966). A mechanism involving hydroxyl ion addition followed by rate-limiting proton transfer from enzyme would appear most unlikely since model hydration reactions with vinyl ethers have not been observed to be base catalyzed (Salomaa et al., 1966). In addition, a carbanion intermediate in maltal hydration would presumably lack the resonance stabilization observed in base-catalyzed reactions (Blanchard & Cleland, 1980).

The observed protonation of maltal from a direction opposite that generally assumed [cf. Thoma et al., (1971) and French (1975)] for the protonation of starch β-amylase raises the question of the nature of the catalytic group responsible for the protonation of maltal. The presence of a pair of ionizable groups at the active site of β-amylase has been reported. In the sweet potato enzyme these are considered (Thoma et al., 1965) to be an imidazole (p$K$ 7.5) and a carboxyl group (p$K$ 3.7), and for soybean β-amylase (Isoda & Nitta, 1988), either an imidazole group or an ε-amino group of lysine (p$K_a$ 8.2) and a carboxyl group (p$K_a$ 3.5). The imidazole group has been assumed to be the source of protonation of the glycosidic oxygen atoms of starch (Thoma et al., 1971; French, 1975). Scheme I reflects the possibility that maltal (which we envision as binding to β-amylase in the same orientation as a terminal maltosyl residue of a starch chain) may be protonated by the carboxyl group. Proximity of the hydrophobic enolic bond to this group could elevate its p$K_a$ as suggested earlier for the hydration of D-glucal by glucosidases (Hehre et al., 1977).

In any case, the observation that β-amylase protonates maltal with a different stereochemistry than starch is evidence that the enzyme's catalytic groups have the ability to function flexibly in response to substrates of different types. The protonation of a glycal and/or related enitol differently from a glycosidic substrate has also been shown for α- and β-glucosidases (Hehre et al., 1977; Chiba et al., 1988; Weiser et al., 1988), β-galactosidase (Lehmann & Zieger, 1977), several cellulases (Kanda et al., 1986; Claeyssens et al., 1990), and trehalase (Weiser et al., 1988). On the other hand, glucoamylases and glucodextranase have been found to protonate D-glucal (Chiba et al., 1988), and β-galactosidase, to protonate D-*galacto*-octenitol (Lehmann & Schlesselmann, 1983), in the same manner as their natural substrates. Why enolic and glycosidic substrates should be protonated differently in some but not all instances is not understood at present. Sinnott (1990) suggests that enolic substrates are highly reactive

compounds and that reactions of some kind are to be expected if such compounds are bound in an environment rich in catalytic functionality. In our experience, enolic glycosyl substrates are quite stable under conditions used to study enzymically catalyzed reactions and require an external source of protonation for reactivity. Maltal, for example, was found to undergo hydration at an extremely slow rate (0.03%/h) in aqueous buffer at pH 5.0 and 35 °C (Hehre et al., 1986).

Of fundamental significance is the finding that whereas both sweet potato and soybean β-amylase protonate maltal from a direction opposite that for protonating amylaceous substrates, they convert this prochiral substrate to a hydration product of the same (β) anomeric configuration as that formed on the hydrolysis of chiral substrates. As noted above, the functional flexibility of the catalytic groups of β-amylase provides for a plastic (substrate-dependent) phase of catalysis concerned with substrate protonation. On the other hand, the formation of β-2-deoxymaltose from maltal and β-maltose from starch strongly suggests that product configuration in each case arises by way of a separately controlled (substrate-independent) means for conserving steric outcome. The formation of β-2-deoxymaltose is not the consequence of an inversion of substrate configuration since maltal has no α- or β-anomeric configuration to invert. Rather, configuration of the hydration product must be created de novo—with the formation of the β- (as opposed to the α-) anomer presumably governed by protein structures that limit the direction of approach of water to the reaction center in the presence of bound substrate. The same structures would be present and likewise control the steric outcome in starch hydrolysis.

β-Amylase is not alone in the ability to create product configuration independently of either the mode of protonation or the configuration of substrate. Evidence for the existence of separate "plastic" electrophilic and "conserved" nucleophilic phases of catalysis has been obtained for various other glycosylases, based on the stereochemistry of their reactions with prochiral (enolic) substrates. Moreover, the existence of separate electrophilic and nucleophilic phases of catalysis has recently been confirmed in an independent way. The α-glucosidase of *A. niger*, purified to homogeneity and crystallized, was found to catalyze the slow hydrolysis of the "wrong" anomer of a small chiral glycosyl compound, β-D-glucopyranosyl fluoride, forming α-D-glucose from it as would be the case from an α-D-glucosidic substrate (Hehre et al., 1990).

Present findings show the specificity of β-amylase to be more strictly expressed with respect to product configuration then with respect to substrate structure or configuration. Product configuration in more than 20 reactions catalyzed with enolic substrates by a variety of other glycosylases has been reported [Chiba et al., 1988; Weiser et al., 1988; Hehre (1989) and references cited therein]. In every instance, including the present observations with β-amylase, the steric outcome has been found to match that of reactions of the same type catalyzed with chiral substrates.

## ACKNOWLEDGMENTS

We thank Professor Yuhei Morita and Dr. Bunzo Mikami for samples of purified isozyme 2 of soybean β-amylase.

**Registry No.** β-Amylase, 9000-91-3; maltal, 32447-71-5; deuterium, 7782-39-0.

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# Fluorescence Spectrum of Barnase: Contributions of Three Tryptophan Residues and a Histidine-Related pH Dependence

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*Received December 6, 1990; Revised Manuscript Received March 21, 1991*

ABSTRACT: Fluorescence spectra of wild-type barnase and mutants in which tryptophan and histidine residues have been substituted have been analyzed to give the individual contributions of the three tryptophan residues. The spectrum is dominated by the contribution of Trp-35. The fluorescence intensity varies with pH according to an ionization of a $pK_a$ of 7.75. This $pK_a$ is close to that previously determined by NMR titration of the C2–H resonances of His-18 as a function of pH (Sali et al., 1989). This histidine residue is close to Trp-94. The pH dependence of the spectrum is abolished when either His-18 or Trp-94 is mutated, and so appears to be caused by the His-18/Trp-94 interaction. The spectral response of this interaction can serve as a probe of the folding pathway and of electrostatic effects within the protein. Changes in the fluorescence spectra on substitution of Trp-94 and His-18 suggest that there is net energy transfer from Trp-71 to Trp-94.

The extracellular ribonuclease barnase (Hartley & Barker, 1972) produced by the prokaryote *Bacillus amyloliquefaciens* is being used extensively as a model protein for biophysical studies on protein folding and stability (Kellis et al., 1988, 1989; Matouschek et al., 1989, 1990; Serrano & Fersht, 1989; Serrano et al., 1990; Bycroft et al., 1990; Horovitz et al., 1990). It is a small (110 residues, 12 382 Da), monomeric, single-domain enzyme, formed by a 5-stranded, twisted antiparallel $\beta$-sheet and 2 $\alpha$-helices (residues 6–18 and 26–34), the first of which packs against the $\beta$-sheet (Mauguen et al., 1982).

The major probe that is being used to chart the folding and unfolding pathways of barnase is its fluorescence emission spectrum (Kellis et al., 1989). It is important, therefore, to understand the factors that contribute to this spectrum in the native protein. Tryptophan residues make the major contribution to the fluorescence emission spectra of proteins when present. The fluorescence properties of these residues are particularly sensitive to the environment of their side chains. There are three tryptophan residues in barnase, at positions 35, 71, and 94 (Figure 1). Trp-35 is near the C-terminal end of the second $\alpha$-helix and relatively far away (22–25 Å) from the other two tryptophans. Trp-71 is in a hydrophobic region at the beginning of the second strand of the $\beta$-sheet. Trp-94 is at the beginning of the fourth strand of the $\beta$-sheet and only 10 Å apart from Trp-71. The fractions of solvent-exposed surface of the side chains of the three tryptophans are 0.075 (Trp-35), 0.029 (Trp-71), and 0.219 (Trp-94). There are numerous close contacts between Trp-94 and a histidine residue (His-18) that lies at the C-terminal end of the first $\alpha$-helix.

We have mutated each of the three tryptophans into significantly less fluorescent residues in order to assess the contribution of individual tryptophans to the fluorescence emission spectrum of barnase. Three mutant proteins were constructed, in each of which a single tryptophan residue was replaced by another aromatic amino acid. To study the specific contribution of Trp-94 to the total fluorescence spectrum and the influence of His-18, two more mutant proteins were made; one in which His-18 was replaced by glycine and one in which Trp-94 was replaced by leucine.

We report here that the major contributor to fluorescence intensity in the protein is Trp-35 and that there is probably energy transfer between the two other tryptophan residues. We also report that there is a pH dependence of fluorescence intensity caused by a tryptophan–histidine interaction, and discuss the use of this effect to study electrostatic interactions within the protein.

## Materials and Methods

### *Materials*

Radiochemicals were from Amersham International plc., Buckinghamshire, U.K. The buffers used in the fluorescence experiments, Tris [tris(hydroxymethyl)aminomethane] and Bis-Tris ([bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane]), and Torula yeast RNA were obtained from Sigma, St. Louis, MO. SP-Trisacryl-M was purchased from IBF biotechnics, Villeneuve La Garenne, France. Dialysis tubing was purchased from Spectrum Medical Industries Inc., Los Angeles, CA. All other reagents were of analytical grade and were purchased either from Sigma or from Amersham.

*Escherichia coli* BL21(DE3) pLysS cells were a gift from Dr. F. W. Studier. The plasmid pTZ18U and the helper phage M13KO7 were obtained from Pharmacia, Uppsala, Sweden. The wild-type barnase gene was cloned into the plasmid pUC9 by Paddon and Hartley (1987) and donated to us. A 1.4-kb *Eco*RI–*Hin*dIII restriction fragment of this recombinant plasmid containing the structural gene for barnase fused to the promoter and signal sequence of the *E. coli* alkaline

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0006-2960/91/0430-6775$02.50/0 