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The effect of ^2H_2O on the activity of α-amylase

Acid-catalyzed acetal hydrolysis is believed to proceed by a mechanism which involves a fast pre-equilibrium protonation of the acetal followed by its slow unimolecular rate-determining decomposition to an alcohol and a carbonium ion and then to products[1]. This sequence, designated as an A-1 path, has been experimentally confirmed by an examination of substituent effects on the rate of acetal hydrolysis[2]. Deuterium isotope studies have revealed that acetal hydrolysis proceeds at a faster rate in ^2H_2O than in H_2O and rate ratios, $k^{2}_{H_2O}/k_{H_2O}$, between 2 and 3 have been observed[3]. It thus appears that proton transfer is not involved in the rate-determining step. The faster rate in ^2H_2O is apparently caused by the difference in the steady-state concentration of the protonated and deuterated acetals and by the fact that the latter is the weaker acid of the two[3].

These results are in contrast to the deuterium isotope effects observed in enzyme-catalyzed acetal hydrolysis reactions, which show faster rates in H_2O than in ^2H_2O (ref. 4). Thus, on the basis of isotope data, the enzyme-catalyzed reactions must proceed by a different path.

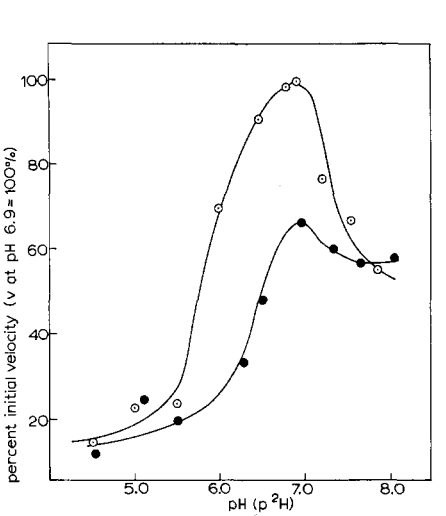
In this report we support this view by comparing pH and temperature effects on hog pancreatic α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) activity in the two solvents, H_2O and ^2H_2O.

Twice-crystallized porcine pancreatic α-amylase (Worthington Biochemical Corp.) having an activity of approx. 1000 μmoles maltose/min per mg enzyme at 30° was used without further purification. Buffer solutions (20 mM) were prepared at a constant ionic strength of 0.04, and they were 0.006 M with respect to NaCl. Sodium glycerophosphate or sodium phosphate buffer was used in the pH region 6.0 to 7.5 while acetate buffers were used from pH 4.5 through 5.5.

All deuterated solutions using 99.86% ^2H_2O (Volk Radiochemicals, Inc.), and stored in tightly sealed containers under nitrogen were prepared just as the corresponding water solutions except that the pH was adjusted 0.4 unit higher in order to obtain the correct p^{2}H (refs. 5, 6). Initial velocities were determined during the first min of reaction by analyzing for reducing groups released from starch using a modification of the 3,5-dinitrosalicylic acid colorimetric assay procedure[7]. Unless indicated, incubations were at 30°. The initial velocities approach the maximum velocity since less than 10% of reaction had occurred during the first min and thus the enzyme remained saturated with substrate.

The isotope rate ratio for α-amylase, $v_{H_2O}/v^{2}_{H_2O}$, at 30° was 1.50±0.13 S.D. (average of 6 runs). This observed ratio is consistant with that which we obtained for yeast invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) 1.4, and with β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) 1.21 (ref. 4). Similar rate data for other carbohydrases have been reported[8].

Rate ratios such as these do not implicate a proton transfer in the rate-determining step of the enzyme reaction in which case the ratios would be about 3, but rather they suggest the participation of a secondary isotope effect. The pH(p^{2}H) rate profile data shown in Fig. 1, supports this view since the pH and p^{2}H optima are identical. The expected shift in the p^{2}H optimum, in a reaction involving proton transfer in the rate-limiting step, would be toward a higher pH by about 0.6 unit



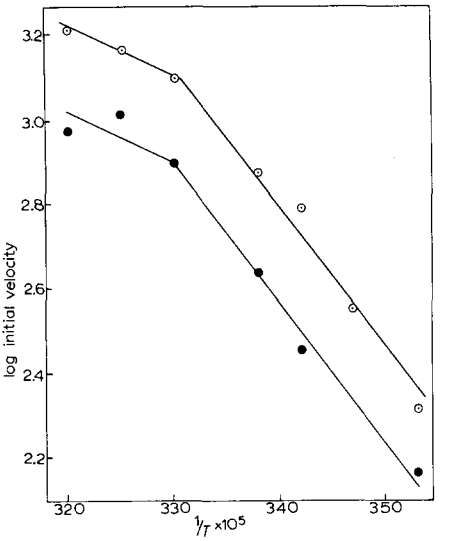


Fig. 1. Effect of pH on the activity of α-amylase in H_2O (⊙—⊙) and ^2H_2O (●—●). A stability curve was run under the same conditions after storing the enzyme at a given pH for 1 min, then transferring aliquots to pH 6.9 solutions for assay. The enzyme was unstable at the extremes of the pH range, *i.e.*, the activity dropped to 50% at pH 4.5, but it retained over 95% of its activity within 0.5 pH unit of 6.9.

Fig. 2. The effect of temperature on the activity of α-amylase in H_2O (⊙— ⊙) and ^2H_2O (●—●).

(ref. 9). This change would be caused by an increase in the pK values of the functional groups. Although two proton bearing groups, carboxyl and imidazole, have been implicated at the active side of α-amylase[10], the decrease in acidity of these groups in ^2H_2O relative to H_2O is not reflected in the p^{2}H rate profile.

Secondary isotope effects have been accounted for in part by assuming differences in solvation and/or hydrogen bonding in the ground and transition states[9]. BENDER[11] has also pointed out that deuterium oxide as a solvent can affect the conformation of the enzyme, and of the enzyme–subtrate complex by solvation. The similarity of the isotope rate ratios for a variety of carbohydrases points to a common enzymatic hydrolysis mechanism, which must be markedly different from acid-catalyzed acetal hydrolysis.

Further support for the idea is contained in the energy of activation data. In Fig. 2 the effect of temperature on α-amylase activity in H_2O and ^2H_2O is shown. For the temperature range 10–30° in H_2O and in ^2H_2O the activation energy, E_a, was found to be 14.9 and 15.1 kcal/mole, respectively; while for the range 30–40° the E_a was 5.4 and 5.6 kcal/mole, respectively. Such changes in activation energy with temperature have been observed in amylolytic reactions and are common for enzyme reactions in general[12].

The slightly greater activation energy in the ^2H_2O solvent is consistent with a faster rate in H_2O than in ^2H_2O. In contrast, the high $k^2_{H_2O}/k_{H_2O}$ rate ratio in acid-catalyzed acetal hydrolysis was related to an energy of activation in ^2H_2O which was about 500 cal/mole lower than that in H_2O (ref. 3).

The fact that the shapes of the two curves are similar lends further support

to the idea that a bond to hydrogen (or deuterium) is not broken in a rate-limiting step, since greater differences in activation energies would have been observed.

Although the isotope data presented here do not lead to a mechanism of action theory for α-amylase, it appears to rule out an A-1 mechanism which operates in acid-catalyzed acetal hydrolysis. Furthermore, secondary isotope effects of the magnitude observed are consistent with various mechanisms for amylase catalysis which have been proposed to account for both the retention of configuration by α-amylase and the inversion by β-amylase.

One of these, an Sn1 displacement, was proposed by MAYER AND LARNER[13] in 1959. The enzyme surface would restrict attack by water on the carbonium ion so as to retain or invert configuration, depending on which enzyme is present.

KOSHLAND in 1953 postulated an Sn2, or double displacement, resulting in retention of configuration for α-amylase, and an Sn1 or single displacement in the β case resulting in inversion[14].

More recently KOSHLAND proposed an ion-pair mechanism for both amylases where orientation of the water molecule determines the observed product configuration[4].

Both the ion-pair and Sn1 mechanisms are plausible. A necessary condition for both mechanisms, namely, similar ^2H_2O effects for α- and β-amylase appears to have been satisfied.

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