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A Kinetic Study of Enzyme Catalyzed Glucose Mutarotation at Variable Pressure and pH

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The enzyme mutarotase (5.1.3.3.) acting on the α/β-anomerisation of glucose has been studied at pressure from 1 to 1 000 bar and over the pH range from 5.50 to 6.75. In contrast to the spontaneous, acid-base catalyzed mutarotation, the enzymatic mutarotation was found to be independent of pressure, invalidating the generally accepted view that the two reactions are identical in mechanism. The p*H* sensitivity of the Michaelis parameters K_m and V supports the idea of histidine as a necessary component of the active center in the enzyme.

The spontaneous, acid-base-catalyzed mutarotation of glucose and certain other sugars has been intensively studied for more than 100 years. An introduction to the relevant literature may be found in a previous paper.[1] That the same reaction could proceed catalyzed by an enzyme, mutarotase found in Penicillium notatum was discovered in 1949[2] and in 1952.[3] Keston[4] found the enzyme in animal tissues and described in later papers[5,6] the distribution of the enzyme in animals and its possible role in the sugar transport. The Penicillium enzyme is thoroughly discussed by Bentley and Bhate.[7,8] The p*H* sensitivity curve for the Penicillium enzyme[9] and for the animal enzyme[10] are similar. So far no differences between the fungus and the animal enzymes have been demonstrated.

The p*H* sensitivity curve for the spontaneous reaction is quite different from that of the enzymatic reaction but in spite of this it is generally suggested that the two reactions operate through similar mechanisms.[8,10] The present paper describes experiments with the aim of elucidation of the catalytic reaction mechanism.

EXPERIMENTAL

In a previous paper[1] the experimental equipment and procedure have been described. The only change is that the Bourdon manometer was replaced with a manganin cell, calibrated by Harwood Engineering.

Experiments at variable pressure. The experiments were carried out at (25.00±0.05) °C in the previously described pressure cell. Phthalate buffer, 0.005 M with respect to potassium phthalate, p*H*=6.50 was used. The p*H* was controlled by a p*H*-meter PHM62 before and after each experiment. The pressure varied from 1 to 1 000 bar.

Experiments at variable pH. The experiments were carried out in a standard 10.00 cm tube at (25.00±0.01) °C in 0.005 M phthalate buffer. The p*H* in the different series of measurements varied from 5.50 to 6.75 and was controlled before and after each experiment. At each p*H* value a series of experiments were performed with 5 different glucose concentrations. The enzyme used was "Mutarotase Sigma" from porcine kidney in crystalline suspension, 5.8 mg protein/ml, in 3.2 M $(NH_4)_2SO_4$, p*H* approximately 6.0. The enzyme was dialyzed against water at 4 °C for 24 h. In each experiment 10 μl of the dialyzed enzyme was used in a total volume of 10 m*l*. Since the dialyzed enzyme was rather unstable, a standard procedure was used to calculate the relative activity.

In all experiments where enzyme solution is added to the reaction mixture, the kinetic data obtained reflect two simultaneously proceeding reactions:

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$$\alpha\text{-glucose} \underset{\rightleftharpoons}{\overset{\text{(spont.)}}{\rightleftharpoons}} \beta\text{-glucose} \qquad \text{(I)}$$

$$\alpha\text{-glucose} \underset{\rightleftharpoons}{\overset{\text{(E)}}{\rightleftharpoons}} \beta\text{-glucose} \qquad \text{(II)}$$

The observed rate constant k_{obs} has contributions from both reactions. However, we know from earlier experiments that the rate constant for reaction I is independent of pH in the pH-range used in our experiments. Thus the contribution from reaction I has a constant value. Concerning the experiments at constant pH and variable pressures, the pressure influence on the speed of reaction I was determined in the preceding paper.[1] The rate constant depends on the pressure according to

$$\frac{\partial \ln k}{\partial P} = -\Delta V^{\neq} \cdot (RT)^{-1}$$

and for the forward and backward reaction in reaction I a value of $\Delta V^{\neq}$ around $-10\ \text{cm}^3 \cdot \text{mol}^{-1}$ was found. Deviation from this $\Delta V^{\neq}$ in experiments where reactions I and II proceed simultaneously is a result of pressure influence on II.

RESULTS

Experiments at variable pressure. The experimental procedure and the calculating program for this type of experiment were given in the preceding paper[1]. See results and Fig. 2 in that paper.

The results of present investigation at variable pressure are shown in Fig. 1.

The results at 1 bar demonstrate (Fig. 1) that the velocity increases linearly with the amount of enzyme. This was to be expected since the concentration of glucose exceeds that of enzyme by several orders of magnitude. That this linearity is also found at elevated pressure is seen from the other straight lines in Fig. 1. The extrapolation of these lines to the vertical axis gives the values of k for the spontaneous reaction since the enzyme concentration here is zero. Calculation of $\Delta V^{\neq}$ from the four intersections yields the same value as that which was found in Ref. 1. The fact that the lines are parallel demonstrates that the same increase of velocity with pressure is found for any amount of enzyme. Obviously the veloc-

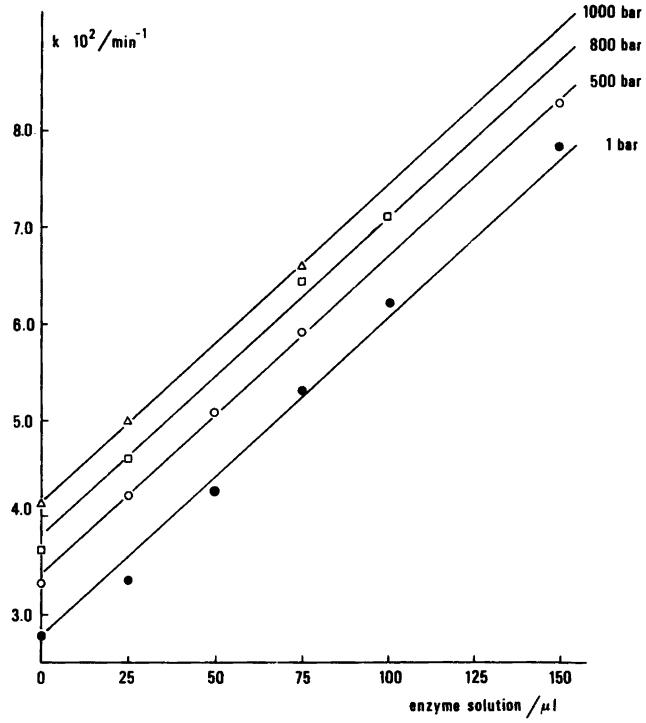

Fig. 1. The observed rate constant k_{obs} as a function of enzyme concentration and pressure. pH=6.50, 25 °C.

Table 1. k_{obs}/min^{-1} as a function of pressure, p*H*: 6.50, 25 °C. Experiment 1: No enzyme added. Experiment 2 and 3: same amount of same enzyme preparation added, but 3 is carried out 2 weeks after 2. See text.

P/bar	1	2	3
1	0.0277	0.0358	0.0330
800	0.0376	0.0458	0.0429
Δk_{obs}	0.0099	0.0100	0.0099

ity only increases as a result of pressure influence on reaction I. The conclusion is that the enzyme reaction is not influenced by pressure, which means that the resulting $\Delta V^{\neq}$ is near zero.

As mentioned the dialyzed solution of enzyme used is not stable. To ensure that the progressive decrease in activity has not influenced the results, a simple experiment at 1 and 800 bar was carried out, the results of which are given in Table 1. Experiment 1 represents the spontaneous reaction I since no addition of enzyme was made. In experiments 2 and 3 the same amount of enzyme was added from the same preparation. But whereas the runs of experiment 2 were made within 48 h after preparation of the enzyme solution the latter was aged for more than a fortnight before being used in the run of experiment 3. The loss of activity in 3 relative to 2, does not alter the finding of an activation volume near 0 for the enzymatic reaction.

Table 2. The Michaelis parameters at various values of p*H*. Pthalic acid buffer, 25 °C, 1 bar. After correction for influence of the spontaneous mutarotation the parameters were calculated from Hanes plot.

p*H*	K_m/M	V/M min^{-1}
5.50	0.130	0.007
5.50	0.165	0.008
5.75	0.107	0.004
5.75	0.112	0.004
6.00	0.027	0.005
6.00	0.028	0.003
6.00	0.019	0.003
6.25	0.015	0.005
6.25	0.021	0.005
6.25	0.013	0.006
6.50	0.018	0.006
6.50	0.021	0.006
6.75	0.045	0.007
6.75	0.045	0.006

Experiments at different values of pH. These experiments were carried out in the p*H* range 5.50 to 6.75 where the mutarotase action is increasing to its maximum. For each value of p*H* used, 5 experiments with different starting concentrations of glucose were made, allowing the determination of the initial velocity as a function of glucose concentration, s. Before calculating the Michaelis parameters K_m and V for the enzymatic reaction, a correction for the contribution from the spontaneous mutarotation was carried out. Then the parameters were calculated, based on a least squares fitting of a plot of s/v against s (Hanes plot). The results are found in Table 2. Obviously there is a minimum value for K_m in the p*H* range around 6.25, while V is not influenced to the same degree.

DISCUSSION

The accepted suggestion of identical reaction mechanisms for the acid-base catalyzed reaction and the enzyme catalyzed reaction, is not very probable in the light of the experiments described here.

High pressure experiments on the acid-base catalyzed reaction [1] demonstrated a difference in volume of the reactants and the activated complex of $-10\ cm^3 \cdot mol^{-1}$, supporting the accepted idea of a concerted displacement reaction. Opposite to this is the finding of a volume difference near zero for the enzyme catalyzed reaction indicating a different type of mechanism.

From the experiments at different values of p*H* it could be proposed that mutarotase belongs to the group of enzymes in which histidine plays an important role as a constituent of the active site. This has also been suggested in other papers.[8,10,11] From Table 2 one can see the variation in the Michaelis' parameters with p*H*. Assuming the validity of the simple Michaelis model we have

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E+P$$

and $v=V/[(K_m/s+1]$

We know from other experiments[10] that in the p*H* range from 5 to 7 the initial velocity v increases with a maximum around 6.25. This could be due either to a decrease in K_m or a rise in V. From Table 2 it can be seen that K_m decreases from 5.50 with a minimum around 6.25 while the maximal velocity in the same range remains fairly constant. Thus the increasing velocity is due to an increase in the association constant k_1 while k_2 is not altered appreciably. It seems that the binding between enzyme and substrate demands deprotonation of a group in the enzyme with a p*K* around 6.25, most probably the imidazolyl group of histidine.

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