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HIGH PRESSURE ENZYME KINETICS OF DEXTRANSUCRASE

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The pressure dependence of enzymatic dextran formation has been observed up to 1000 at for several substrate concentrations. First order denaturation effects could be separated from the thermodynamic effects, which lead to a volume of 30.4 to 44.0 ccm per mole for the formation and −13.6 ccm per mole for the activation of the enzyme–substrate complex. Denaturation depends on the substrate concentration. This leads to the conclusion that only the free enzyme is denatured, whereas the ES complex is stable.

1. Introduction

High hydrostatic pressure can influence enzyme reactions in two entirely different ways. Often the enzyme is denatured either reversibly or irreversibly by high pressure. In other cases the enzymes are stable enough, so that the pressure-induced change of the rate constants k can be measured. The respective volume of activation $\Delta V^{\ddagger}$ can be calculated according to

$$\mathrm{d} \ln k/\mathrm{d}p = -\Delta V^{\ddagger}/RT. \qquad (1)$$

Older publications on these two effects have been almost completely reviewed by Laidler [1, 2], Curl and Jansen [3], and Matthews, Dow and Anderson [4]. Later Suzuki and co-workers have published a series of papers on different properties of enzymes under pressure; these are discussed in [5]. Furthermore, the publications [6–11] give information on deactivation of enzymes caused by pressure, whereas [11–14] deal with the effects of pressure on the catalytic process itself. Only in [13] and [14] are values given for several substrate concentrations, thus allowing the evaluation of the step of formation as well as the step of activation of the enzyme–substrate complex.

The present paper deals with the influence of different pressures on the reaction rate of the enzyme dextransucrase over a wide range of substrate concentrations, so that the volumes of activation of both steps can be calculated. In the pressure range used in this work denaturation and rate of reaction changes occur simultaneously. By separating the effect of denaturation, however, it is possible to determine the rate of reaction of these enzyme molecules which are still active.

2. Experimental methods

The enzyme is produced as described in ref. [15], using bacteria Leuconostoc mesenteroides B 512 F *. It is purified by a methanol precipitation and a pH-precipitation, and by centrifugation at 12000 rpm. It is then dissolved in a 0.05 molal acetate buffer of pH = 5.2, which contains 0.02 per cent Na-azide in order to prevent microbial growth. The enzyme solution is stored at 3°C.

Normal sugar is taken as substrate, since tests have shown, that biochemical grade sucrose gives identical results.

The course of the reaction is observed by measuring the product fructose using the method of Nelson and Somogyi [16, 17]. All reaction rates are measured as initial rates, i.e., when only very little substrate is converted into product. The enzyme concentrations used

* A gift of Knoll AG, Ludwigshafen, FRG.

in our experiments are approximately 0.03 to 0.06 enzyme units per ml. An enzyme unit is defined as the amount of enzyme, which produces one micromole of fructose per min at optimum conditions (25°C, 0.05 molal acetate buffer pH 5.2, 150 mmol/ℓ substrate).

Fig. 1 shows a schematic diagram of the high-pressure apparatus. The enzyme is added to the solution at 25°C and stirred. The mixture which has already started to react is injected into the autoclave by a syringe through a high pressure valve A. The autoclave is equipped with a stirrer and has a volume of 410 ccm. The temperature of 25°C is kept constant by water flowing through a cooling coil. Samples are taken via the high-pressure valve B. In the first minutes of the reaction, samples are taken at normal pressure to determine the reaction rate v_0. To raise the pressure, acetate buffer is pumped into the system by a high pressure pump (W.C. t'Hart, Rotterdam) until the desired pressure is attained. In order to get a pressure of 1000 at, 15 ccm buffer have to be added to the reaction vessel, i.e., the solution has a mean compressibility of 3.6×10^{-2} per 1000 at. Hence a compressibility correction of 3.6 per cent per 1000 at has to be taken into account. The pressure is raised at a rate of about 60 at per second. The temperature rise of 1.68°C per 1000 at is annuled within less than 30 s, so that the data, taken over periods of 10 to 150 min, are influenced only in the time immediately after the pressure rises. Taking samples causes a total loss in volume of less than 10 ccm during the whole experiment. This loss is compensated by pumping in buffer solution. Temporary pressure drops (20 to 40 at) cannot be avoided. The resulting error, however, is negligible.

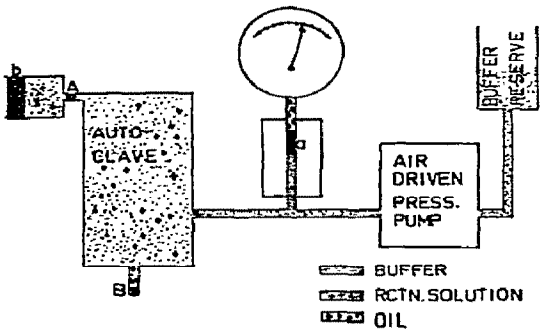

Fig. 1. Schematic diagram of the high-pressure apparatus. A and B are high-pressure valves as described in the text. Movable pistons are described by a and b.

All pressures are measured by a steel spring manometer calibrated by the manufacturer (Dreyer, Rosenkranz und Droops, Hannover) for a range of 0 to 2500 at.

All parts in contact with the buffer or the reaction solution are made of V2A-steel, except for some joints, which have parts made of copper alloy.

3. Results

3.1. General features of the enzyme

Dextransucrase catalyses the reaction

$$(\text{anhydroglucose})_n + \text{sucrose} \rightarrow (\text{anhydroglucose})_{n+1} + \text{fructose},$$

where n is the degree of polymerisation of the anhydroglucose dextran. The formation of the dextran proceeds by an insertion mechanism [18, 19]:

$$EP_n + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ESP_n \xrightarrow{k_2} EP_{n+1} + F, \qquad (2)$$

(E: enzyme, S: sucrose, F: fructose, P_n: dextran chain.) The polymer molecules can be released from the enzyme–substrate complex by the so-called acceptor reaction (20):

$$A + ESP_n \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} AESP_n \xrightarrow{k_4} AP_n + ES, \qquad (3)$$

$$ES \xrightarrow{k_2'} EP_1 + F. \qquad (3a)$$

When there is no special acceptor A in the solution, the substrate itself acts as acceptor.

The above scheme leads to the Michaelis–Menten equation with the additional term $K_a/(K_a + [S])$ for the acceptor reaction,

$$v([S]) = \frac{v^{max}[S]}{K_m + [S]} \frac{K_a}{K_a + [S]}, \qquad (4)$$

where $v^{max} = k_2[E]_0$; $[E]_0$ is the total enzyme concentration;

$$K_m = \frac{k_{-1} + k_2}{k_1}, \qquad K_a = \frac{k_{-3} + k_4}{k_3}.$$

The term $K_a/(K_a + [S])$ affects the reaction at high substrate concentrations. In the present work no pressure influence on this term is found. Thus this term can be neglected when pressure effects are considered. That justifies the use of the simple form of the Michaelis–Menten equation

$$v([S]) = v^{max}[S]/(K_m + [S]), \quad (4a)$$

for all following considerations. For the sake of simplicity E and ES are substituted for EP_n and ESP_n in the following text.

3.2. Denaturation of the enzyme

The concentration of the product fructose is shown as a function of time in fig. 2 for an experiment at 1000 at and [S] = 150 mmole/ℓ. After about 150 min the concentration C of the product approaches a final value C_{end}. This means, the enzyme has become inactive. The denaturation is irreversible. On the assumption that a first-order denaturation occurs, the simple relation

$$[E](t) = [E]_0\, e^{-t/\tau} \quad (5)$$

holds, where τ is the time constant of denaturation. The same dependence has also been found to be true for the pressure denaturation of lactic dehydrogenase [11] and α-amylase [21]. Since the reaction rate is proportional to the concentration of the still active enzyme molecules, the following relation is valid:

$$v_p(t) = v_p\, e^{-t/\tau}, \quad (6)$$

where v_p is the reaction rate at pressure p and time zero, i.e., the time of pressure onset.

Integration gives the concentration of the product as a function of time

$$C(t) = \int_0^t v_p\, e^{-t'/\tau}\, dt' = C_{end} - v_p\, \tau\, e^{-t/\tau}, \quad (7)$$

with $C_{end} = v_p\, \tau$. In logarithmic scale eq. (7) converts to

$$\ln(C_{end} - C(t)) = \ln(v_p\, \tau) - t/\tau. \quad (8)$$

Fig. 3 shows the time dependence of the concentration of the product in a semilogarithmic plot according to eq. (8) using the values from fig. 2. The linearity of the plot confirms the assumption that the denaturation is of first order. The slope gives τ = 34.6 min. The method discussed above for the determination of τ can be applied advantageously to those experiments in which taking samples causes a release of pressure resulting in an additional denaturation of the enzyme. This is the case for the dextransucrase. Fig. 4 shows the difference in reaction rate, where in one case taking a sample causes a smooth decompression and in the other case

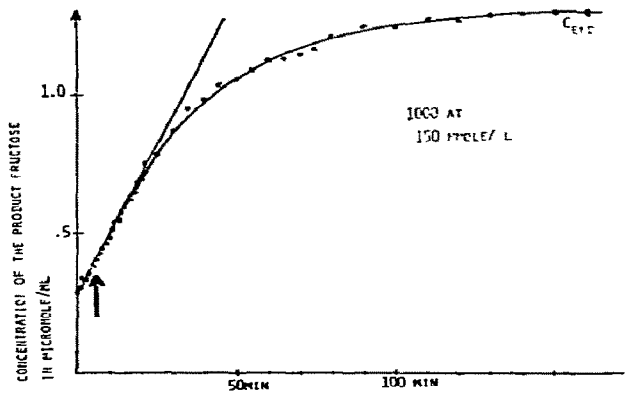

Fig. 2. Product concentration as a function of time at 1000 at. Substrate concentration [S] = 150 mmole/ℓ. The arrow indicates the time of presssure onset. The straight line shows the expected course at normal pressure.

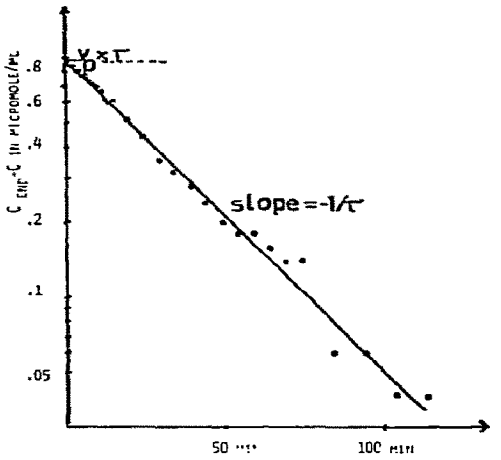

Fig. 3. Semilogarithmic plot of $(C_{end} - C(t))$ versus time for the data of fig. 2. The slope gives $-1/\tau$, the ordinate gives $C_{end} = v_p\tau$.

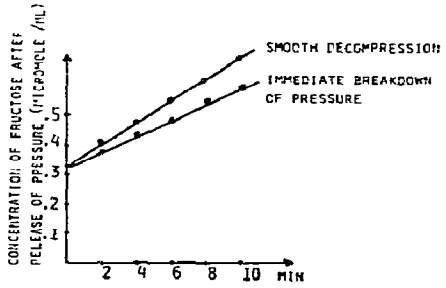

Fig. 4. Residual activity of the enzyme after release of pressure for smooth decompression and for immediate breakdown of pressure.

an immediate breakdown of pressure. Both enzyme samples have been kept under a pressure of 1000 at for 12 min. Whereas the residual activity of the enzyme can be influenced by the mode of sampling, the concentration of the product is not affected (the values at $t = 0$ in fig. 4 coincide). Therefore the determination of τ from the residual activity would cause errors, whereas the use of product concentrations yields reproducible results.

For pressures below about 500 at the time constant of denaturation becomes very large. Therefore practically no denaturation occurs. As an example, fig. 5 shows the time course of the product concentration at 400 at and [S] = 20 mmole/ℓ. There is no denaturation. Only a reversible change in the reaction rate occurs.

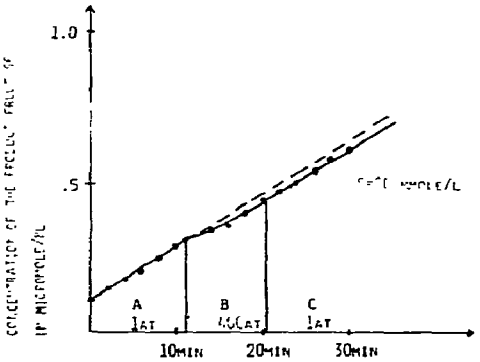

Fig. 5. Product concentration as a function of time for the case, where no denaturation occurs. In region A the enzyme is under normal pressure, in region B the pressure is 400 at. In C, pressure is released to normal pressure, in order to investigate reversibility. [S] = 20 mmole/ℓ.

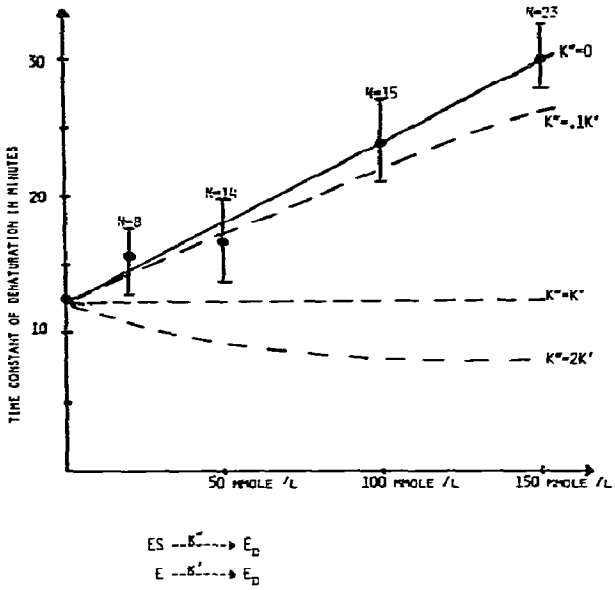

Fig. 6. Time constant of denaturation as a function of substrate concentration. The straight line and the dotted curves are theoretical curves for several assumed values of k'' (see Discussion). The points are mean values of N experiments. N is written at the upper end of the bars. These are given by $(\tau_{max} - \tau_{min})/N$ according to simple statistical rules.

Fig. 6 shows τ as a function of the substrate concentration for 1000 at. Since the values of τ fluctuate strongly due to reasons which are not yet understood, mean values of several experiments under the same conditions have been taken.

3.3. Pressure dependence of the reaction rate

From the slope and the ordinate of fig. 3 $\tau = 34.6$ min and $v_p \tau = 0.88$ micromole/ml are obtained; therefore $v_p = 0.0254$ micromole/ml min. Together with the value of v_0 determined by the slope of the curve in fig. 2 before the pressure has been raised, the ratio of the reaction rate at pressure p and normal pressure, v_p/v_0, becomes 1.18. This has to be corrected by 3.6 per cent as mentioned above, so that the correct value is 1.22. Table 1 gives values of v_p/v_0 for different pressures and concentrations. Although the values of v_p/v_0 have been obtained simultaneously with τ from the same experimental data, the v_p/v_0 values are nearly

Table 1
Values of v_p/v_0 for different pressures and substrate concentrations

Pressure in at	Substrate concentration in mmole/ℓ						
	20	50	100	150	300	600	1200
100	0.95	–	–	1.04	–	–	–
200	0.93	1.08	(1.23)	1.11	–	–	–
400	0.95	1.01	1.21	1.21	–	–	–
600	0.85	0.89	1.18	1.19	–	–	–
800	0.69	0.94	1.09	1.27	–	–	–
1000	0.56	0.77	1.05	1.22	1.37	1.56	1.57

constant at given [S] and p compared to those of τ, which fluctuate strongly as mentioned above.

Fig. 7 shows a semilogarithmic plot of v_p/v_0 versus pressure for different substrate concentrations. A problem occurring when the pressure dependence of an enzymatic reaction is measured only at a single substrate concentration must be pointed out. As one recognizes from fig. 7, one obtains positive, zero or negative slopes for the graphs depending on the substrate concentration chosen. The concentration and pressure dependent slopes represent volumes of activation (see eq. (1)) that are extremely difficult to interpret. Furthermore the non-linearity in the curves of fig. 7 simulates very different compressibilities of the activated complexes and the reactants. In fact this curvature is caused by the changes of k_2 and K_m with pressure.

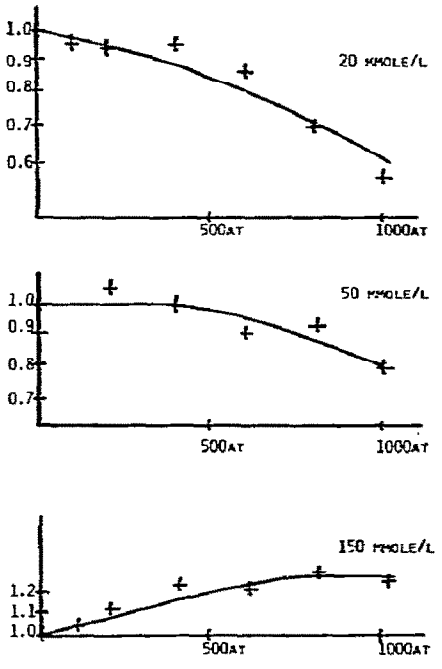

Fig. 7. Semilogarithmic plot of v_p/v_0 as function of pressure for [S] = 20, 50 and 150 mmole/ℓ. The curves are fitted to the experimental points using eq. (9).

3.4. The pressure dependence of K_m and v^{max}

On the assumption that even at high pressures a Michaelis–Menten mechanism holds, eq. (4a) is valid and one obtains the following relation:

$$\frac{v_p}{v_0} = \frac{v_p^{max}}{v_0^{max}} \frac{K_m + [S]}{K_p + [S]}, \tag{9}$$

where K_m, v_0^{max} and K_p, v_p^{max} are the Michaelis constants and maximum rates, respectively, at normal pressure and at pressure p. K_m for the enzyme used in these experiments is determined by the method of Lineweaver and Burk [22] to be 14 ± 2 mmole/ℓ. Using the new variable

$$Y \equiv \frac{v_p/v_0}{K_m + [S]},$$

which contains the experimental data, eq. (9) converts to

$$[S]\, Y = \frac{v_p^{max}}{v_0^{max}} - K_p Y. \tag{10}$$

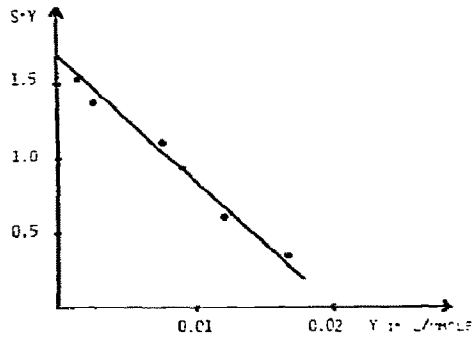

Fig. 8. [S] Y versus Y plot for 1000 at. The negative slope gives K_p, the ordinate gives v_p^{max}/v_0^{max}. The straight line is obtained by linear regression.

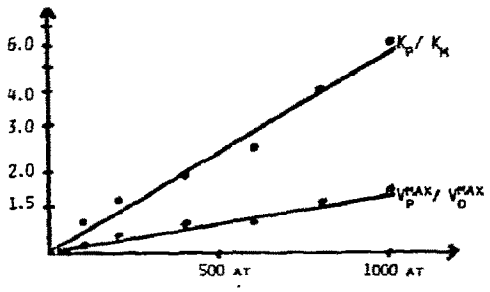

Fig. 9. Semilogarithmic plot of K_p/K_m and v_p^{max}/v_0^{max} versus pressure. The slopes represent the respective volume of activation.

Fig. 8 shows a plot of [S] Y versus Y for 1000 at. The points represent substrate concentrations of 20, 50, 100, 150, 300 and 1200 mmole/ℓ. The ordinate gives $v_p^{max}/v_0^{max} = 1.71$, the negative slope $K_p = 86$ mmole/ℓ and thus $K_p/K_m = 6.14$. The dependence of K_p/K_m and v_p^{max}/v_0^{max} on pressure is shown in a semilogarithmic plot in fig. 9. In both cases the results can be represented by straight lines, although small deviations from linearity might be possible.

Finally, fig. 10 shows the dependence of the reaction rate on the substrate concentration for 1000 at and normal pressure, calculated with the data of fig. 9. The reaction is accelerated by high pressure when high substrate concentrations are used (i.e., the catalytic process itself is accelerated). For low substrate concentrations, however, the rate is inhibited by high pressure (i.e., the access of substrate to the enzyme is inhibited). This explains why, in fig. 7, negative, zero and positive activation volumes are simulated.

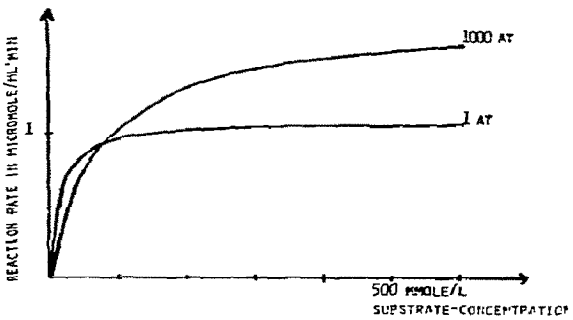

Fig. 10. Theoretical Michaelis–Menten plots for normal pressure and for $p = 1000$ at.

4. Discussion

4.1. The kinetic effect of pressure

Eq. (1) can be rewritten

$$\Delta V^{\ddagger} = -RT \, \mathrm{d} \ln(k_p/k_0)/\mathrm{d}p \,. \tag{11}$$

For enzyme reactions of the Michaelis–Menten type the following volumes can be obtained:

(a) $\Delta V_2^{\ddagger}$ (obtainable from the pressure dependence of k_2 or v^{max}) is the change in volume, which occurs, when the ES complex becomes the activated complex $(ES)^{\ddagger}$.

(b) $\Delta V_K^{\ddagger}$ (obtainable from the pressure dependence of $1/K_m$) is a composite volume, since $1/K_m = k_1/(k_{-1} + k_2)$. Laidler [1] has shown, that the following relation holds:

$$\Delta V_K^{\ddagger} = \Delta V_1^{\ddagger} - \frac{k_{-1}\Delta V_{-1}^{\ddagger} + k_2 \Delta V_2^{\ddagger}}{k_{-1} + k_2} \,. \tag{12}$$

$\Delta V_K^{\ddagger}$ has an obvious meaning, when $k_2 \ll k_{-1}$. Then $\Delta V_K^{\ddagger} = \Delta V_1^{\ddagger} - \Delta V_{-1}^{\ddagger}$ which is the change in volume for $E + S \rightarrow ES$.

(c) $\Delta V_0^{\ddagger} = \Delta V_2^{\ddagger} + \Delta V_K^{\ddagger}$ is given by

$$\Delta V_0^{\ddagger} = \Delta V_1^{\ddagger} - \frac{k_{-1}}{k_{-1} + k_2} (\Delta V_{-1}^{\ddagger} - \Delta V_2^{\ddagger}) \tag{13}$$

and gives the volume change $\Delta V_1^{\ddagger}$ for the transition $E + S \rightarrow (E + S)^{\ddagger}$ in case $k_2 \gg k_{-1}$ or the volume change for the whole step $E + S \rightarrow (ES)^{\ddagger}$ in case $k_2 \ll k_{-1}$.

The slopes of the straight lines in fig. 9, obtained by linear regression, represent the following volumes of activation:

$$\Delta V_2^{\ddagger} = -13.6 \text{ ccm/mole} \,,$$

$$\Delta V_K^{\ddagger} = 44.0 \text{ ccm/mole} \,,$$

$$\Delta V_0^{\ddagger} = 30.4 \text{ ccm/mole} \,,$$

According to eqs. (12) and (13) the interpretation of $\Delta V_K^{\ddagger}$ and $\Delta V_0^{\ddagger}$ depends on the values of k_2 and k_{-1}. As we have no exact information on these values, only the limiting cases $k_2 \ll k_{-1}$ and $k_2 \gg k_{-1}$ are discussed. These are represented in fig. 11, which shows the volume changes during the course of the reaction. On the assumption, that the volume of the activated state $(E + S)^{\ddagger}$ lies between the volumes of E + S and

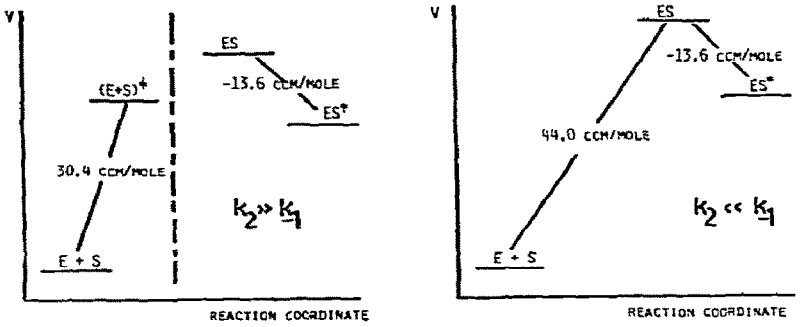

Fig. 11. Models of the volume changes during the reaction.

ES, one gets the same sign for $\Delta V_2^{\ddagger}$ and $\Delta V_{-1}^{\ddagger}$. From eqs. (12) and (13) as well as from fig. 11 it follows that the volume during the formation of the ES complex increases strongly, independent of the values of k_{-1} and k_2.

In chemical reactions large volume changes are often caused by solute–solvent interactions based on electrostatic or hydrophobic effects. Electrostatic forces are sensitive to the ionic strength of the solution. According to the Debye–Hückel theory there should be a dependence of a rate constant k on the ionic strength J:

$$\ln k = \ln k_0 + 1.02\, z_A z_B \sqrt{J}, \qquad (14)$$

where z_A and z_B are the charge numbers of the reacting particles. Neither an influence on v^{max} nor on K_m was found for the dextransucrase, when varying J from 0.005 to 0.2 mole/ℓ, i.e., $z_A z_B$ must be about zero. Thus electrostrictive effects have no significance.

Alternatively, if hydrophobic groups of the enzyme become inaccessible to the solvent during the transition E + S → ES, the volume change and the increased dissociation of ES into E + S could have similar explanations to those given by Penniston [7] for the pressure-induced dissociation of enzymes into subunits. He suggests formation of a hydrophobic ice around non-polar side chains of the enzyme. However, other unpublished experimental results on the ultrasonic denaturation of dextransucrase and calculations using the temperature dependence of K_m lead to the conclusion that the formation of the ES complex causes a decrease in entropy. Since for hydrophobic as well as for electrostatic effects $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ should have equal signs, another explanation seems to be more probable: The formation of the ES-complex leads to a conformational change of the enzyme, resulting in a shape having a larger volume. Such a volume increase of about 40 ccm/mole or 66 Å^3 per molecule changes the volume of the enzyme (100 000 Å^3) by less than 0.1 per cent.

4.2. Discussion of the denaturation effects

The evident linear relationship in fig. 6 between τ and [S] can be explained as follows:

As the enzyme exists in two forms during reaction (E and ES) and as the denaturation of the two forms can proceed with two different rate constants, the denaturation may be expressed by the following relationship:

$$-\frac{d[E]_{tot}}{dt} = k'[E] + k''[ES] = k_d[E]_{tot}, \qquad (15)$$

where $[E]_{tot} = [E] + [ES]$ is the total concentration of the enzyme molecules which are still active.

The second part in eq. (15) gives the experimental rate constant:

$$k_d = k' \frac{[E]}{[E]_{tot}} + k'' \frac{[ES]}{[ES]_{tot}}. \qquad (15a)$$

According to the Michaelis–Menten kinetics the following relation holds:

$$\frac{[E]}{[E]_{tot}} = \frac{K_p}{K_p + [S]} \quad \text{and} \quad \frac{[ES]}{[E]_{tot}} = \frac{[S]}{K_p + [S]}. \qquad (15b)$$

Consequently eq. (15a) becomes

$$k_d = k' \frac{K_p}{K_p + [S]} + k'' \frac{[S]}{K_p + [S]}, \quad (15c)$$

or

$$\tau = 1/k_d = \frac{K_p + [S]}{k' K_p + k'' [S]}. \quad (16)$$

If $k'' = 0$, i.e., when the ES complex is protected against the denaturing influence of pressure, eq. (16) reduces to

$$\tau = \frac{1}{k'} + \frac{1}{k' K_p} [S], \quad (17)$$

or with $1/k' = \tau_0$:

$$\tau = \tau_0 + \frac{\tau_0}{K_p} [S]. \quad (17a)$$

In fig. 6 possibilities other than $k'' = 0$ are also considered. For the linear case, which gives the best description of the results, τ_0 is 12.25 min and K_p = 103 mmole/ℓ. In spite of the large errors of τ the result for K_p is in rather good agreement with the value obtained from the v_p/v_0 data (86 mmole/ℓ). Thus the model described can be regarded as true. Especially, the assumption that only the free enzyme is denatured, whereas the ES complex is stable, seems to be confirmed.

Acknowledgement

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