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VOLUME CHANGES DURING ENZYME REACTIONS. THE INFLUENCE OF PRESSURE ON THE ACTION OF INVERTASE, DEXTRANASE AND DEXTRANSUCRASE

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The pressure dependence of the maximum velocities and the Michaelis constants for the enzymes invertase and dextranase was measured up to 1400 bar. The corresponding activation volumes $\Delta V_C^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ proved to be independent of pressure. Together with data from other sources the meaning of $\Delta V_C^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ is established and the volume profiles of the reactions are constructed. These profiles are similar in contour to the volume profile of the dextran formation catalyzed by the enzyme dextransucrase, but the amount of the volume changes is very much larger for dextransucrase. The evaluation of salt effects shows, that for all three enzymes solvent interactions are not important in explaining the results. The reaction mechanisms seem to be governed by conformation changes of the enzymes. The larger effects in dextransucrase are explained by the produced dextran chain remaining tightly bound to the enzyme and being transported relative to the enzymes position in each reaction cycle.

1. Introduction

In a previous work [1] we reported the pressure dependence of the enzyme-catalyzed formation of dextran by dextransucrase. In this reaction sucrose is split into fructose and a glucosyl-residue, which is inserted into a polymer-chain (dextran) [2]. According to eq. (1)

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

where $\Delta V^{\ddagger}$ is the activation volume and k the rate constant of a reaction step, we obtained large activation volumes, especially for substrate binding, in comparison to the numerous data of non-enzymatic reactions [3].

The aim of the present work is to find out if for other enzymes acting on carbohydrates such large volume changes can also be detected and to elucidate their sources. We chose the enzymes invertase and detranase, because their substrates are sucrose and dextran, which are substrate and product in the reaction of dextransucrase.

The pressure dependence of the action of invertase on sucrose at high substrate concentrations has already been reported by Eyring et al. [4]. In order to include the formation of the ES-complex in this investigation additional high pressure experiments at low substrate concentrations had to be performed.

2. Experimental

Invertase (β-fructosidase, EC 3.2.1.26) was obtained from Boehringer Mannheim (Nr. 104922) and dextranase (endo-dextranase, EC 3.2.1.11) was from Sigma (C 1508, grade 1). Dextransucrase was prepared according to [5]. Household sucrose and dextran 2000 (Pharmacia) were used as substrates.

The experiments were performed at 25°C in 0.05 M acetate buffer. The pH was 4.7 in the case of invertase and 5.2 in the case of dextranase and dextransucrase. The enzyme solutions were stored at 4°C. Storage at room temperature in the case of dextranase caused a reversible decrease of activity. If the enzyme was refrigerated again, it recovered within approximately 12 hours.

The reactions were observed by measuring the produced reducing sugars by the method of Nelson [6] and Somogyi [7]. The glucose content was determined enzymatically [8]. The reaction rates follow the Michaelis–Menten equation. The Michaelis-constants K_m were determined by the method of Lineweaver and

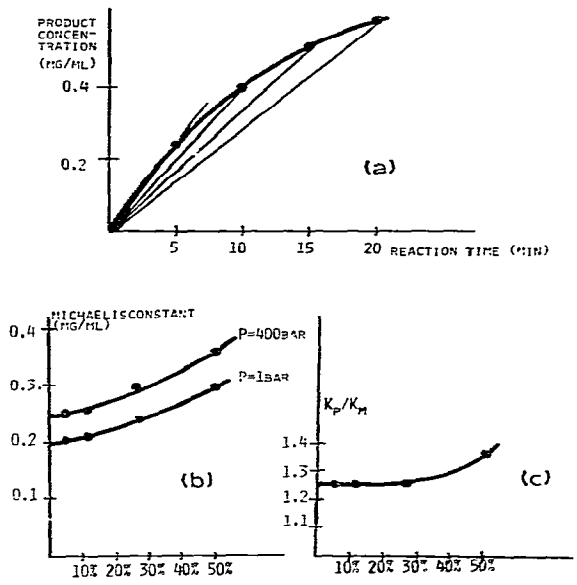

Fig. 1. a) Product concentration as function of time for the reaction of dextranase with 0.7 mg/ml dextran 2000. Apparent initial rates at four reaction times (i.e. four degrees of substrate consumption) are determined by the slopes of straight lines. b) Michaelis constants at two pressures as function of substrate consumption. The constants are calculated on the basis of apparent initial rates using eight substrate concentrations (0.7, 0.8, 1.0, 1.2, 1.5, 2.0, 3.0 and 4.0 mg dextran 2000/ml). c) Ratio of the two Michaelis constants of b).

Burk using initial rates of at least 6, usually 8 concentrations.

For dextranase K_m is very low (0.2 mg dextran/ml [9]). At substrate concentrations of the order of K_m product-time curves showed fairly strong curvature. Thus the smallest concentration used for the determination of K_m was 0.7 mg/ml. But even then a slight curvature in the product time curves caused errors in the determination of initial rates due to consumption of substrate. Thus K_m-values were determined for several degrees of substrate consumption. The true value of K_m was then evaluated by extrapolation to a substrate consumption of zero as shown in fig. 1a and 1b. Fig. 1c shows that it is easier to determine the ratio of two K_m-values at different pressures, because errors cancel each other to a certain degree. Up to a substrate conversion of about 30%, K_p/K_m remains nearly constant (K_p is the Michaelis constant at pressure p).

The high pressure techniques were similar to those described in [1]. In order to prevent denaturation of the enzymes by contact with metal surfaces, the reaction solutions were poured into small glass tubes with elastic lids. Eight tubes were introduced simultaneously into the autoclave filled with water. The elastic lids equilibrated pressure differences between the water and the reaction solution. No denaturation occured at pressures up to 1400 bar.

The volumes of the overall reaction were evaluated by measuring the density changes during reaction. The densities were determined by counting the oscillations of a U-tube filled with reaction-solution (apparatus: Heraeus-Paar DMA 60 and DMA 601).

3. Theory

3.1. The mechanisms of the enzymes

For invertase the mechanism is well accepted [10]:

$$E + Su \rightleftharpoons ESu \rightleftharpoons \underset{G}{EF} \rightarrow E + F, \qquad (2)$$

Su = sucrose, F = fructose, G = glucose, E = enzyme.

For dextranase we assume a similar mechanism

$$E + G_n \rightleftharpoons EG_n \rightleftharpoons \underset{G_m}{EG_{n-m}} \rightarrow E + G_{n-m}, \qquad (3)$$

G_n, G_m, G_{n-m} are polyclucosides with $n, m, n-m$ units $n \geqslant m + 2$.

These reactions may be compared with the action of dextransucrase [11] resulting in the formation of the high polymer dextran G_n:

$$EG_n + Su \rightleftharpoons ESuG_n \rightleftharpoons \underset{F}{EGG_n} \rightarrow EG_{n+1}. \qquad (4)$$

The three mechanisms can be more generally described:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{P_1}{\overset{k_2}{\rightleftharpoons}} EP_2 \overset{k_3}{\rightarrow} E + P_2, \qquad (5)$$

where S is the substrate, P_1 and P_2 are the products.

According to [12] the reaction rate is given by

$$v = (k_c \cdot [E]_0 \cdot [S])/(K_m + [S]), \quad (6)$$

where [S] = substrate concentration and $[E]_0$ = enzyme concentration,

$$k_c = (k_2 k_3)/(k_2 + k_3), \quad (7)$$

$$K_m = (k_{-1} + k_2)\, k_c/(k_1 k_2). \quad (8)$$

3.2. The pressure dependence of K_m and k_c

The pressure dependence of k_c can be described according to eq. (1) by the activation volume $\Delta V_c^{\ddagger}$ (see e.g. Laidler [12])

$$\Delta V_c^{\ddagger} = \Delta V_2^{\ddagger} + (\Delta V_3^{\ddagger} - \Delta V_2^{\ddagger})/(1 + k_3/k_2). \quad (9)$$

Similarly $\Delta V_{Km}^{\ddagger}$, the activation volume describing the pressure dependence of K_m, can be obtained:

$$\Delta V_{Km}^{\ddagger} = -\Delta V_1^{\ddagger} + \Delta V_c^{\ddagger} - (\Delta V_2^{\ddagger} - \Delta V_{-1}^{\ddagger})/(1 + k_2/k_{-1}). \quad (10)$$

Pressure dependent values of $\Delta V_c^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ may be interpreted by compressibility effects. They can be pressure dependent, however, even if the activation volumes $\Delta V_i^{\ddagger}$ ($i = 1, -1, 2, 3$) of the single steps are independent of pressure. This becomes evident, if one inserts the pressure dependent forms of the single rate constants k_i into equations (9) and (10):

$$\Delta V_c^{\ddagger} = \Delta V_2^{\ddagger} + \frac{\Delta V_3^{\ddagger} - \Delta V_2^{\ddagger}}{1 + (k_3^0/k_2^0)\exp[-(\Delta V_3^{\ddagger} - \Delta V_2^{\ddagger})p/RT]}, \quad (11)$$

$$\Delta V_{Km}^{\ddagger} = -\Delta V_1^{\ddagger} + \Delta V_c^{\ddagger} - \frac{\Delta V_2^{\ddagger} - \Delta V_{-1}^{\ddagger}}{1 + (k_2^0/k_{-1}^0)\exp[-(\Delta V_2^{\ddagger} - \Delta V_{-1}^{\ddagger})p/RT]}, \quad (12)$$

k_i^0 ($i = 1, -1, 2, 3$) are the rate constants at normal pressure.

The influence of pressure on $\Delta V_c^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ corresponding to equations (11) and (12) can be larger by orders of magnitude than the influence caused by compressibility effects, which are expected to be not more than a few percent per 1000 bar. On the other hand, $\Delta V_c^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ can be pressure independent only, if the denominator in the last term of eqs. (11) and (12) equals one or becomes very large or if the numerator vanishes.

From eq. (9) and (11) then follows

$$\Delta V_c^{\ddagger} = \Delta V_2^{\ddagger} \quad \text{if } k_2 \ll k_3 \text{ or if } \Delta V_2^{\ddagger} \approx \Delta V_3^{\ddagger},$$

$$\Delta V_c^{\ddagger} = \Delta V_3^{\ddagger} \quad \text{if } k_2 \gg k_3.$$

From eq. (10) and (12) analogeously follows

$$\Delta V_{Km}^{\ddagger} = -\Delta V_1^{\ddagger} + \Delta V_c^{\ddagger} \quad \text{if } k_2 \gg k_{-1} \text{ or if } \Delta V_2^{\ddagger} \approx \Delta V_{-1}^{\ddagger},$$

$$\Delta V_{Km}^{\ddagger} = -\Delta V_1^{\ddagger} + \Delta V_c^{\ddagger} - \Delta V_2^{\ddagger} + \Delta V_{-1}^{\ddagger} \quad \text{if } k_2 \ll k_{-1}.$$

The relations for the rate constants must be satisfied over the whole measured range.

4. Results

4.1. pH and pressure dependence of the kinetic constants

Fig. 2 shows the pressure dependence of ratios of the Michaelis-constants K_p/K_m and the maximum rates V_p/V_0 for invertase (a) and dextranase (b). The respective curves for dextransucrase are given in [1]. The semilogarithmic plots are linear within the experimental errors. Thus the activation volumes are independent of pressure.

Fig. 3 shows the ratio V_p/V_0 for dextranase (p = 1000 bar) at different pH-values. The ratio is independent of pH in the range from pH = 4.5 to pH = 6.0, which is also the pH-plateau at normal pressure. A similar result for invertase has been reported in [4]. Additionally the ratio K_p/K_m for dextranase is independent of pH in the range from pH = 5.2 to 5.65. Consequently, pressure shifts of pH (0.18 pH-units per 1000 bar in acetate buffer [13]) do not cause errors, as all pressure experiments were performed at pH-values in the center of the pH-plateaus.

4.2. Salt effects

For the three enzyme reactions K_m and V at normal

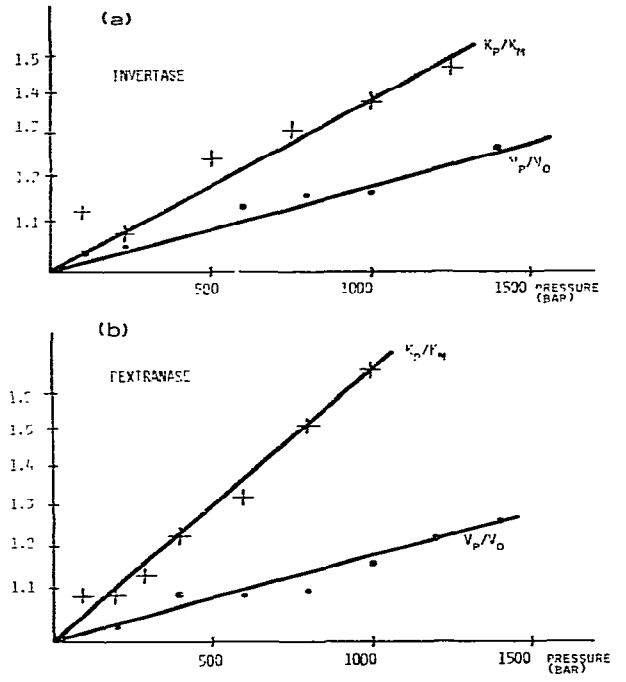

Fig. 2. Semilogarithmic plots of K_p/K_m and V_p/V_0 as function of pressure for invertase (a) and dextranase (b). The slopes of the lines are determined by linear regression. The activation volumes obtained from the slopes using eq. (1) are listed in table 2. The statistical errors of the activation volumes are of the order of 10%.

pressure are not dependent on the ionic strength J in the range from $J = 0.007$ mole/l to $J = 0.1$ mole/l (acetate buffer).

The reactions are inhibited by large KCl concentrations the largest effect occurring in the case of dextransucrase. The ratios K_p/K_m and V_p/V_0, however, are not influenced significantly by KCl, as indicated in table 1.

Table 1
Ratios of V_p/V_0 and K_p/K_m with 0.15 mole/l KCl (0.3 mole/l for dextranase) and in absence of KCl (values in brackets).

	Invertase	Dextranase	Dextransucrase
V_p/V_0	1.26 (1.17)	1.14 (1.17)	1.62 (1.70)
K_p/K_m	1.29 (1.37)	1.70 (1.66)	5.10 (5.25)

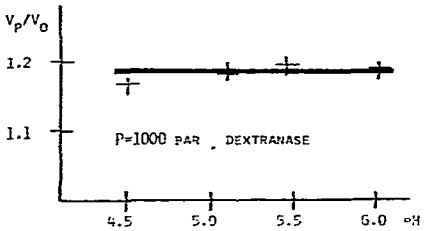

Fig. 3. V_p/V_0 at 1000 bar for dextranase as function of pH (acetate-buffer).

4.3. Volumes of the overall reactions

The reaction volumes ΔV_R in cm^3/mole are obtained using

$$\Delta V_R = (1000/c)\,(\rho_i/\rho_f - 1), \qquad (13)$$

where c is the concentration of converted substrate in mole/l, ρ_i the initial density and ρ_f the density after conversion of the substrate. Fig. 4a shows the change in density for the inversion of sucrose by invertase. A similar curve is obtained for the dextransucrase catalyzed formation of dextran from sucrose. The reaction volumes, calculated by eq. (13) are

ΔV_R (dextransucrase) = −4.1 cm^3/mole,
ΔV_R (invertase) = −7.3 cm^3/mole.

In the case of dextranase this simple method is not very accurate. Dextranase catalyzes the degradation of dextran into smaller dextrans and finally isomaltose according to eq. (3). With a smaller reaction rate, however, isomaltose is converted into glucose in the last phase of the reaction. The glucose formation can not be taken into consideration, as all our kinetic experiments are based on initial rates. Therefore the initial reaction volume has to be determined. Fig. 4b shows the density curve of the dextranase catalyzed reaction. The concentration at the onset of the reaction is 68.42g/l. After 15 min (the end of the linear section in fig. 4b), the concentration of isomaltose and higher dextrans was determined as 101 mmole/l. This value and the corresponding density were used in eq. (13). The result is $\Delta V_R = -2.8$ cm^3/mole. A second experiment with a dextran concentration of 17.1 g/l yielded −3.2 cm^3/mole.

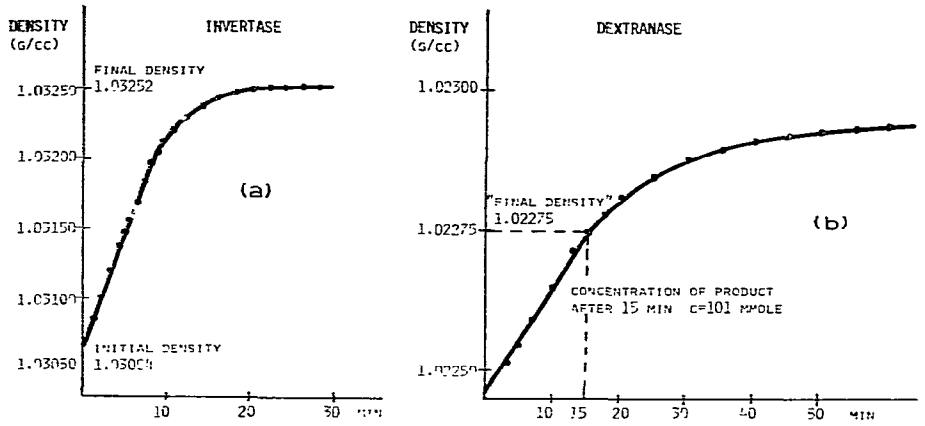

Fig. 4. a) Density of the reaction solution as function of time for invertase with an initial sucrose concentration of 250 mmole/l. b) Density curve for dextranase with an initial dextran concentration of 68.42 g/l.

The mean value is

ΔV_R(dextranase) = −3.0 cm^3/mole.

Table 2 summarizes the obtained volumes of reaction and activation.

5. Discussion

5.1. The volume profiles

All experimentally obtained $\Delta V^{\ddagger}$-values are independent of pressure within the experimental errors. Together with results of other publications the meaning of the experimental activation volumes is as follows:

a) Invertase:

pH-data indicate, that $k_2 \ll k_{-1}$ and $k_2 \ll k_3$ [14], i.e. $K_m \approx k_{-1}/k_1$ and $k_c \approx k_2$. It follows $\Delta V_c^{\ddagger} \approx \Delta V_2^{\ddagger}$ and $\Delta V_{Km}^{\ddagger} \approx -\Delta V_1^{\ddagger} + \Delta V_{-1}^{\ddagger}$.

Table 2
Experimentally obtained volume changes in cm^3/mole.

	$\Delta V_c^{\ddagger}$	$\Delta V_{Km}^{\ddagger}$	ΔV_R
Invertase	−4.2	−8.2	−7.3
Dextranase	−4.4	−13.1	−3.0
Dextransucrase	−13.6	−44.0	−4.1

b) Dextranase:

From pH-data it is probable, that $k_{-1} \ll k_2$ [14], i.e. $K_m \approx k_c/k_1$. Thus $\Delta V_{Km}^{\ddagger} = -\Delta V_1^{\ddagger} + \Delta V_c^{\ddagger}$. $\Delta V_c^{\ddagger}$ is either $\Delta V_2^{\ddagger}$ or $\Delta V_3^{\ddagger}$. The numerical value of $\Delta V_c^{\ddagger}$ is nearly identical with that of invertase. As both enzymes catalyze the hydrolysis of a glycosidic bond, it may be concluded, that the identical $\Delta V_c^{\ddagger}$ values describe the same step in both cases. It follows $\Delta V_c^{\ddagger} \approx \Delta V_2^{\ddagger}$.

Of particular interest is the volume change $\Delta V_{ES} = \Delta V_1^{\ddagger} - \Delta V_{-1}^{\ddagger}$ of the substrate binding step E + S $\rightleftharpoons$ ES. ΔV_{ES} is estimated to be ≈ 13 cm^3/mole as follows: In many reactions of the type A + B $\rightleftharpoons$ AB the activation volume is about $\frac{2}{3}$ of the reaction volume [3], i.e. $\Delta V_{ES} \approx \frac{3}{2}\Delta V_1^{\ddagger} = \frac{3}{2}(\Delta V_c^{\ddagger} - \Delta V_{Km}^{\ddagger})$.

c) Dextransucrase

Studies of the kinetics in the pre-steady-state resulted in $k_{-1} \approx k_2 \approx k_3$ [11]. Together with this the pressure independent $\Delta V_c^{\ddagger}$ and $\Delta V_{Km}^{\ddagger}$ lead to $\Delta V_{-1}^{\ddagger} \approx \Delta V_2^{\ddagger}$ and $\Delta V_2^{\ddagger} \approx \Delta V_3^{\ddagger}$. Therefore $\Delta V_{Km}^{\ddagger} \approx -\Delta V_1^{\ddagger} + \Delta V_{-1}^{\ddagger}$ and $\Delta V_c^{\ddagger} \approx \Delta V_2^{\ddagger} (\approx \Delta V_3^{\ddagger})$.

The overall volume change of the dextran formation is connected with the reaction volumes for the hydrolysis of sucrose and dextran, because dextransucrase splits sucrose and forms a new α-1, 6 glucosidic bond. Therefore the relation

ΔV_R (invertase) − ΔV_R (dextranase)

= ΔV_R (dextransucrase)

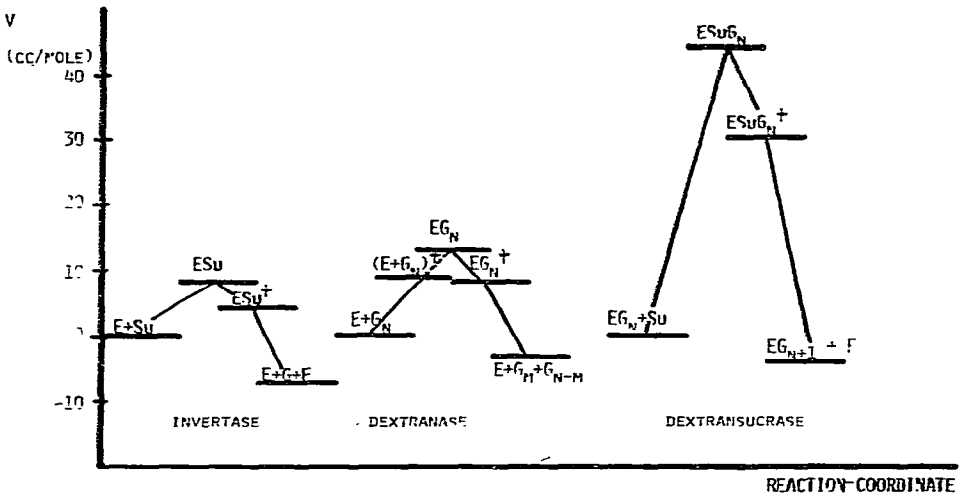

Fig. 5. Volume profiles of invertase, dextranase and dextransucrase.

approximately should hold. The result is $-7.3 + 3 = -4.3$ cm^3/mole, which is in agreement with the experimental value of -4.1 cm^3/mole (see table 2).

Fig. 5 shows the volume profiles of the three reactions. The shapes of the profiles are remarkably similar.

5.2. *Causes of changes in volume*

The activation- and reaction volumes may be caused by the volume changes of the substrates and the enzymes themselves or by the varying interactions with the solvent during the course of reaction. The latter possibility includes: The reaction of two species that are charged or are dipoles, the formation and decay of ion pairs, the accumulation and spread of charges. If during these processes the solvent has free access, electrostrictive volume effects occur. If this is so, then according to the Debye–Hückel-theory the rate constants are dependent on the ionic strength J of the solution, e.g. in water at 25°C $\ln k = \ln k_0 + 1.02 \times Z_A Z_B \sqrt{J}$, where Z_A and Z_B are the charge numbers of two reacting particles. We did not find any dependence on ionic strength (section 4.2) consequently electrostrictive volume effects can be neglected.

Another kind of volume change by solute–solvent interactions originates in conformational changes of a protein, which are accompanied by the transfer of charged or hydrophobic groups from the more hydrophobic interior of the globular protein to the aqueous solution or vice versa. The transfer of CH_4 from hydrophobic surroundings to water, for example, causes a decrease in volume of 22 cm^3/mole [15]. If the water contains salt, e.g. KCl, the water structure is influenced and the volume effect is of a different degree. Consequently, changed exposure of enzyme groups to the solvent is detected by testing the influence of KCl on activation volumes [16]. Such salt effects could not be found, but only a reversible inhibition of the activity by KCl, which is nearly pressure independent, i.e. $\Delta V^{\ddagger}$-values remain constant. The conclusion is, that conformation changes of the enzymes are not accompanied by an altered exposure of enzyme groups to water.

As solvent effects are not important in the investigated group of enzymes, the volume changes have to be explained by variations in enzyme conformation without participation of the solvent and/or by the altering bonds of the converted substrates. In all three reactions a glycosidic bond has to be loosened at the beginning of the reaction. This demands an activation volume of about +10 cm^3/mole [3]. If loosening of

the bond accompanies the formation of the enzyme-substrate complex, this would explain the volume increase for invertase and dextranase in part. The large volume increase for dextransucrase, however, is not explainable by bond loosening. There is an increase in volume of the enzyme molecule caused by a substrate induced conformation change. During the following course of the reaction this initial volume increase is reversed bringing the enzyme back to the start. The similarity of the volume profiles in fig. 5 may indicate similar conformational changes for invertase and dextranase as it does in the case of dextransucrase. The very much larger volume changes of dextransucrase are explainable by the particular mechanism of the enzyme (chapter 3.1): The produced high polymer dextran is tightly bound to the enzyme during a great number of reaction cycles [2], [17]. In each reaction cycle the dextran chain is moved one glucosyl unit relative to the enzyme. It is supposed, that this transport function of the enzyme is connected with large conformational changes.

Acknowledgement

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