Biochimica et Biophysica Acta, 525 (1978) 392—398

BBA 68501

INFLUENCE OF VISCOSITY ON ENZYMATIC REACTIONS STUDIED WITH GLUCOAMYLASE OF *ASPERGILLUS NIGER*

BERNHARD HINSCH and MARIA-REGINA KULA

Gesellschaft für Biotechnologische Forschung mbH., Mascheroder Weg 1, D-3301 Braunschweig-Stöckheim (G.F.R.)

(Received December 28th, 1977)

Summary

Viscosity can be interpreted in terms of transport of momentum and, therefore, it should influence the kinetics of enzyme reactions. A theory, developed by Somogyi and Damjanovich ((1975) J. Theor. Biol. 51, 393—401), is based on this idea. Transport of momentum must always be accompanied by the transport of mass and this second influence of viscosity is a limiting factor for fast reactions in the liquid phase. A third aspect is, that the chemical potentials of the components of viscous solutions are altered. This paper reports experiments concerning the influence of the viscosigens (compounds that increase the viscosity of solvents), alginate, sucrose, and maltose on the kinetic behaviour of glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3). The observed invariance of V and the decrease of K_m are explained by the increase of chemical potentials and restriction of momentum transport.

Introduction

The high viscosity effect, found in most natural systems, is often not taken into account during metabolic studies. Diffusion of all molecules is restricted under viscous conditions and the catalytic properties of enzymes are also affected. Somogyi and Damjanovich [1] suggest a new kind of enzyme regulation generated by the surrounding medium. There is a strong interest in this question as enzymes are increasingly used in industrial processes; for example, production of glucose from starch and isomerization to fructose are reactions which both take place in highly viscous solution.

Theory

An enzyme-catalyzed reaction can be represented by

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P \qquad (1)$$

and the initial rate kinetics of this process can be described by

$$v = V/(1 + k_m/[S]) \tag{2}$$

with

$$K_m = (k_{-1} + k_2)/k_{+1} \tag{3}$$

and

$$V = k_2[E] \tag{4}$$

(E, S, ES, P are enzyme, substrate, enzyme-substrate complex and product, respectively; k is a rate constant; v, V, and K_m are reaction rate, maximum reaction rate and Michaelis constant, respectively).

In case of competitive inhibition instead of Eqn. 2 Eqn. 5 can be derived for the formation of product

$$d[P]/dt = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i}\right)} \tag{5}$$

with the inhibitor constant K_i. If an inhibitor I is acting non-competitively

$$d[P]/dt = \frac{V}{\left(1 + \frac{K_m}{[S]}\right)\left(1 + \frac{[I]}{K_m}\right)} \tag{6}$$

The viscosity η of the medium can directly alter the reaction rates, if a mechanism of diffusion control [2] is valid. Alberty and Hammes [3] in their investigations on fumarase showed that this can well be applied to enzyme reactions. There exists another possible mechanism: in analogy to reactions in the gas phase, the ES complex could be activated by collisions with surrounding molecules. A model was developed for enzyme reactions assuming that on the surface of the protein there are certain sensitive sites [1]. From these sites upon excitation by collisions, energy is directly transferred to those bonds in the active site which are to be cleaved. The solution is treated as a quasi-crystalline network. The molecules are assumed to reach the next site in this network by a jump in the time τ

$$\tau = l^2/6D \tag{7}$$

where l is the distance between neighboring sites and D the diffusion constant.

On the basis of these assumptions, the authors concluded that the breakdown rate constants of the ES complex must be inversely proportional to viscosity in either direction. It is not only viscosity, but also the mass or radii distribution of the solution, that determines the rate constants. The latter effect influences the preexponential portion of the rate constants, as well as the activation energies, in a manner that cannot be quantitated at present. Viscosity changes can be induced by the addition of several types of compounds, such as polymers, which cause appreciable increase of viscosity at low concentrations, or low molecular weight substances. For the purpose of this investigation, it was important to select viscosigens (compounds that increase the visco-

sity of solvents) which least alter the physicochemical properties of the solution and which, at the same time, do not undergo specific interactions with the enzyme. Specific interactions can be detected if one applies the models of competitive and non-competitive inhibition (Eqns. 5 and 6), but these are applicable only at constant viscosity. Other physicochemical parameters of importance are ionic strength, dielectric constants and chemical potentials. The first can readily be controlled. Experimental evidence suggests that the second should not effect reactions with non-ionic participants [4—6]. Variations of chemical potentials may be important when using polymeric compounds to increase viscosity. Rod-shaped macromolecules (e.g. polysaccharides) exert exclusion on spherical molecules according to

$$1/f = K_{av} = \exp(-L(r_c + r_s)^2) \tag{8}$$

f is the activity coefficient of a sphere with the radius r_s in a solution of randomly suspended rods of radius r_c, the sum of their length per ml being L. K_{av} is that portion of the volume of the solution which is available to the spheres. It must be mentioned that in Eqn. 8 only steric effects are considered and energetic effects are neglected. Altered activity coefficients will affect K_m and V, as can be seen from Eqns. 9 and 10:

$$K_m^{app} = K_m \frac{f_{ES}}{f_E f_S} \tag{9}$$

$$V^{app} = k_2[E]f_E \tag{10}$$

Indices of the activity coefficient f are used as explained in connection with Eqns. 1—4. If the substrate is a small molecule, f_{ES} and f_E will have approximately the same values. For these investigations, the enzyme glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) was chosen. It acts usually on polymeric substrates, although it can attack substrates as small as maltose. Glucose always is the only product of the reaction.

Materials and Methods

Glucoamylase (free of α-amylase) from *Aspergillus niger* was purchased from Merck (Darmstadt). The enzyme was assayed polarimetrically with maltose as a substrate, using 0.1 M sodium acetate buffer (pH 5.0) at 40°C (for maltose and sucrose as viscosigens) or 50°C (for alginate). The effects of mutarotation of the sugars were corrected adequately. Before starting the reaction, all solutions were preincubated at the reaction temperature. Mutarotation was also taking place during the reaction and this fact has not been recognized by many workers. The enzyme neither used nor produced the anomers in amounts that were consistent with their equilibrium concentrations. It follows that mutarotation did not cease during the reaction. If, however, the reaction conditions were set to make the catalytic reaction rate the controlling factor, then as mutarotaion equilibrium is approached the experiment can be relevant.

The polarimeter used in these investigation had an accuracy of 1/1000° rotational angle (Perkin-Elmer, Uberlingen, PE 214). Viscosities were measured with an Epprecht type instrument, which provides for the extrapolation of vis-

cosity to zero shearing force (Contraves AG, Zürich, Rheomat 30 with Low-shear 2). Dielectric properties were determined with a Dekameter 30 (Wissenschaftlich Technische Werkstätten, Weilburg/Obb.), an instrument working on the resonance principle in the MHz region.

The purity of sugars was determined enzymatically or by paper chromatography. It was found that only maltose was not pure. It contained 3.7% of glucose as judged by the hexokinase method [10]. The other compounds were glucose (Merck, biochemical and microbiological grade), sucrose (Merck, density gradient centrifugation grade), sodium alginate (Roth, Karlsruhe, purum grade); other materials were of highest purity commercially available.

We used a non-linear optimization method minimizing the squared differences between experimental values and values which were calculated with estimated coefficients. We then applied a weighted linear regression for the inversed initial velocities on inversed substrate concentrations [11]. The results were tested for statistically significant deviation from the results in solutions free of viscosigen.

Experiments and Results

Reaction kinetics of glucoamylase were studied using three different substances. First, the substrate maltose served as viscosigen in concentrations up to

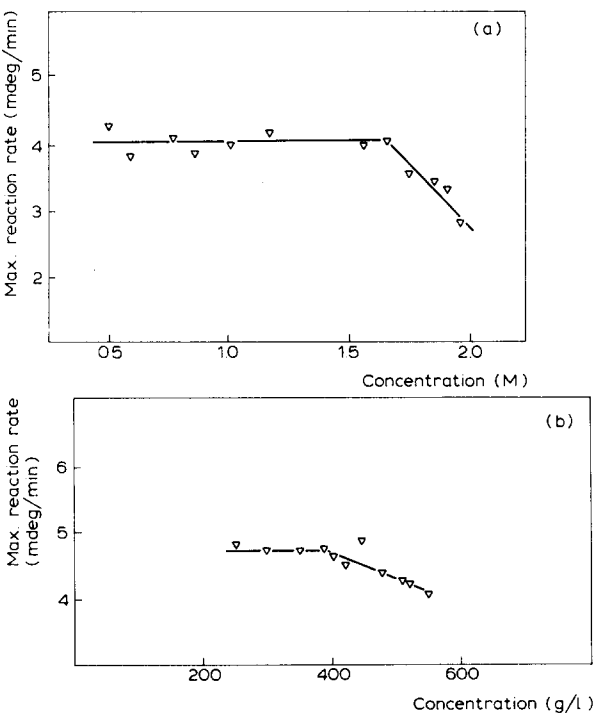

Fig. 1. Glucoamylase with (a) maltose and (b) sucrose as viscosigens. The maximum reaction rate V in millidegrees rotational angle per min at various concentrations of (a) maltose and (b) sucrose. The reaction was monitored polarimetrically at 40°C. In case b the substrate concentration is 0.61 M maltose. The decrease of V above concentrations of 1.7 M maltose and 370 g/l sucrose, respectively, is explained by the fact that at the temperature of the assay the solutions were supersaturated.

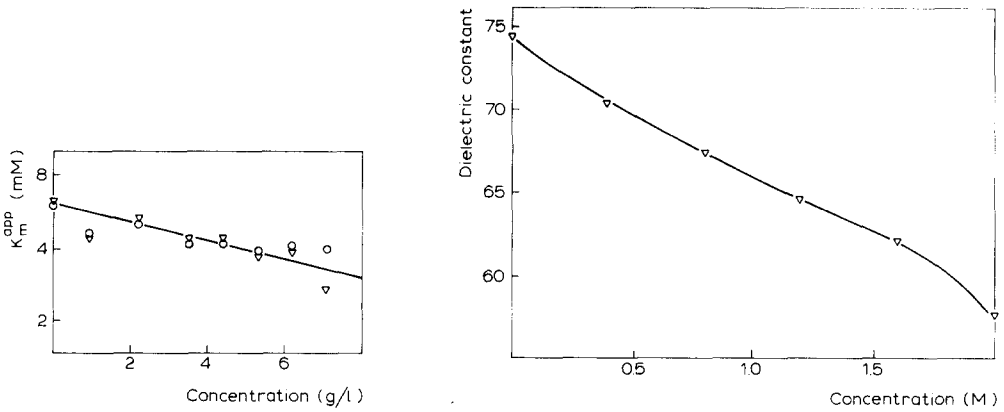

Fig. 2. Glucoamylase with alginate as viscosigen. The apparent Michaelis constant K_m^{app} as a logarithmic function of the alginate concentration. Evaluation of K_m^{app} by weighted linear regression (○) and by non-linear optimization (△).

Fig. 3. Dielectric constant of maltose solutions. Dielectric constant of maltose in H_2O at 40°C as a function of maltose concentration.

2 M. Viscosities were found between $0.8 \cdot 10^{-3}$ and $18 \cdot 10^{-3}$ Pa · s^{-1}. In order to find out if sucrose produced the same effects as maltose, we assayed the enzyme with this second compound and 0.61 M maltose as substrate, under the same conditions. In this case, viscosities ranged from $4 \cdot 10^{-3}$ to $18 \cdot 10^{-3}$ Pa · s^{-1}. Sucrose is only a very poor substrate for glucoamylase. From these experiments, we can only observe the effect of these changes on the maximum velocity, V (Fig. 1). For the determination of K_m, we elevated the temperature to speed up mutarotation. Sodium alginate, in concentrations up to 7 g/l, was used as viscosigen. Ionic strength was maintained at a constant level by addition of KCl. Fig. 2 shows the resulting parameters.

Further experiments were designed to probe the dielectric properties. As the conductivity of alginate solutions was very high, solutions were demineralized before measurements. For alginic acid we could not obtain more than three values at 16°C. Alginate solutions were prepared in water at 0, 0.203 and 0.346 g/l and the dielectric constants measured at 16°C to be 82.5, 85 and 86, respectively. The dielectric constants of maltose solutions are shown in Fig. 3. Values for sucrose are published elsewhere [12].

Discussion

The results of this investigation can be discussed under five aspects: The viscosigens could cause specific interactions with the protein or electrostatic interactions, diffusion control, exclusion effects and activation by collisions to be responsible for the observed effects.

Specific interactions are not involved, as the kinetic data do not fit Eqns. 5 or 6. Electrostatic potentials do not influence the kinetic parameters, in agreement with experiments by other workers [4—6]. The results with glucoamylase are identical for all viscous agents, although they exhibit opposite dielectric properties (Fig. 3). The invariance of V (Fig. 1) is confirmed by statistical evi-

dence. This was established by first applying an F-test which showed whether the variances were comparable. The results were then compared to those obtained in pure buffer by Student's t-test. In no case could we find a significant difference ($P < 0.10$). The decrease in the maximum velocity with maltose and sucrose at high concentrations (Fig. 1) is explained by the fact that these solutions were supersaturated at 40°C. This presumably resulted in an altered structure of the solution, which was also shown by the effects on the dielectric constant (Fig. 3).

The rates of forward and reverse reactions (k_{+1} and k_{-1}, respectively) are not diffusion controlled, as shown by the low K_m values. If the combination of enzyme and substrate to ES is diffusion controlled, one would expect an increase of the apparent K_m (Eqn. 3), which is not found. Also an estimation of the minimum values of the rate constants leads to the conclusion that there is no diffusion control.

In order to explain the observed effects on the Michaelis parameters, we have only to consider exclusion effects and activation by collisions. In the case of glucoamylase, both of these mechanisms apparently apply. Activation by collision is restricted so that the maximum reaction rate should decrease with $1/\eta$, whereas, because of exclusion effects. a linear increase with viscosigen concentration c_V is expected. If we assume, in a first approximation, an exponential relationship between η and c_V, both effects cancel each other and thus the invariance of V (Fig. 1) is explained. The assertion, $\eta \sim e^c$, is purely empirical, as an adequate theory for concentrated solutions has not been developed yet. But Fig. 4 shows that it can be used as a fairly good approximation for solu-

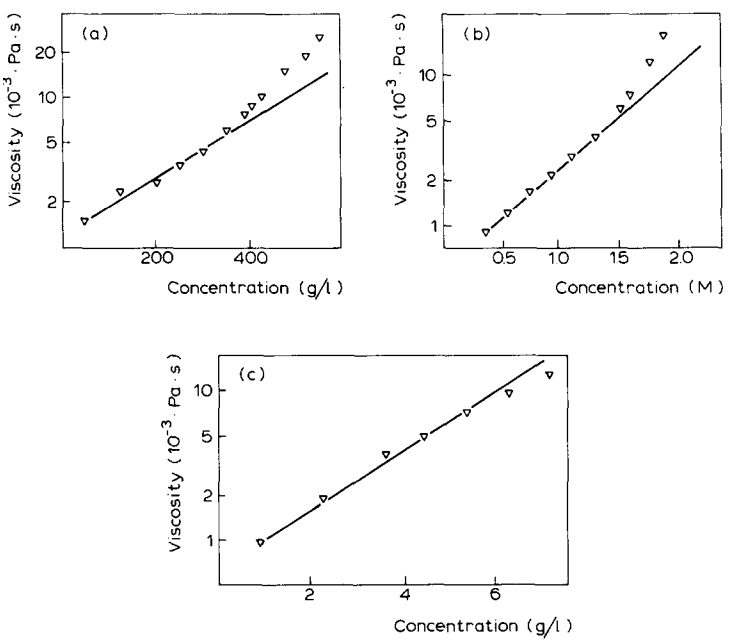

Fig. 4. Dependence of viscosity on concentration of (a) sucrose, (b) maltose, and (c) alginate solutions. Viscosity can be treated as a logarithmic function of the concentration of (a) sucrose, (b) maltose, and (c) alginate solutions in a limted range.

tions of maltose, sucrose, and alginate at the concentrations used in our experiments. The Michaelis constant should vary with reciprocal viscosity because of the collision activation mechanism. Exclusion principles suggest a proportionality to e^{-c} and, with the same approximation as above ($\eta \sim e^{c}$) these predictions are identical. Plotting K_m^{app} logarithmically against c_V (Fig. 2) confirms this view.

Other experiments, using glucose isomerase and α-glucosidase, led to different results (unpublished data) demonstrating that the observed influence of viscosity on reactions catalyzed by glucoamylase cannot be used as a generalization.

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