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Enzyme activity in self-evolving microenvironment. Differential microcalorimetry, UV spectrophotometry, HPLC and X-ray scattering studies [1]

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Abstract

Enzymatic activity of β-D-glucosidase is studied in self-evolving microstructured environments. Activity and microenvironment changes are followed by UV spectrophotometry, isothermal differential microcalorimetry, HPLC and small angle X-ray scattering. The original medium composed of octyl-β-D-glucoside (OG) and water is progressively converted through the enzymatic activity to mixtures of OG–glucose–octanol and water. The influence of the microenvironment on enzymatic activity is demonstrated and quantified for different initial OG compositions and enzyme contents. Phase transitions are simultaneously monitored by calorimetry and spectrophotometry and correlated with studies by other techniques. Lamellar phase appearance is observed by X-ray scattering.

INTRODUCTION

Molecular enzymology devoted to the study of free enzymes in water is useful to elucidate the structure of catalytic centers and physicochemical mechanisms governing biocatalysis. Most experiments are usually done in water with enzymes, isolated from living cells, in a very pure form. However, the properties observed in vitro in aqueous solutions are not directly linked to functioning of these enzymes in vivo, since cells are made of different compartments and organized assemblies, in which subcellular

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structure and the compartmentalization of enzymes play an important role in the regulation of metabolism [1–4]. One way of approach to model enzymes functioning in vivo is to incorporate them into microheterogeneous media formed by surfactant–organic solvent–water mixtures [3,5,6]. Classical studies in micellar enzymology are carried out when the enzyme is placed inside a well characterized microstructure. Conversely, in our case, enzymatic activity is used to create a self-evolving microenvironment in which enzyme kinetics are studied.

In the first case, surfactant–organic solvent–water mixtures spontaneously form different phases, the nature of which depends only on the relative ratio of the components. Enzymes are catalytically active in such media and have been studied in different microenvironments such as direct micelles, reversed micelles, microemulsions or lyotropic liquid crystals [7–9]. When enzymes are placed within a microstructure, activity towards a substrate is measured, and microenvironment structure remains the same during kinetic experiments. In this study, using self-evolving systems, the enzyme environment changes during the progress of reaction [10,11]. Products and substrates modify the enzyme microenvironment, which can cause a phase transition. A model system of octyl-β-D-glucoside (OG)–water (or glucose in water)–octanol plus the enzyme β-D-glucosidase is studied. Enzymatic activity and microenvironment changes are followed by UV spectrophotometry, isothermal differential microcalorimetry, HPLC and small angle X-ray scattering. Using these techniques, microstructure modifications and related kinetic events are characterized.

EXPERIMENTAL

Enzymatic reaction

β-D-glucosidase catalyses the reaction

$$OG + H_2O \rightarrow \text{octanol} + \text{glucose}$$

The original medium composed of OG and water is progressively converted via enzymatic activity to mixtures of OG, glucose, octanol and water. Enzyme substrates (OG–water) and products (glucose–octanol) are the components of the pseudohomogeneous system.

The phase diagram OG–water–octanol shows two monophasic regions. First, L_1 is a micellar region of OG in water (with or without octanol). The critical micellar concentration of OG at 30°C is 22.3 mM (0.65% by mass) (data not shown). The second region L_2 is a microemulsion containing a large proportion of octanol. The L_α region is a lamellar phase. For the X region, structures have not yet been determined (Fig. 1A). During enzymatic hydrolysis of OG, glucose and octanol are formed, so glucose appears as a new constituent of the ternary system. The diagram OG–10% glucose

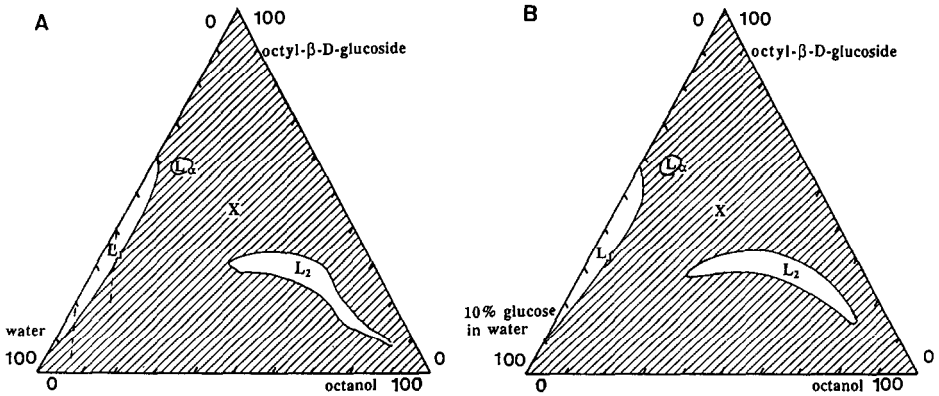

Fig. 1. Phase diagrams for surfactant–water–organic solvent system. The monophasic domains and phase boundaries are shown for octyl-β-D-glucoside–water–octanol (A) and for octyl-β-D-glucoside–10% glucose in water–octanol (B). L_1 is a micellar solution of octyl-β-D-glucoside in water, L_2 is a microemulsion, L_α is a liquid crystalline phase with a lamellar texture and X is a state with a non-determined structure. The broken lines indicate the enzymatic exploration lines followed by the two mixtures examined (20 and 40% OG).

in water–octanol is shown on Fig. 1B. When glucose in water is used instead of water, the domains remain the same and phase boundaries are similar.

The standard buffer in all experiments was 50 mM citrate–phosphate, pH 5.25. As a reference, β-D-glucosidase was assayed on salicin in the same buffer at 37°C. Enzymatic activity was found to be 11 I.U. (μmol glucose min^{-1} per mg solid). β-D-glucosidase activity for OG hydrolysis was determined following glucose production by an HPLC method, as described in [10].

Spectrophotometry

Continuous monitoring of the phase transition, as the enzymatic reaction proceeded, was performed by recording the turbidity at 400 nm using a double beam spectrophotometer (Perkin Elmer λ2). Experiments were carried out at 30°C in 10×10 mm^2 cuvettes on non-agitated reaction mixtures.

Differential calorimetry

Isothermal differential microcalorimetry experiments were performed using an Arion microcalorimeter (sensitivity 95 μV mW^{-1} at 40°C); the temperature was maintained at 30 ± 0.5°C with temperature drift less than 0.05°C. Sample holders of 1.5 and 0.5 ml [12], containing 1.1 and 0.4 ml respectively, were used for 20% and 40% octyl-β-D-glucoside experiments

respectively. Apparatus calibration was performed using the Joule effect, peak surface being measured by weighing [13].

Differential calorimetry measurements were carried out simultaneously with spectrophotometry from the same reaction mixtures. In this regard, reaction mixtures were prepared at 30°C by mixing the OG solution (1.5 ml) with freshly prepared enzyme solution (100 μl), divided into two parts placed in the spectrophotometer cuvette and in the calorimeter sample holder. Therefore, before sample temperature equilibration, calorimetric recording is meaningless.

X-ray scattering

Small angle X-ray scattering experiments were made on the D24 line of L.U.R.E. (Orsay, France) at 1.608 Å. The reaction mixture containing the enzyme was placed inside a thin X-ray capillary of about 1 mm external diameter. The sample holder temperature was thermoregulated at 30 ± 0.1°C.

RESULTS

Enzymatic catalysis was used for exploration of the phase diagram OG–water–octanol. Starting from the micellar region L_1, two initial points were studied: 20% and 40% of OG in buffer. β-D-glucosidase hydrolyses OG, forming octanol and glucose, and leading to an evolution of the reaction mixture which may result in a phase transition.

Reaction progress was continuously monitored by differential calorimetry. Calorimetry is a non-specific technique suitable for monitoring chemical or biochemical processes with which thermal effects are associated [14]. The heat effect (dQ/dt J s^{-1}) for a reaction, catalysed by an enzyme, is given by the relationships (1) and (2) for an enzyme showing Michaelis–Menten kinetics [15]

$$S \gg K_m \quad dQ/dt = \Delta H_R k_0' \nu \tag{1}$$

$$S \ll K_m \quad dQ/dt = \Delta H_R k_1' \nu S \tag{2}$$

ΔH_R, k_0', k_1', ν and S are respectively the molar reaction enthalpy (J mol^{-1}), the zero-order rate constant (V'_{max} mol s^{-1} ml^{-1}), the first-order rate constant (V'_{max}/K_m s^{-1}), the volume of the calorimeter holder (ν ml) and the substrate concentration.

The thermal effect associated with enzymatic OG hydrolysis was recorded as a function of time by differential calorimetry, as described above. An experiment without enzyme enables one to quantify the heat effect resulting from calorimeter equilibrium. Results obtained for 40% OG with two different enzyme concentrations (6.4 and 12 mg of enzyme per g) are shown

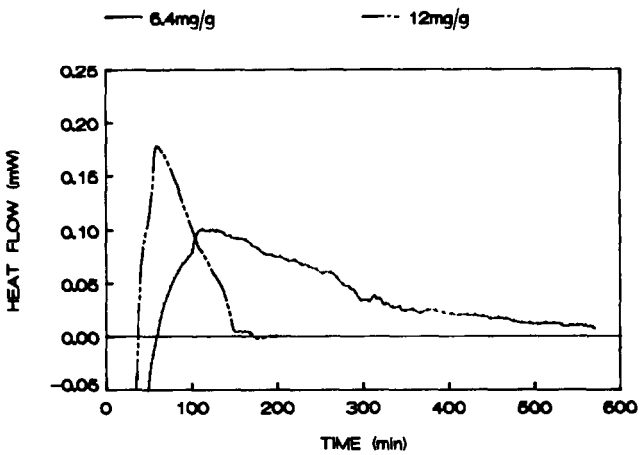

Fig. 2. Microcalorimetry recordings of enzymatic activity. Experiments were made at 30°C with 40% OG micellar solutions with enzyme concentrations of 6.4 and 12 mg g^{-1} (mg of enzyme per g of substrate solution). Negative heat flow values observed at the beginning of the recording correspond to the end of temperature equilibration, and are meaningless.

in Fig. 2. During enzymatic catalysis, heat production increases to a maximum and then decreases until the end of the reaction. The two curves exhibit the same shape and show similar events, especially a steep increase just before the maximum. When the enzyme concentration is approximately doubled, the amplitude of the peak increases by a factor of two whereas the corresponding time is divided by two. The amplitude of the peak and the time scale are in agreement with enzyme concentration. Heat produced is a direct measurement of enzyme activity.

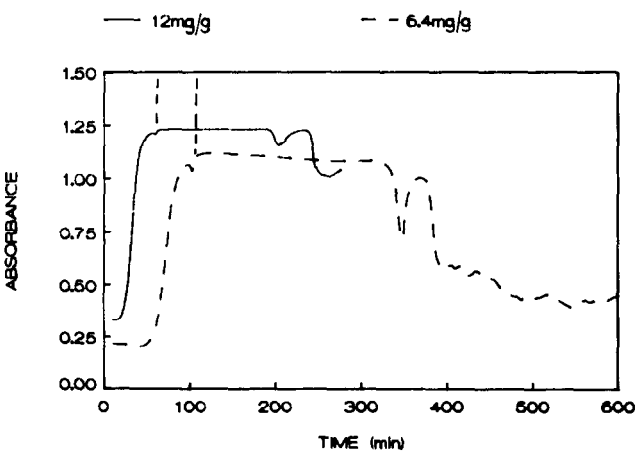

Fig. 3. Turbidity monitoring during phase diagram exploration catalysed by enzymatic activity. Conditions as in Fig. 2; simultaneous recordings of microcalorimetry and turbidity.

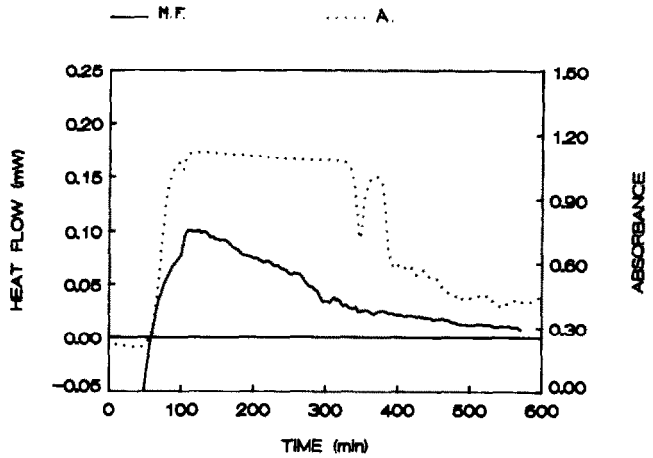

Fig. 4. Comparison of microcalorimetry and light scattering recordings. Simultaneous experiments were made at 30°C with 40% OG solutions and enzyme concentration of 6.4 mg g^{-1}.

Turbidity recordings at 400 nm were performed in the same conditions on the same reaction mixtures (Fig. 3). Optical density is independent of enzyme concentration but, as it was for calorimetry experiments, the time scale–enzyme concentration relationship is respected. Reaction rate is almost proportional to enzyme concentration. Low values at the beginning correspond to the concentrated micellar solution turbidity. Optical density increases in two consecutive steps; the first one, which is presently under

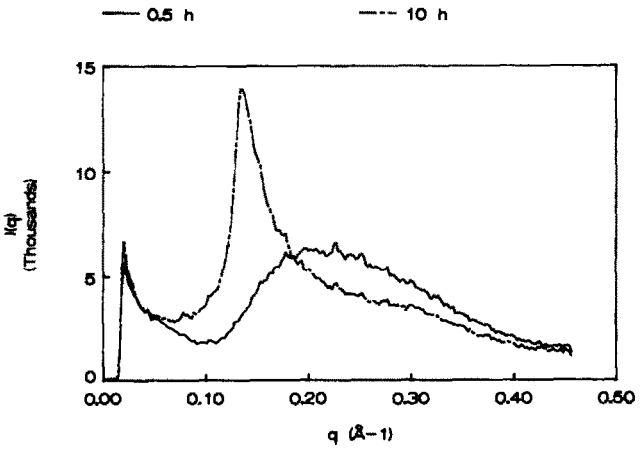

Fig. 5. Small angle X-ray scattering. Spectra obtained from 40% OG micellar solution show the appearance of a new phase (probably lamellar) during the course of phase diagram exploration catalysed by enzymatic activity (30°C, enzyme concentration 6.4 mg g^{-1}).

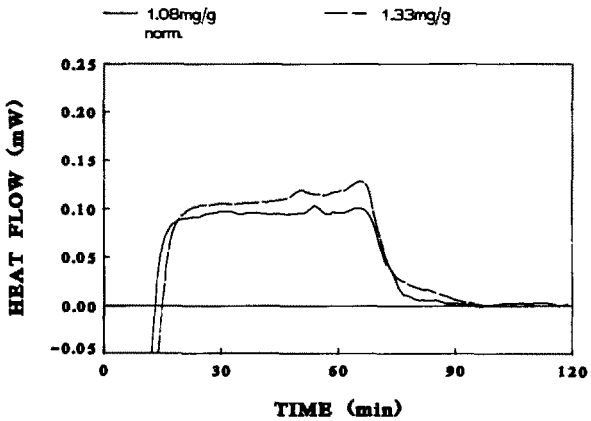

Fig. 6. Correspondence of the phase diagram explorations recorded by calorimetry and obtained from two different enzyme concentrations starting from 20% OG micellar solution.

study, is not yet explained, but coincides with the exit from the micellar phase, the second (vertical dotted line) is linked to the formation of the lamellar phase L_α observed by X-ray scattering measurements. The following events are probably due to the crossing of different boundaries.

Comparison of calorimetry and light scattering recordings show that the maximum of heat flow corresponds to the minimum recorded before the dotted line (appearance of L_α) (Fig. 4). In contrast, later events, mainly attributed to phase separation, observed by both techniques do not seem to be correlated.

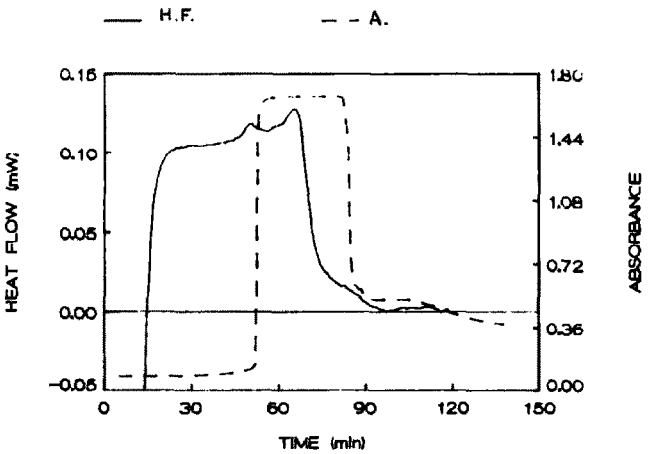

Fig. 7. Heat flow and turbidity correlations. Phase diagram exploration catalysed by β-D-glucosidase at 30°C was made starting from 20% OG micellar solution.

Small angle X-ray scattering has been recorded for 40% of OG under the same conditions. Formation of a lamellar phase after several hours of enzymatic reaction is shown in Fig. 5. The typical micelle scattering pattern corresponds to the initial concentrated micellar solution at 30 min, whereas the Bragg line shown at 10 h corresponds to the lamellar phase first order.

Similar experiments were performed for two enzyme concentrations (1.08 and 1.33 mg g^{-1}) and starting from 20% of OG. The same phenomena as for 40% OG experiments are observed. Figure 6 shows calorimetric profiles normalized to the same enzyme concentration (1.33 mg g^{-1}); a great similarity of heat flow events is observed. The same is also true for light scattering experiments (data not shown). Figure 7 represents calorimetry and turbidity recordings obtained for 1.33 mg g^{-1} of enzyme.

DISCUSSION

The elapsed time at which the heat production peak is obtained for the 40% OG experiment corresponds exactly to an increase in enzymatic reaction rate, as seen by HPLC glucose formation measurements [10]. Isothermal differential calorimetry recordings give a direct representation of enzymatic activity modulations occuring during phase transitions; moreover, the signal amplitude represents enzymatic reaction heat flow.

For the 20% OG experiment, heat flow is approximately constant during the first hour, which indicates a constant reaction rate during the first period of the reaction. From these considerations, a mean value of 80 mW mg^{-1} g^{-1} energy flow was measured for 20% of OG and enzyme concentrations of about 1 mg g^{-1}. HPLC measurement of glucose production indicates a reaction rate of 1.78% of glucose per hour, which allows calculation of an enzymatic catalysed reaction molar enthalpy of 2.8 kJ mol^{-1} of OG.

HPLC experiments give discrete measurements of glucose production as a function of time but enzyme catalysed phase transition boundaries cannot be characterized by this method. Except for microcalorimetry, physicochemical methods such as light scattering or X-ray diffraction do not allow monitoring of the experiment on the phase diagram representation, because there is no direct relationship between these measurements and glucose production or reaction rate. Thus, a combination of these techniques is necessary to study enzyme catalysed phase transitions.

Exploration patterns found for 20% and 40% OG are quite different, and involve differences in the succession of phase transitions and also in enzyme kinetics. A larger study in exploration of the phase diagram is in progress. Nevertheless, the results obtained with different techniques show that subtle enzymatic exploration of a phase diagram is possible when recording a physicochemical parameter sensitive to phase changes or to reaction rate modifications.

The peak observed by X-ray scattering after recording for 10 h (Fig. 5) may correspond to the first order of a lamellar phase. In this case, the mean observed d spacing of 46.5 Å will result from an important swelling of the lamellae compared with values found by Chung and Jeffrey [16] for pure OG (28.5 Å) and other alkylglucoside lamellar phases. Incorporation of octanol in the OG layers may be responsible for the mean d spacing increase.

Different techniques developed to follow enzyme activity in microstructured media have been used with success for the study of OG–water (or glucose in water)–octanol plus β-D-glucosidase model system. Combination of these techniques with structural studies is necessary to study enzyme microenvironment dynamic interactions.

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