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Enzyme-microenvironment dynamic interactions in microstructured media

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Abstract

A new approach for the study of an enzyme's relationship with its own reaction medium is described. The microheterogeneous (micellar and liquid crystalline) media octyl-β-D-glucoside /water (or glucose in water)/octanol plus the enzyme β-D-Glucosidase were used to study enzyme-microenvironment dynamic interactions.β-D-Glucosidase hydrolyses octyl-β-D-glucoside to form glucose and octanol. So doing, it could cause a transition from a one-phase domain of the diagram to a polyphasic system. Starting from each homogeneous region of the phase diagram, the enzyme, due to its catalytic activity, was responsible for different phase changes. The physicochemical properties of the medium changed in proportion to the progress of the enzymatic reaction. The modifications of the microenvironment were correlated with kinetic patterns specific for each phase exploration. Enzymatic reaction rate modifications occuring during the transitions were function of the different microstructured phases in presence but they were also dependent on local concentration and disponibility of octyl-β-D-glucoside monomer.

Studies devoted to free enzymes in aqueous open media do not reflect *in vivo* situation, since subcellular structures and/or compartmentalization of enzymes play an important role in metabolism regulation [1, 2]. Also, most enzymes are believed to work at/or near a water organic medium interface. Such statement is obvious for membrane bound enzymes but also for macromolecular complexes and for enzymes involved in different compartimentalized metabolic pathways such as : glycolysis, Krebs cycle, pentose phosphate pathway [3].The main structural pattern of biological membranes is the flat bilayer of phospholipids as represented by the fluid mosaic model [4] which show membrane bound and incorporated proteins standing inside or near an amphiphilic environment. However, this model is not the only way of organisation and rearrangement of lipids. Many biological membranes are containing a high percentage of non bilayer prefering lipids [5,6]. Evidence for incorporation of different proteins(cytochrome c, methemoglobin, protein from membrane erythrocyte) in such structures has been obtained. Morever, for ATPase and manosyl transferase maximum activity has been found when the formation of intramambraneous particles occured. For lipolitic enzymes (lipases and phospholipases), in some cases, non bilayer lipids have been shown to be the best substrates [7].

Artificial systems composed of surfactant/water/organic solvent mixtures spontaneously form different microstructured phases which are similar to those obtained with natural lipids [8, 9]. In order to reproduce *in vivo* conditions, enzymes may be introduced into these pseudohomogeneous systems. More than 40 enzymes have been studied in reverse micellar systems, for reviews see [10, 11, 12] . As a general rule, kinetics of enzymatic catalysed reactions in reverse micelles obeys the classical Michaelis-Menten scheme [13]. Nevertheless, some enzymes show completely different catalytic properties compared to those in aqueous solutions [14,15]. Catalytic constants are function of the ratio W_0 =[water]/[surfactant] which is mainly responsible for the size of reverse micelles [10]. The catalytic constant can be affected more significantly ; in the case of chymotrypsin a direct relationship between W_0/ kcat and mobility of the protein has been shown [14] .

Apart from reverse micelles other microstructured phases have been used for enzymatic studies. Different enzymes (phosphatases, peroxidase) were placed in lyotropic structures such as lamellar, reverse hexagonal or reverse cubic phases [16]. Enzymatic activity toward a substrate was studied as a function of the microenvironment. Michaelis-Menten kinetic constants have been related to the different microstructures.

I. MICROENVIRONMENT/ENZYMATIC ACTIVITY RELATIONSHIP

In all cases reported so far, microenvironment structure remains unmodified during all kinetic experiments. Moreover, for enzymes which function is to modify structures, such as lipolytic ones, the characterization of the dynamic relationship between the enzyme and the structuration of its microenvironment is seldomly considered [17, 18].The aim of this paper is to study a system in which the enzyme directly modifies its microenvironment and the latter affects itself the enzyme activity. A new approach for the study of an enzyme's relationship with its own reaction medium has been developped [19]. The ternary system octyl-β-D-glucoside /water (or glucose in water)/octanol plus the enzyme β-D-Glucosidase were used. The enzyme hydrolyses the surface active substrate in such a way that the relative ratio among the different components changes, producing a transition from a one phase domain to a polyphasic system which allow the study of enzyme-microenvironment dynamic interactions.

The ternary system octyl-β-D-glucoside/water (or glucose in water)/octanol is used. Enzyme substrates (octyl-β-D-glucoside/water) and products (glucose/octanol) are the components of the pseudohomogeneous system. The enzyme β-D-Glucosidase catalyses the reaction :

$$\text{octyl-}\beta\text{-D-glucopyranoside} + H_2O \longrightarrow \text{octanol} + \text{glucose}$$

The pseudoternary diagram octyl-β-D-glucoside/10%glucose in water/octanol which is shown Fig.1 exhibits two monophasic domains and a liquid crystalline region. The first domain, L_1, is an aqueous micellar solution of octyl-β-D-glucoside. The second domain, L_2, located in the middle of the diagram, is a microemulsion. The third region, L_α, is a liquid crystalline region that appears to have a lamellar texture. Between two monophasic domains, as L_1 and L_α, the delimited region is composed of these two phases in equilibrium.For more details one can refer to[19]. In this system, microenvironment modifies enzymatic activity. Changes observed may be caused by modifications of the substrate availability and more generally by restricted diffusion processes as well as direct influence of the structure on protein activity.

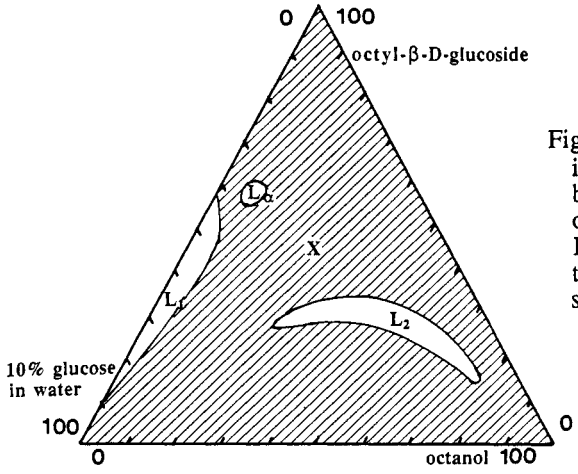


Fig 1 : Phase diagram octyl-β-D-glucoside/10% glucose in water/octanol. The monophasic domains and phase boundaries are shown . L_1 is a micellar solution of octyl-β-D-glucoside in water, L_2 is a microemulsion, L_α is a liquid crystalline phase with a lamellar texture and X is a state with a non-determinated structure (mostly polyphasic domains).

II. WHAT PARAMETERS DO CAUSE ENZYMATIC ACTIVITY MODIFICATIONS?

In order to answer this important question, enzymatic activity has been examined in different domains with different techniques.

(a) L_1 phase: a monophasic domain

In L_1 phase, the micellar solution of octyl-β-D-glucoside is composed of monomer molecules coexisting with micelles. Enzymatic activity has been found affected by a factor of 6 from diluted to concentrated micellar solutions. Which is the physicochemical form of octyl-β-D-glucoside hydrolysed by β-D-Glucosidase : micelle, monomer or both ?

The critical micellar concentration of octyl-β-D-glucoside determined in 50 mM phosphate/citric acid buffer (pH 5.25) at 30°C is equal to 22.3 mM. A micellar solution of octyl-β-D-glucoside formed spontaneously above this value while the monomer concentration remained equal to the critical micellar concentration[22]. Enzymatic activity measurements for octyl-β-D-glucoside hydrolysis were performed by following glucose production, the rate of which was determined for octyl-β-D-glucoside from 3 mM to 100 mM ; enzyme concentration was 0.1 mg/ml. Reaction rate versus

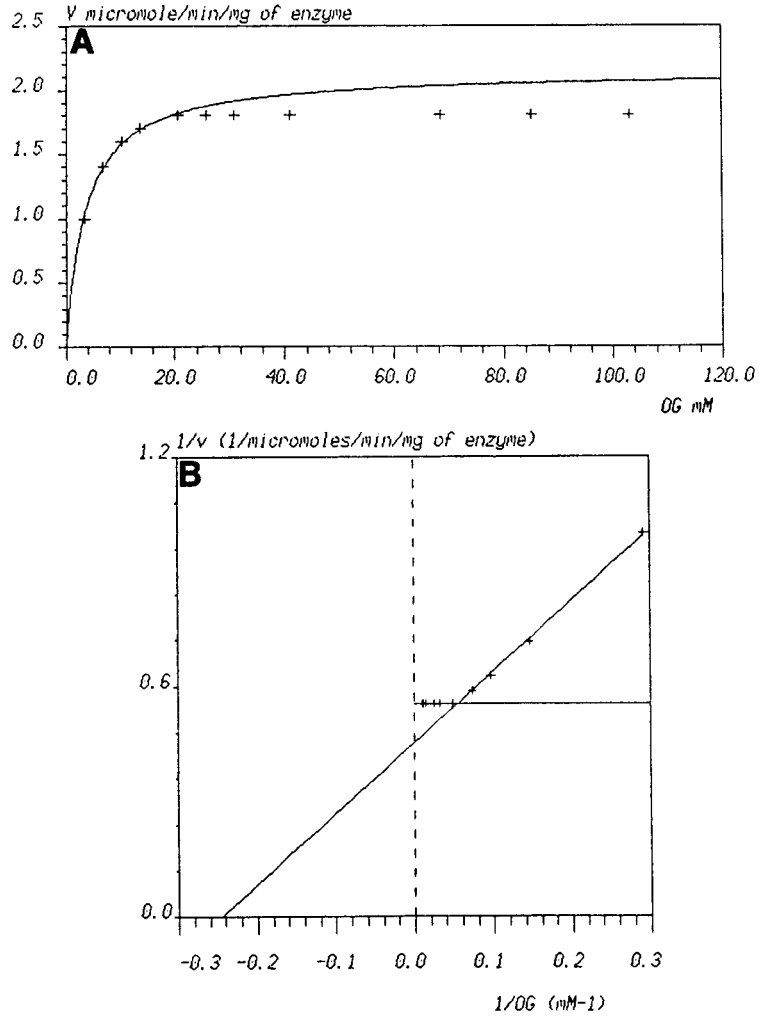


Fig 2 : Determination of β-DGlucosidase actual substrate form.
(A) Reaction rate of glucose production was measured for different octyl-β-D-glucoside concentrations. Full line represents Michaelis-Menten curve obtained with kinetic parameters calculated from Hanes-Woolf plot.
(B) Lineweaver-Burk representation for octyl-β-D-glucoside hydrolysis.

octyl-β-D-glucoside concentration is plotted on Fig. 2A. For octyl-β-D-glucoside concentrations from 3 mM to 20 mM, the relation between the rate of the reaction and substrate concentration followed a classical Michaelis-Menten scheme (Fig. 2B). Apparent kinetic constants were determined from Hanes-Woolf representation [S/V=f(S)]. In these experimental conditions V_{max}= 2.1 µmol. min^{-1}/mg enzyme and K_m = 3.7 mM.

The maximum rate obtained on Fig. 2A was 1.8 µmol. min^{-1}/mg enzyme and was reached for 19 mM as shown by the Lineweaver-Burk representation (Fig. 2B). Substrate disponibility was limited by the critical micellar concentration. It is believed that the low solubility of octanol in water leads to its preferential insertion in octyl-β-D-glucoside micelles and to the formation of mixed micelles. However, in these measurements we neglected the influence of octanol and glucose on the critical micellar concentration.This value is in excellent agreement with the 22.3 mM value, taking into account that the critical micellar concentration of mixed micelles is lower than the one of pure micelles [21]. For concentrations below the critical micellar concentration, the enzyme followed classical kinetics which can be described by the Michaelis-Menten equation. Above 19 mM the rate was limited by the actual monomer octyl-β-D-glucoside concentration so the enzyme does not follow Michaelis-Menten kinetics. At low octyl-β-D-glucoside concentrations the monomer is the actual enzyme substrate and micelles act as a reservoir of octyl-β-D-glucoside molecules. As soon as one molecule of octyl-β-D-glucoside was hydrolysed, one was released by the micelles. At higher octyl-β-D-glucoside micellar concentrations enzymatic activity is reduced (for 20 and 40% octyl-β-D-glucoside reaction rates from 0.93 to 0.28 µmol.min^{-1}/mg of enzyme have been found). Viscosity of the L_1 phase which increases with octyl-β-D-glucoside concentration may explain this activity reduction.

(b) Phase diagram exploration

When β-D-Glucosidase was added to a ternary mixture octyl-β-D-glucoside/ buffer/octanol, octanol concentration increased and the relative ratios of the components were altered such that texture of the reaction medium changed .The enzyme was found to change the relative ratios among water (or glucose in water), octanol and octyl-β-D-glucoside, in such a manner that the physicochemical structure of the mesophases changed continuously as the enzymatic reaction proceeded and eventually resulted in phase changes[19, 22].

For practical purposes, phase diagram exploration was started from each identified homogeneous region (L_1, L_2 and L_α). Inside the L_1 domain, values from 20 % to 55 % of octyl-β-D-glucoside were used. Only one initial composition was studied in L_2 and L_α. Experiments were done at different enzyme concentrations. After a certain period of time, the enzyme was present in a biphasic system. In most of the experiments enzymatic activity was studied from a one phase to a biphasic system. Starting from the monophasic domains, different possible transitions are shown :

a)L_1 ----> $L_1 + L_2$; b)L_1 ----> $L_1 + L_\alpha$; c) L_2 ----> $L_2 + X$. d)L_1 ----> $L_1 + L_\alpha$ and e)L_α ----> $L_1 + L_\alpha$; (Fig. 3). β-D-Glucosidase activity has been studied for the different transitions. Arrows on Fig. 3 represent the different explorations of the phase diagram. Glucose appearance was followed by HPLC, kinetic measurements were performed for each phase transition. These phase diagram explorations

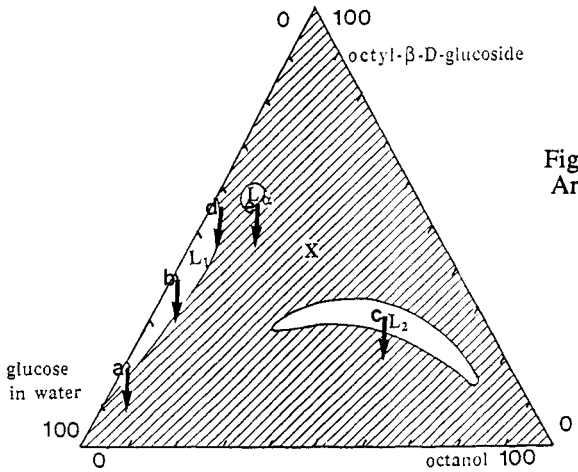


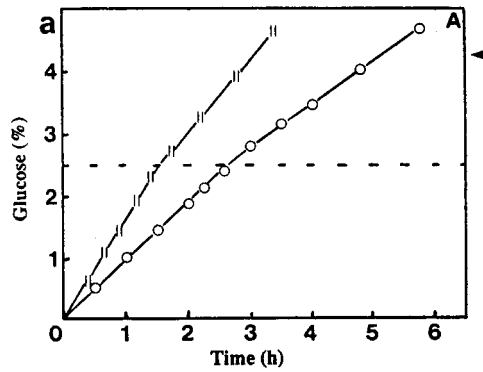
Fig 3 : β-D-Glucosidase catalysed phase transitions. Arrows show enzymatic catalysed phase transitions.

leaded to different β-D-Glucosidase activity recordings, shown on Fig. 4. Both glucose formation (A)(%total mass) and reaction rate (B)(%h-1) were plotted as a function of time. For each initial point, glucose production increased with enzyme concentration (Fig.4a, b and c). For two concentrations of octyl-β-D-glucoside in buffer, lower concentrations of octyl-β-D-glucoside resulted in faster formation of glucose (Fig.4a and b) mainly due to an increase of viscosity as octyl-β-D-glucoside concentration increased. In all cases, as the medium structure changed, the velocity of glucose production also evolved.

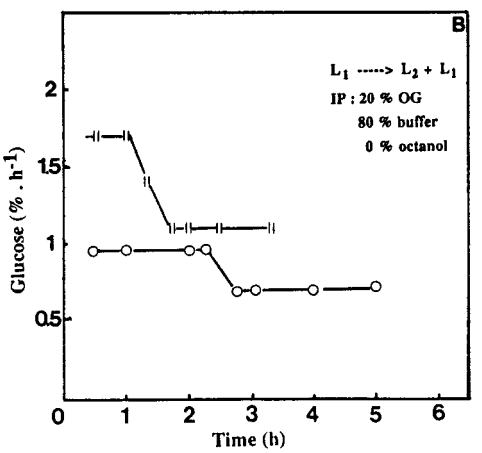
For 20% octyl-β-D-glucoside experiments (Fig 4a), as the micellar boundary was reached, the rate of the reaction decreased. The continuous decreasing of velocity correspond to the transition from L_1 to $L_1 + L_2$.The two curves of Fig 4a show that enzymatic activity is directely related to enzyme concentration. For 40 % octyl-β-D-glucoside experiment (Fig. 4b), the rate of the enzymatic reaction was significantly affected when the phase transition occured. The rate of the reaction decreased and increased rapidly at the phase boundary, corresponding to a peak observation ; the amplitude of this peak increased with the enzyme concentration and was particular to the transition from L_1 to $L_1 + L_\alpha$.

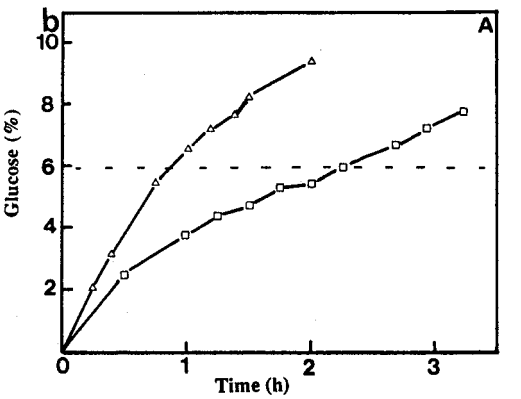
β-D-Glucosidase also catalysed the exit from the microemulsion region, through the transition L_2 ----> $L_2 + X$ (Fig. 4c). The kinetic pattern obtained is an intermediate between the continuous decrease observed for 20% octyl-β-D-glucoside in buffer and the jump in velocity obtained when the lamellar phase is involved in the phase transition.

For 55% octyl-β-D-glucoside, it was possible to study two different initial compositions corresponding to different homogeneous phases. The enzyme was responsible for the two transitions L_1 ----> $L_1 + L_\alpha$ and L_α ----> $L_1 + L_\alpha$. It was possible to generate the L_α phase and to provoke its demixtion. In both cases, the kinetic patterns were similar to the jump obtained with 40% octyl-β-D-glucoside in buffer. When the lamellar texture was involved in the transition, the kinetic pattern showed a jump at the phase boundary.



← Fig. 4a





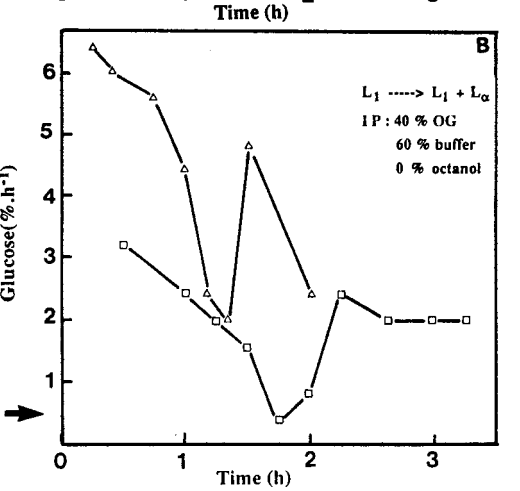
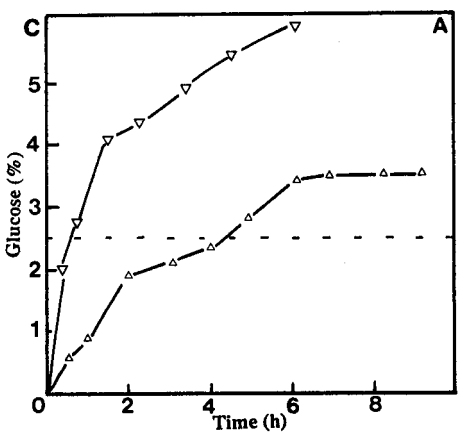


Fig. 4b →



← Fig. 4c

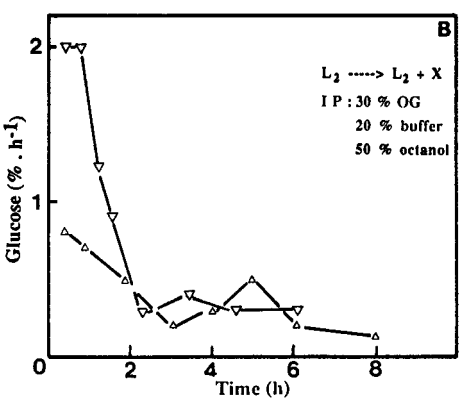


Fig. 4 : β-DGlucosidase activity for different phase transitions. For each phase transition, kinetic measurements are performed. β-D-Glucosidase activities for the different transitions are shown :

Fig. 4a, 4b and 4c corresponding to arrows on Fig. 3. A : Glucose(% mass) was measured as a function of time (h). B : The rate of reaction (% glucose.h^{-1})was plotted as a function of time (h). Enzyme concentrations were :

(- ○ -) 0.55 mg / g, (-|| -) 1.1 mg / g, (- □-) 10 mg / g, (- △ -) 20 mg / g ,(- ▽-) 30 mg / g.

The dashed line (----) symbolyzes the percentage glucose at the phase boundary. IP gives the initial point composition.

These results could be explained by the medium microstructure evolution which resulted from the enzyme activity. Thus, it was possible to observe the differences in reaction velocities before and after the phase transitions. Moreover, the modifications of the medium are correlated to the kinetic patterns characteristic of each phase transition. For the transition L_1 ----> $L_1 + L_2$, the continuous decreasing in velocity as the transition proceeded reflects the difference of velocity observed when the enzyme was located in each phase. Conversely, the peak observed for the transitions L_1 ----> $L_1 + L_\alpha$ and L_α ----> $L_1 + L_\alpha$ is not a linear function of the velocities in L_1 and L_α and should then be due to mesophase reorganization. In such concentrated substrate regions β-D-Glucosidase did not obey Michaelis -Menten scheme as shown on Fig. 2. For a same initial point, rates are not proportional to enzyme concentration. Also it is difficult to quantatively compare the different enzyme catalysed phase transitions. Reaction rates in experiments with concentrated octyl-β-D-glucoside in buffer were from 0.5 to 6% of glucose per hour for enzyme concentrations from 0.5 to 20 mg/ml of solution which correspond to 0.926 and 0.277 µmol. min^{-1}/mg of enzyme, respectively [one % of glucose per hour correspond to 926 µmol. $min^{-1}.l^{-1}$]. Diffusion and solubility of octyl-β-D-glucoside monomer in the different microstructures could be responsible for this decrease of 2 to 6-fold in reaction rate compared to the maximal rate obtained at the critical micellar concentration. Reaction rate modifications as a phase transition took place, were probably due to local concentration and disponibility of octyl-β-D-glucoside monomer at the boundary although the direct influence of the structure on enzyme activity cannot be ruled out.

In order to corroborate the rapid rate modifications observed at the boundaries and to compare different enzyme catalysed phase transitions we correlated kinetic patterns and phase transition events in a continuous manner.

(c) Enzymatic activity monitoring

β-D-Glucosidase is responsible for different phase transitions as discussed from Fig.3 and 4, which show specific kinetic patterns. HPLC measurements of glucose formation only gave discrete values from which it is difficult to deduce precise kinetics. In order to define and precisely correlate kinetic events and phase transitions, we developed methods to continuously monitor the reaction progress. Phase transitions were followed by recording turbidity at 400 nm while reaction progress was continuously monitored by differential calorimetry.

Calorimetry is a non specific technique suitable for monitoring chemical or biochemical processes as soon as thermal effects are associated [23]. Combination of both techniques allowed us to continuously follow phase transitions and kinetic events simultaneously on the same reaction mixtures.
Experiments were performed for 20 and 40% octyl-β-D-glucoside in buffer corresponding to the transitions L_1 ----> $L_1 + L_2$(a) and L_1 ----> $L_1 + L_\alpha$ (b) respectively. Fig 5A shows results obtained for 20% octyl-β-D-glucoside.

The phase transition corresponds to the rapid increase in optical density when the transition L_1 ----> $L_1 + L_2$ occurs, after crossing the boundary light scattering resulted in a high absorbance value. Heat production was approximately constant during the first hour. This constant rate of glucose production observed by both HPLC and microcalorimetry attests that there is no influence of both glucose and octanol concentrations on kinetics and confort the non-participation of the micellar octyl-β-D-glucoside as actual enzyme substrate. From these considerations, a mean value of 80mW/mg/g energy flow was measured for 20% OG and enzyme concentrations of about 1mg/g. HPLC measurement of glucose

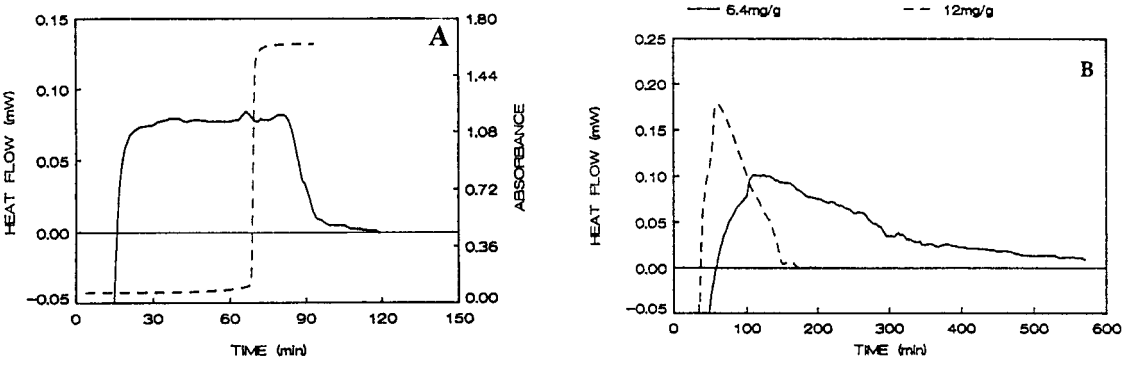


Fig.5 : Continuous monitoring of β-D-Glucosidase catalysed phase transitions
Dotted line represents turbidity at 400 nm as a function of time. Full line shows the corresponding calorimetric profile. (A) Initial point : 20% octyl-β-D-glucoside in buffer ; enzyme concentration was 1.08 mg /g. (B) Initial point : 40% octyl-β-D-glucoside in buffer ; enzyme concentrations were 6.4 and 12 mg / g. First event recorded by calorimetry correspond to calorimeter thermal equilibration.

production indicate a reaction rate of 1.78 %glucose/h which allow to calculate an enzymatic catalysed reaction molar enthalpy of 2.8 kJ/mole of octyl-β-D-glucoside.When the transition occured, a bump reflected a little increase in the reaction rate. After this bump, heat production came back to the precedent level. Finally, the reaction rate decreased as in the correspondingHPLC experiment. Calorimetry is more sensitive and allow to monitor reaction rates.

Results obtained for 40% octyl-β-D-glucoside are shown on Fig. 5B. Calorimetric profile showed a heat production peak occuring when the lamellar phase appeared. After this, heat production slowly decreased down to the baseline which can result from phase separation. Heat production profile has the same shape as the curve showing reaction rate evolution as a function of time Fig.4b-B ; the peak observed from calorimetric measurements corresponds to the jump described from HPLC experiments. Exploration patterns found for 20 and 40% OG are quite different, and involve differences in the phase transitions succession and also in enzyme kinetics. A larger study of the phase diagram exploration is under 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.

CONCLUSION

The microheterogeneous (micellar and liquid crystalline) media octyl-β-D-glucoside /water (or glucose in water)/octanol plus the enzyme β-D-Glucosidase was used to study enzyme-microenvironment dynamic interactions. The physicochemical properties of the medium changed in proportion to the progress of the enzymatic reaction. Starting from each homogeneous region of the phase diagram, the enzyme due to its catalytic activity, was responsible for different phase transitions. Using techniques such as differential microcalorimetry and turbidity measurements, the modifications of the microenvironment were correlated to kinetic patterns corresponding to each phase transition in a continuous manner. Furthermore, we determined that octyl-β-D-glucoside monomer was the actual enzyme substrate. The variations of the enzymatic reaction rates observed during the phase transitions are a function of the different phases involved at the boundary but they also depend on local concentration and disponibility of octyl-β-D-glucoside monomer in these restricted environments.

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