Influence of Environment Modifications on Enzyme Catalysis

Comparison of Macromolecular and Molecular Effects of Cosolvents on Lipoxygenase Reactions

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INTRODUCTION

With the emergence of biocatalysis in nonconventional media, it has been pointed out that enzymatic events are highly sensitive to modifications of the microenvironment and that the nature and state of the solvent greatly influence the reactivity of enzymatic proteins.[1] Cosolvent-containing media may thus be used to elucidate the relationship between the catalytic activity of the protein and the surrounding aqueous solution and may provide a possible model to approach *in vivo* catalysis.

Soybean lipoxygenase-1 had previously been studied in low-hydrated media with linoleic acid as a substrate and several significant differences were reported[2,3] in relation to changes of the reaction medium: either an amplification or decrease of the dioxygenation reaction, variations of specificity, and modifications of secondary reactions. Those results indicated a similar qualitative behavior of the enzyme regardless of the cosolvent used, whereas quantitative differences implied the identity of the additive. Both macroscopic and microscopic influences may be evoked: the former deals with a general effect of the additives on the structuration of the medium and its physicochemical and thermodynamical properties; the latter implies an effect of the cosolvents, at the molecular level, on the structure of reacting molecules.

The purpose of this work is to compare the effects of water-soluble cosolvents at molecular and macroscopic levels and to contribute to the determination of the relation between their presence in the reaction medium and the catalytic behavior of soybean lipoxygenase-1.

MATERIALS AND METHODS

Type-1 lipoxygenase was purified from soybean seeds as previously described.[4] Linoleic acid and arachidonic acid were purified and used at a final concentration of

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300 μM. Dioxygenation activity was measured using a polarographic method convenient for lipoxygenase activity measurement in cosolvent-containing media.[5] Immobilization was performed with a co-cross-linking method using bovine serum albumin and glutaraldehyde at subzero temperature.

RESULTS

Macroscopic Effects

First, the consequences of the addition of water-soluble cosolvents (glycerol, sucrose, and salts) to the aqueous reaction medium were considered at a macroscopic level.

Water Activity

Water activity is a thermodynamical parameter that reports on the global effect of a compound on the availability of water. Lipoxygenase (LOX) was chosen as a model enzyme as it is very active ($k_{cat}/K_M = 5 \times 10^6\ M^{-1} \cdot s^{-1}$) and does not use water as a reactant. Soybean lipoxygenase-1 activity was reported as a function of the reaction-medium water activity (FIGURE 1). When the additive concentration increases, the medium water activity decreases. The curves show a maximum, whereas an increasing function is usually observed. Moreover, the highest lipoxygenase activity was not obtained at a single water activity value, but the maximum depended on the identity of the additive. However, a high a_w (> 0.85) is required to observe a significant activity and a narrow range of high water activity (0.94 to 0.98) contained all the observed maxima. The same behavior was observed for linoleic and arachidonic acids, even though the two substrates differ in their carbon number and unsaturation.

Viscosity

The tested additives are viscosigens. Previous studies demonstrated that enzyme activity may be very sensitive to the medium viscosity. The limiting effect of viscosity on lipoxygenase activity was high for viscosity values $\geq$ 20 mPa·s (FIGURE 2). For lower values, the activity rate was not affected by viscosity, with activities even higher than in buffer being observed; the maxima lay between 2.5 and 9 mPa·s. The influence of viscosity was dependent on which substrate was used: the reaction was less affected by a viscosity increase with arachidonic acid than with linoleic acid.

pH and Ions

Type-1 soybean lipoxygenase is known to present a maximal activity with a single peak at pH 9.0, whereas other enzymes are more efficient at pH 7.0.[6] Moreover, using iron EPR, the sensitivity of the enzyme to ions has been demonstrated.[7] We tested the influence of pH without buffering the reaction medium by using diluted solutions

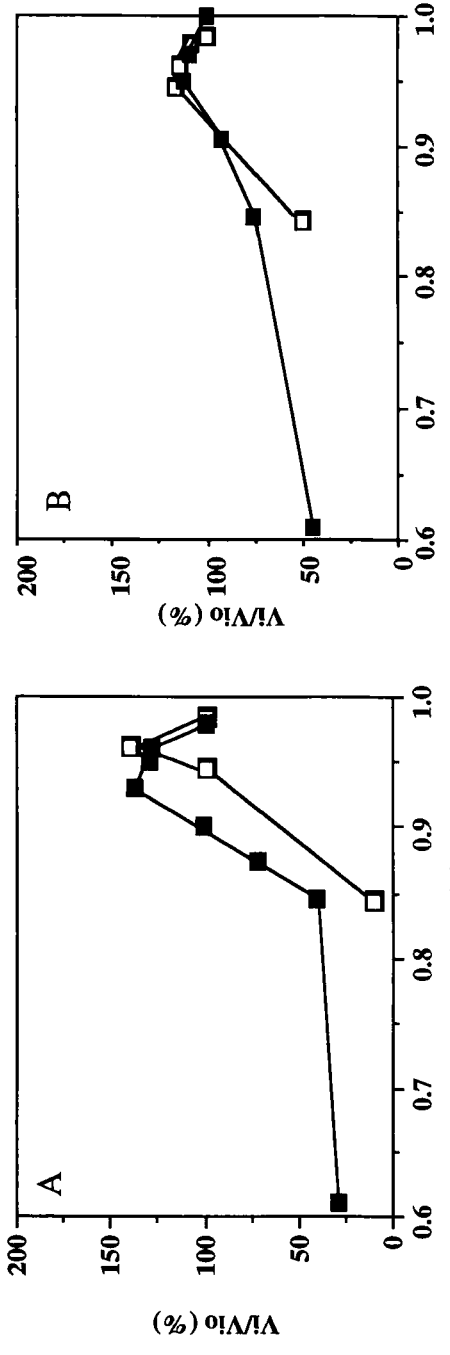

FIGURE 1. Relative activity of soybean lipoxygenase-1 as a function of water activity of the reaction medium containing glycerol (■) or sucrose (□). Substrate: (A) linoleic acid; (B) arachidonic acid. V_{i0} is the initial actrvity in the absence of cosolvent, equal to 120 μmol/min·mg LOX.

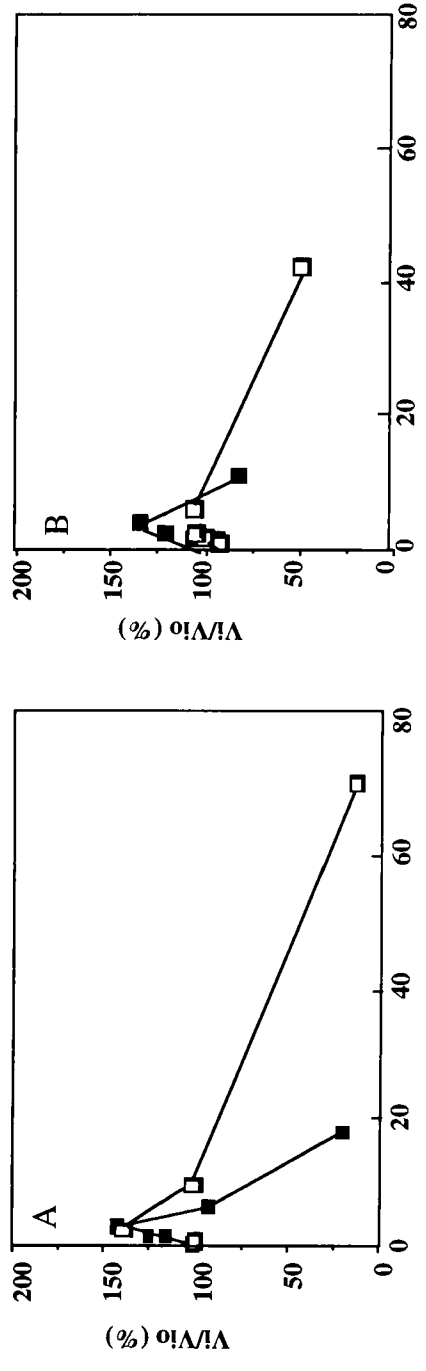

FIGURE 2. Relative activity of soybean lipoxygenase-1 as a function of viscosity of the reaction medium containing sorbitol (■) or sucrose (□). Substrate: (A) linoleic acid; (B) arachidonic acid. V_{i0} is the initial activity in the absence of cosolvent, equal to 120 μmol/min·mg LOX.

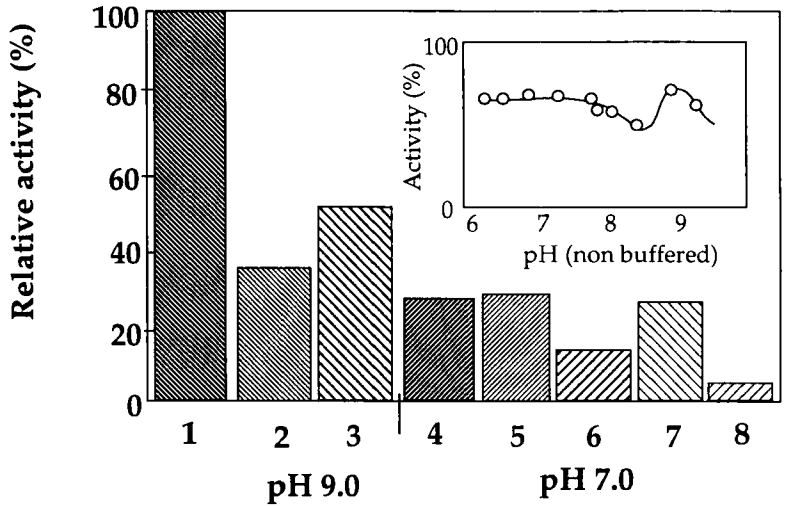

FIGURE 3. Lipoxygenase relative activity as a function of buffer at pH 9.0 for buffers 1, 2, and 3 and at pH 7.0 for buffers 4 to 8; 100% activity is equal to 120 μmol/min·mg LOX. Buffers—1: 0.1 M pyrophosphate buffer; 2 and 5: NaOH; 3 and 4: 0.04 M Veronal buffer; 6: 0.1 M Na-phosphate; 7: 0.1 M Tris-maleate; 8: 0.1 M K-phosphate. (Inset) Lipoxygenase activity as a function of reaction medium pH in nonbuffered media.

of NaOH and HCl; no pH variation is induced by the reaction itself. The usual pH dependence is not observed and maximal activity was measured at both pH 7.0 and pH 9.0. Several buffers were thus compared (FIGURE 3). The influence of ions is more important than the one of pH.

Although a macromolecular effect may drastically affect lipoxygenase catalysis for high cosolvent concentrations, a low salt, sugar, or polyol content may implicate a direct effect on the enzyme at the molecular level.

Effects at the Molecular Level

The influence of cosolvents on enzyme structure was considered. In a previous study,[3] slight differences in enzyme conformation induced by the addition of polyol to pyrophosphate buffer were evident. A modification of the position of aromatic residues was observed as well as an activation of the enzyme. Here, immobilization was used to further investigate the relationship between activity and microenvironment. It has been verified that immobilization restrains molecular movements. Lipoxygenase was immobilized in one buffer and used in another (TABLE 1). To exert a positive influence on lipoxygenase, pyrophosphate must be located in its microenvironment. As this influence is conserved after immobilization when used in another buffer, as previously observed for sorbitol,[2] we may suppose that it proceeds through similar slight variations in structure.

DISCUSSION

In order to evaluate the role of the reaction medium on the catalysis of lipoxygenase at a macroscopic level, the relation between various physicochemical parameters and enzyme activity was explored. Soybean lipoxygenase presented unusual dependences on these parameters; an optimal cosolvent concentration was observed for each substrate. The global parameters describing the reaction medium are not sufficient to totally control the catalytic behavior of the enzyme. However, by varying both the additive used to modify the environment and the substrate, this allowed for the determination of a critical range for each parameter corresponding to a maximal enzyme activity. The important point is not the actual value of these physicochemical parameters, but the fact that they are indicative of a particular organization of water around the enzyme, the substrate, the cosubstrate, and the cosolvent molecules. All the activity maxima correspond to a single range of additive concentration varying between 25 and 40 g of additive/100 g of solution.

For high concentrations of additives (above 40 g of additive/100 g of solution), reactant hydration and diffusion are not optimal—as indicated by water activity and viscosity measurements—which explains the decrease of enzyme activity. For low concentrations of additives (below 25 g of additive/100 g of solution), the media present almost the same water activity, viscosity, or surface tension regardless of the additive used. Thus, the quantitative differences observed in enzyme activity may implicate the identity of the used additives. The various effects of ions and pH are other illustrations of the limitation of global parameters to describe lipoxygenase behavior. The different cosolvents could exert different influences at the molecular level as already evoked.[2]

Using a spectroscopic method, it has been shown[3] that some slight structural modifications are induced by cosolvents, with the amplitude of the variation depending on the used additive. This observation is confirmed by fluorescence studies. Immobilization with covalent bonding was here used as a tool to "freeze" the enzyme

TABLE I. Influence of 0.01 M Pyrophosphate on Lipoxygenase Activity in Various Conditions

Immobilization and Catalysis Conditions	Activity (μmol/min·mg LOX)	Relative Activity (%)
No immobilization; activity measured at pH 9.0 in 0.04 M Veronal buffer	87	100
No immobilization; activity measured at pH 9.0 in 0.01 M pyrophosphate buffer	123	140
LOX immobilized in 0.04 M Veronal buffer, pH 9.0; activity measured at pH 9.0 in 0.01 M pyrophosphate buffer	15	100
LOX immobilized at pH 9.0 in 0.01 M pyrophosphate buffer; activity measured at pH 9.0 in 0.04 M Veronal buffer	21	138

structure. These experiments have shown that the variations of enzyme structure are directly implicated in the activation of the enzyme and that ion-induced structural variations are additive to the ones produced by sugars and polyols.

Both macromolecular and molecular effects seem to be involved in the modification of lipoxygenase catalysis induced by the addition of water-soluble cosolvents to the reaction medium. It now appears unavoidable to consider that cosolvents exert two opposing effects on the enzyme:

(1) even at low concentrations, they induce slight conformational variations that may be favorable for enzyme activity;
(2) over a threshold of high additive concentration, the macroscopic influences are significant and they prevent the enzyme from expressing its activity normally.

The net influence of the additives on enzyme activity is thus dependent on the relative importance of these two antagonistic effects, directly related to the cosolvent chemical nature and concentration.

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