Influence of the hydrophobicity of lipase isoenzymes from *Candida rugosa* on its hydrolytic activity in reverse micelles

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Received 15 December 1994; revised version received 25 January 1995

Abstract Two isoenzymes of *Candida rugosa* lipase, having the same mol.wt., size and similar aminoacid sequence, were studied in reverse micelles of AOT. The results demonstrated the relevance of lipase hydrophobicity in reactions in anionic micelles. This is a key factor in mitigating the inhibition effect of charged micelles. The more hydrophobic isolipase A was a better biocatalyst for hydrolytic processes in these systems. Its α-helix content increased from 31% to 49% of the total structure in reverse micelles. A fluorescence study indicated a more apolar environment for the more hydrophobic isolipase A. Emission spectra of this isolipase in the AOT systems were blue shifted. At ω_0 values where each isolipase presented its maximum activity, a decrease of the emission intensity of Trp was found. An enzyme and substrate dependence of optimal ω_0 is reported. The different interaction of isolipases A and B with the micellar system produced an opposite ω_0 dependence to their stabilities. The more hydrophobic lipase A had higher stability at higher droplet sizes.

Key words: Candida rugosa; *Candida cylindracea*; Lipase; Reverse micelle; AOT; Fluorescence; Circular dichroism

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes which 'in vivo' hydrolyze the long chain aliphatic esters of triglycerides [1]. The potential of lipases for enantioselective [2] and regioselective [3–7] processes is becoming increasingly recognized. Lipase activity is greatly increased at the lipid–water interphase [8]. The interfacial activation is associated with a conformational change in the lipase molecule [9, 10]. This hypothesis is in accordance with the structure of lipases [11–14]. In three of these lipases, the catalytic triad is not exposed to the solvent. X-ray crystallographic studies of *R. miehei* lipase complexed with inhibitors [15,16] showed a large displacement of a loop that covers the active site. This movement gives rise to the exposure of hydrophobic residues which markedly increases the apolarity around the active centre and stabilizes the interaction with the substrate interface. Other lipases contain similar loops. Thus, it is likely that conformational rearrangement of surface loops in these enzymes, in response to the oil–water interphase, is an essential part of their functionality [16]. In previous papers [17–19] we isolated isoenzymes A and B from *Candida rugosa* or *cylindracea* lipase (E.C.3.1.1.3). Lipases A and B have the same molecular mass, similar amino acid content (79%, [18]) and N-terminal sequences, but differ in hydrophobicity, specificity [17,18] and pI [19]. cDNA clones for these lipases have been isolated [20]. Isolipases from *Candida rugosa* have differencies in the regions interacting with interphases and substrates [21].

Reverse micellar systems are formed by nanometer-sized water droplets dispersed in organic media by the action of surfactants [22]. Micellar systems were traditionally considered as models to study the action of enzymes at/or near biological membranes. In the case of lipases, especially active at interphases, it should be interesting to analyse how the micellar media can promote their interfacial activation. This may be studied with pure lipases. Until now it was known that ionic micelles inhibit the activity of lipases, with the exception of lipase B from *Chromobacterium viscosum*, which has higher activity in anionic micelles than in water [23].

In this paper, we compared the hydrolytic activity of the well characterized isoenzymes, lipases A and B from *C. rugosa*, in water and in AOT/n-heptane/water systems. In reverse micelles, factors such as substrate partition and enzyme size are similar for both isolipases. Their different activities in micellar media are discussed considering their distinct hydrophobicities [17–19], and the results of CD, fluorescence and UV-visible studies.

2. Materials and methods

2.1. Materials

Lipase type VII from *Candida rugosa*, *p*-nitrophenyl alkanoates (acetate, butyrate, octanoate and laurate), AOT and the buffers EPPS, MES, BES were purchased from Sigma (St Louis, MO, USA).

n-Heptane (Scharlau, HPLC grade) was dried over type 4A molecular sieve prior to use.

2.2. Lipase purification

The purification of the two extracellular isolipases from *Candida rugosa* (lipase A and lipase B) has been described previously [18].

2.3. Enzyme assays

The hydrolytic activity of isolipases A and B were measured by following the accumulation of *p*-nitrophenol in a Kontron Instruments UVIKON 930 spectrophotometer equipped with thermostated cells (26°C in all experiments). The monitoring wavelength was selected at the isosbestic point (λ_{iso}) of the nitrophenol/nitrophenolate couple [24]. In water solution, pNPB was used as the substrate. The assay mixture (5 ml) consisted of pNPB dissolved in acetone, with the corresponding buffer. The final acetone concentration in the mixture was 4% in a 50 mM buffer solution. Initial rates were estimated by measuring the increase in absorbance at 346 nm (molar extinction coefficient = 4,800 $M^{-1} \cdot cm^{-1}$ [25]). In both systems, the maximum reaction time was 5 min, thus avoiding a possible influence of the enzyme stability. Michaelis–Menten kinetics and its derived Lineaweaver–Burk or Eadie–Hofstee plots were drawn for the estimation of K_m and k_{cat}. From these values, the rate constant, k_2^0 ($k_2^0 = k_{cat}/k_m$) was calculated. The concentration units used for the enzyme and reactants were calculated from the total volume of the micellar system.

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Abbreviations: AOT, Sodium *bis*-(2-ethylhexyl) sulphosuccinate; pNPA, pNPB, pNPO and pNPL, *p*-nitrophenyl acetate, butyrate, octanoate and laurate, respectively; $\omega_0 = [H_2O]/[surfactant]$; $k_2^0 = k_{cat}/K_{m,app}$; CD, far UV circular dichroism.

SSDI 0014-5793(95)00104-2

2.4. Determination of enzyme stability

The stabilities of isolipases A and B were studied at three values of ω_0 (5, 12.5 and 30) in MES buffer (pH 6.1). After incubation of the micellar solutions containing the enzyme at 30°C, aliquots were taken at the indicated times and the residual activity measured. This was assayed in bulk water using *p*-NPB as the substrate.

2.5. Fluorescence study of isolipases

Fluorescence spectra of purified lipases A and B in 1 mM Sodium phosphate buffer, pH = 7.0, were compared with the spectra in micellar systems of AOT at ω_0 values of 5 and 20, and at the corresponding optimal pH of lipases A and B. Protein concentration in the cuvette was 0.017 mg/ml and 0.067 mg/ml of lipases A and B, respectively. Spectra were recorded at 26°C at 280 nm excitation and 450–280 nm emission, in a Perking Elmer fluorescence spectrometer LS 50B. Emission and excitation slit widths were 5 nm. Spectra were uncorrected for the instrument sensitivity, but Raman emission of the solvent – buffer or micellar solution – was subtracted.

2.6. CD studies

CD spectra of isolipase A in 0.1 M NaPi, pH = 7.0 and in AOT micelles (ω_0 = 20) were obtained on a Jovin Yron Mark III dichrograph fitted with a 250 W Xenon lamp. Ellipticity was calculated taking a mean molecular weight per residue of 110. Secondary structure estimation from CD spectra were performed by using a program designed by Menendez-Arias et al. [26] based on the sets of parameters reported by Yang et al. [27].

3. Results and discussion

3.1. Dependence of the hydrolysis rate on ω_0

In the present work, the ω_0 dependence on the reaction rates of isolipases A and B was studied using four *p*-nitophenylalkanoates. Results are expressed by $k_{2,app}$ ($k_{2,app} = v/[E][S]$), where v is the initial rate and [E] and [S] the enzyme and initial substrate concentrations, respectively. The term apparent for the $k_{2,app}$ refers to the non saturated conditions of the substrates due to experimental limitations.

For all substrates, the activity of isolipase A (Fig. 1A) reached a maximum at ω_0 = 5. Higher substrate concentrations were used when the reaction rates were too low (i.e. pNPL). The profile was smoother for the longest chain-length (pNPL) ester; the most hydrophobic substrate. Our results could be fitted to a model in which an enzyme presents its highest activity in the bound water (water involved in the solvation of the surfactant headgroups and their counterions) or in the surfactant apolar tail domains of the micellar system, according to Bru et al. [28]. This behaviour is consistent with the intrinsic nature of lipases, which act at the interphases. A similar dependence of the activity vs. ω_0 was reported for *Rhizopus delemar* lipase [29] and bilirubin oxidase with hydrophobic substrates [30,31].

Martinek et al. reported that when the ω_0 is an optimum the inner diameter of the empty micelle practically corresponds to the size of the molecule of the entrapped enzyme [32–35]. However, there is an ongoing discussion in the literature as to whether the ω_0 dependence of the enzyme activity is related to the size of the enzyme or not. This may be studied with pure lipases A and B (similar size), and different substrates. Isolipase B presented a more complex dependence of $k_{2,app}$ vs. ω_0

Table 1
Kinetic parameters of lipases A and B for the hydrolysis of *p*-nitrophenylalkanoates in aqueous and micellar systems

Enzyme	System	Substrate	pH	$K_{m,app}$* (mM)	k_{cat} (s^{-1})	k_2^0 ($M^{-1} \cdot s^{-1}$)
Lipase	Aqueous	pNPB	6.1	0.18 ± 0.02	1274 ± 110	(6.90 ± 0.05) 10^{+6}
		pNPB	7.2			(8.13 ± 0.09) 10^{+6}
		pNPB	7.4			(11.88 ± 0.18) 10^{+6}
		pNPB	7.5			(11.10 ± 0.21) 10^{+6}
		pNPB	8.0			(10.08 ± 0.04) 10^{+6}
	Micellar	pNPB	5.0			(0.01 ± 0.01) 10^{+6}
		pNPB	5.6			(0.32 ± 0.01) 10^{+6}
		pNPB	6.1	0.22 ± 0.01	50.2 ± 2.1	(0.22 ± 0.01) 10^{+6}
		pNPB	7.1			(0.11 ± 0.01) 10^{+6}
		pNPB	8.0			(0.01 ± 0.01) 10^{+6}
	Micellar	pNPA	6.1	1.16 ± 0.35	40.8 ± 12.3	(0.035 ± 0.001) 10^{+6}
		pNPO	6.1	5.81 ± 0.90	952 ± 170	(0.164 ± 0.001) 10^{+6}
		pNPL	6.1	11.6 ± 2.7	649 ± 152	(0.056 ± 0.001) 10^{+6}
Lipase B	Aqueous	pNPB	5.5			(5.72 ± 0.05) 10^{+6}
		pNPB	6.1	0.17 ± 0.01	1125 ± 58	(6.45 ± 0.05) 10^{+6}
		pNPB	7.2			(6.53 ± 0.08) 10^{+6}
		pNPB	7.4			(4.57 ± 0.02) 10^{+6}
		pNPB	7.5			–
		pNPB	8			(5.38 ± 0.02) 10^{+6}
	Micellar	pNPB	5.0			–
		pNPB	5.6			(3.98 ± 0.08) 10^{+3}
		pNPB	6.1	0.25 ± 0.01	1.61 ± 0.05	(6.45 ± 0.16) 10^{+3}
		pNPB	7.1			(1.98 ± 0.05) 10^{+3}
	Micellar	pNPA	6.1	**	**	(0.45 ± 0.01) 10^{+3}
		pNPO	6.1	1.49 ± 0.07	5.88 ± 0.28	(3.95 ± 0.01) 10^{+3}
		pNPL	6.1	5.04 ± 0.45	4.54 ± 0.41	(0.90 ± 0.01) 10^{+3}

The assays were carried out at 26°C in MES (pH 5.6–6.1), BES (pH 7.1–7.4) and EPPS (pH 8.0) buffers. The concentrations of lipases A and B were 2.7 nM and 24.5 nM, respectively. The hydrolysis of PNPB in water was measured spectrophotometrically at 346 nm, in a 50 mM buffer solution. In the AOT system, the hydrolytic activity of lipases A and B were measured at 317 nm, [AOT] = 0.1 M, buffer 1.8 mM and ω_0 = 5.
*In aqueous system $K_{m,app} = K_m$. **The plot of the pNPA hydrolysis rate against the substrate concentration catalyzed by lipase B did not deviate substantially from linearity. This behaviour is consistent with a $k_{m,app}$ value for this substrate much higher than the studied ester concentrations. Higher substrate concentrations were not studied due to the experimental limitations. Values of k_2^0 were obtained by transformations of data according to the Eadie-Hofstee plot, but the k_{cat} and K_m values could not be determined.

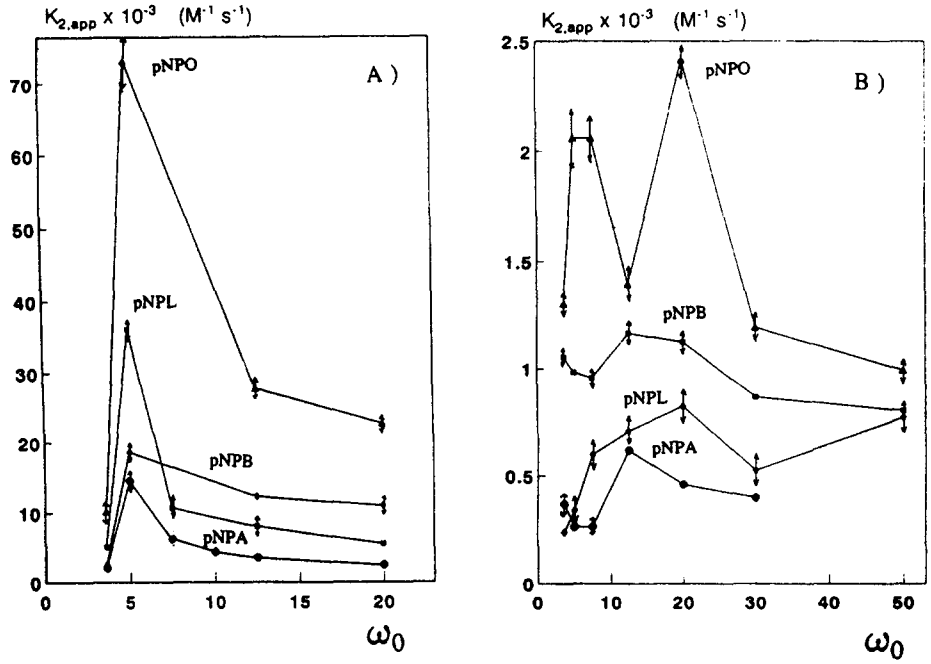

Fig. 1. Dependence of $K_{2,app}$ on ω_0 for different *p*-nitrophenylalkanoates. The hydrolysis reactions, catalyzed by lipases A (a) and B (b), were carried out in 0.1 M AOT, 1.8 mM MES buffer (pH 6.1) at 26°C. The substrates were 0.3 mM pNPA (●), 0.5 mM pNPB (■), 0.4 mM pNPO (▲) and 0.6 mM pNPL (◆) in *n*-heptane. The concentration of lipases A and B were 2.7 nM and 24.5 nM, respectively.

(Fig. 1B) than isolipase A. Distinct maxima were detected with different substrates. These results were reproducible with various enzyme concentrations. The ω_0 dependence of the activities of these two isoenzymes in reverse micelles was not directly correlated to the size of the enzyme, due to their similar molecular weight and size, and since there was also a substrate dependence in Fig 1B.

The dependence of the activity of crude *C. rugosa* lipase on ω_0 was previously studied using olive oil as the substrate (optimal ω_0 = 10.5) [36]. However, it has been reported that when the purity of the preparation increases the optimal water content tends to decrease [37]. Thus, the optimal ω_0 value with pure lipase should be lower.

3.2. Dependence of the rate constant on the pH

The influence of the pH on the rate constant, k_2^0, was determined using pNPB in bulk water and in micellar systems of ω_0 = 5 (Table 1). In aqueous solution almost no variation was found on the k_2^0 values of isolipase B, but in reverse micelles a maximum was obtained at pH 6.1. In the case of isolipase A in an aqueous and micellar system, optimum values could be established at pH = 7.5 and 5.6, respectively. The AOT system shifted the optimal pH to lower values than in water. Shifts in the optimal pH in the acidic direction have been found also for lipase PS from Amano [38], and for *Chromobacterium viscosum* lipase [39] in these AOT systems. This phenomenon is frequently attributed to the physical state of water in micelles and its subsequent effect on the pK_a values of aminoacid residues in the active site of the enzyme [40]. It should be considered that at ω_0 = 5, where the experiments were carried out, no free water exists in the micelle [41,42]. In contrast, non interfacial active enzymes like α-chymotrypsin [42], tyrosinase [43], alkaline phosphatase [44] etc., show shifts to the basic region. The opposite shift in the optimal pH of lipases might be due to the different localization of their active centres into the micelle regions. Thus, changes in the dielectric constant of the environment of the active centres should produce different shifts in their pK_a values.

3.3. Kinetic parameters in bulk water and in microemulsion

The activities of isolipases A and B with pNPB were studied in both aqueous and micellar media (pH 6.1, the optimal pH of lipase B in Table 1; ω_0 = 5). The kinetic parameters, $K_{m,app}$, k_{cat} and k_2^0 are given in Table 1.

In aqueous solution the kinetic constants had similar values for isolipases A and B. However, in reversed micelles k_2^0 was 50 times higher for isolipase A (pH = 5.6) than for isolipase B (pH = 6.1). It is known that ionic micelles inhibit the activity of lipases [45–47]. In this case, the micellar system resulted to be more unfavorable for isolipase B than for isolipase A. Thus, k_2^0 was 1,000 times lower in reverse micelles than in water for isolipase B, but only 37 times lower in the case of isolipase A. The inhibition effect is due not only to the unfavourable distribution of the substrate between the interphase and the organic bulk, but also that the ability of lipases to change to a more active conformation [14–16] might be partially decreased at the ionic interphase. It was shown that amphiphatic compounds inhibit the lipolysis by covering the substrate or the enzyme, making their contact more difficult [48]. However, the substrate distribution should be the same for these two isoenzymes. The lower activity of isolipase B compared with isolipase A must therefore be due to a distinct lipase localization in the micelle, due to their different hydrophobicities [18] (in accordance with the fluorescence study, Fig. 2). Thus, the inhibition effect of isolipases A and B in ionic micelles was related to the enzyme penetration into the interphase, which depended on their hydrophobicities. Changes in the activities and/or selectivities of enzymes induced by the increase in the hydrophobicity of the

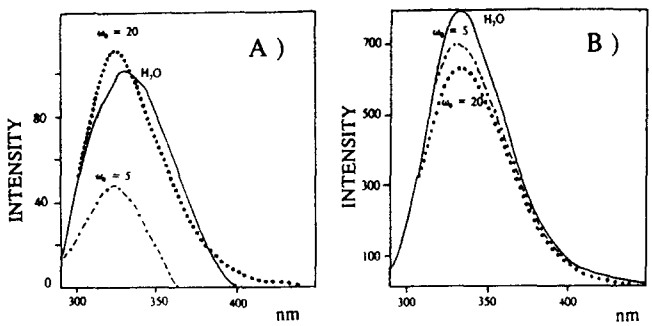

Fig. 2. Fluorescence spectra of lipases A and B, in buffer and micellar systems. (A) lipase A and (B) Lipase B: (—— buffer system; –.–. $\omega_0 = 5$; ···· $\omega_0 = 20$). Conditions: 1 mM sodium phosphate buffer system; micellar systems of 0.1 M AOT; 1.8 mM MES buffer (pH 5.6 for lipase A, and 6.1 for lipase B, their respective optimal pH values in Table 1). The assays were carried out at 26°C. The concentrations of lipases A and B were 0.017 mg/ml and 0.067 mg/ml, respectively.

reaction medium, had been attributed to slight conformational changes in the enzyme and/or substrate molecules [49,50].

3.4. Dependence of the kinetic constants on the chain-length of p-nitrophenyl esters

A small variation of k_2^0 values was obtained with variation in the chain length of the substrates (Table 1). The best substrate for both lipases was pNPB, but the difference between the best and the worst substrate was only a factor of 7 (lipase A) or 14 (lipase B). k_2^0 values were two orders of magnitude higher for isolipase A than for isolipase B. This difference was greater with substrates of longer chain length. The increase of $K_{m,app}$ values as the chain-length of the esters increased can be partially attributed to the unfavorable partition coefficients of the longer chain-length substrates [51–53]. However, pNPA was a poor substrate for both lipases. This should be expected for lipolytic enzymes. In the case of isolipase A, the k_{cat} values for the short-chain substrates (pNPA and pNPB) were almost 20 times lower than their long-chain analogues (pNPO and pNPL). In both isoenzymes, the maximum value was achieved with pNPO.

3.5. Stability of isolipases A and B in AOT/n-heptane microemulsions

The stabilities of isolipases A and B in AOT micelles were markedly different. Lipase A lost 50% of its initial activity after 35, 50 and 80 min at ω_0 values of 5, 12.5 and 30, respectively. The activity of lipase B dropped to 50% in 25 h, 32 min and 19 min at $\omega_0 = 5$, 12.5 and 30, respectively. Thus, while the stability of isolipase A improved as ω_0 increased the opposite was found for lipase B. The most stable lipase in bulk water was also the most stable in reverse micelles of AOT. However, the molecular differences between the isoenzymes, responsible for the different interactions between the protein and the micelle components (see fluorescence study, Fig. 2), produced an opposite variation in their stabilities when the droplet size was changed. Isolipase B mainly solubilized into the water core, resulted more stable in micelles of a size closer to that of the enzyme than isolipase A. The ω_0 dependence of the isolipase B stability resembled that reported by Han and Rhee [36], with crude *C. rugosa* lipase. The similarity could be explained by considering that isolipase B was the main isoenzyme in the crude extracts of *C. rugosa* lipase [17–19].

3.6. Spectroscopic studies of isolipases A and B

(i) *Fluorescence spectra* of isoenzymes A and B in aqueous and micellar systems ($\omega_0 = 5$ and 20) are presented in Fig. 2. These spectra are dominated by tryptophan's absorbance and emission [54].Thus, lipase spectra showed only emission of Trp (λ_{max} = 325–333 nm). *Candida cylindracea* lipase has 5 or 6 Trp of which at least one of them is in the active centre environment [11,20].

The wavelengths maxima were in agreement with the different hydrophobicities of these isolipases. Thus, only spectra of the more hydrophobic isolipase A were blue shifted in reverse micelles (Fig. 2A). This blue displacement of isolipase A spectra indicated an hydrophobic environment of the enzyme in the micelle, while the environment of isolipase B was similar to that of an aqueous medium. Fluorescence spectra of *R. arrhizus* lipase were also blue shifted upon incorporation into AOT micelles [55]. The emission intensities decreased at ω_0 values of maximum activity (see Figs. 1 and 2). These intensities increased with respect to the intensity in water, at ω_0 values where these two isolipases showed lower activities. At $\omega_0 = 20$, the intensity of isolipase A was higher than in the buffer solution. The reverse effect was found for isolipase B. This opposite spectral behaviour of isoenzymes A and B, is further evidence for either a distinct environment of isolipases in the micellar system, or different conformational rearrangement of these proteins at $\omega_0 = 20$. These two options could be expected for these isolipases, considering their molecular differences: i.e. the higher interaction of isolipase A in hydrophobic chromatography [18]. Distinct emission intensities in water and in micelles but without any shift of λ_{max}, have been reported for porcine pancreatic lipase [47] and bovine liver catalase [56].

In spite of the high sequence homology of these isoenzymes, their different amino acids are not homogeneusly distributed in the molecule. The flap covering the active site appears to be quite variable in the *Candida r.* isolipases [21]. The flap is the most variable structure of lipases and it seems to be responsible for the distinct lipase interaction with water/oil interphases

(ii) *CD spectra* of isolipase A showed a conformational change, with an increment of its α-helix content in micelles: 31% α-helix in aqueous buffer and 49% in AOT micelles. In contrast, *R. arrhizus* lipase showed a dramatic increase in β-sheet in the AOT system [55].

(iii) *UV-visible spectra.* The absorbances of isolipases A and B in AOT micelles ($\omega_0 = 5$) were respectively, 2.25 and 1.42 times higher than in water. This effect was not observed at $\omega_0 = 20$ (data not shown).

In this work, lipase hydrophobicity is shown to be a key parameter in mitigating the inhibition effect of charged micelles. Consequently, it should be interesting to consider this in the detergent industry and in their use in synthetic or hydrolytic processes in ionic micelles.

Acknowledgements: We thank Dr. Jesús Pérez Gil of the Universidad Complutense de Madrid for his helpful assistence in the study of CD spectroscopy, including the correct management of the BASIC microcomputer program used. We want to thank Prof. Robert F. Freedman for his support and help during our stays in his Department at the University of Kent. This work has been financed by the spanish CICYT (No. PB92-0495).

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