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LACTOPEROXIDASE-CATALYZED INACTIVATION OF HEXOKINASE

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Summary

The enzymatic activity of hexokinase (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) decreased rapidly when the enzyme was exposed to the lactoperoxidase antimicrobial system (consisting of lactoperoxidase, H_2O_2 and SCN^-). Inactivation did not begin until the reaction of one sulfhydryl group per hexokinase monomer was completed. Loss of enzyme activity accompanied the reaction of at least one additional sulfhydryl group per monomer. Covalent incorporation of ^{14}C-labeled SCN^- into hexokinase increased as the inactivation reaction progressed. The rate of the hexokinase activity loss depended on temperature, pH and the presence of glucose and phosphate ion.

When H_2O_2 and SCN^- were applied to a Sepharose column bearing covalently attached lactoperoxidase, the column eluate inactivated hexokinase. This demonstrated that the lactoperoxidase molecule itself need not be in contact with hexokinase in order to catalyze hexokinase inactivation. The sulfhydryl-reactive oxidation product of SCN^- which is generated by the column is sufficient.

The results are consistent with a two-stage reaction in which the exposed, non-essential sulfhydryl groups on the hexokinase molecule react first to produce an enzymatically active but unstable form of hexokinase. This modified form of hexokinase then undergoes a spontaneous, temperature-dependent structural change, which allows reaction of previously shielded, essential sulfhydryl groups. The phenomenon described here suggests a possible mechanism for the antimicrobial effects of the lactoperoxidase system.

Introduction

The antimicrobial properties of the lactoperoxidase-thiocyanate-hydrogen peroxide system are well documented [1—3]. This antimicrobial system is

found in human saliva, milk and tears [4] where it serves as one of the innate immune defenses. Lactoperoxidase catalyzes the oxidation of thiocyanate (SCN^-) by peroxide (H_2O_2) to produce the $OSCN^-$ anion, an unstable sulfhydryl reactive compound with antimicrobial effects [5—7].

Hexokinase was first suggested as a possible target of the lactoperoxidase system by Oram and Reiter in 1966 [2]. An inhibitory effect on this enzyme was verified in *Streptococcus mutans* [5] where hexokinase activity was reduced 40—70% in cells treated with the lactoperoxidase system. The inactivation of purified preparations of hexokinase has been demonstrated with fluid phase lactoperoxidase and with lactoperoxidase adsorbed to human enamel [8], but no previous reports regarding the mechanism of this reaction have appeared.

The present paper reports studies on the rate and extent of hexokinase inactivation and the effects of temperature, pH and substrate on this reaction. The reaction of hexokinase sulfhydryl groups with $OSCN^-$ and the incorporation of radiolabeled SCN^- into hexokinase were also measured. The results are analyzed in terms of a model which takes into account many of the physical and chemical properties of hexokinase.

Materials and Methods

Materials were of reagent grade unless otherwise noted. Quartz distilled water was used in all experiments.

Lactoperoxidase Lactoperoxidase purified from bovine milk was obtained from Sigma Chemical Co. Sepharose-bound lactoperoxidase was obtained from Worthington Biochemicals. Lactoperoxidase was assayed as described previously [8].

Hydrogen peroxide Peroxide was assayed by measuring the oxidation of *O*-dianisidine at 460 nm in the presence of horseradish peroxidase.

Thiocyanate and $OSCN^-$. SCN^- and $OSCN^-$ were assayed by the method of Hoogendoorn et al. [6].

Hexokinase Yeast hexokinase isozyme A was obtained from Boehringer-Mannheim. Isozyme B was from Worthington Biochemicals. Both were received in $(NH_4)_2SO_4$ slurry and desalted on a Sephadex G-25 column shortly before use. Hexokinase concentration was determined spectrophotometrically at 280 nm. The absorbance × 1.0 is equal to the concentration in mg/ml [9]. Hexokinase activity was assayed by the coupled enzyme method in 1% triethanolamine hydrochloride buffer, pH 7.5 at 37°C. The assay medium contained a saturating excess of hexokinase substrates and NAD as well as approx. 3 I.U. glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), the coupling enzyme, in 2.0 ml medium. (1 I.U. is defined as the quantity of enzyme required to produce 1 μmol/min product at pH 7.4 and 25°C using NAD as cofactor.) The assay was initiated by the addition of hexokinase, and the reaction progress was followed by measuring NADH production at 340 nm [10].

The presence of $OSCN^-$ in the assay mixture did not affect the activity of the coupling enzyme, nor did $OSCN^-$ react with NADH. This was demon-

strated by adding glucose 6-phosphate to the assay medium in the presence and absence of $OSCN^-$.

The inactivation of hexokinase was carried out in 1% triethanolamine hydrochloride buffer and in phosphate buffer. Unless otherwise noted, the system components were lactoperoxidase (0.3 mg/ml), thiocyanate (2.75 mM), and hydrogen peroxide (300 μM). Peroxide was added last to prevent inactivation of lactoperoxidase. This mixture was allowed to incubate 10—15 s to permit complete reaction of peroxide and conversion of SCN^- to $OSCN^-$, and hexokinase at a final concentration of 3 μg/ml was then added. After incubation at 37°C, an aliquot of this solution was mixed with 2 ml assay solution in a cuvette and the absorbance at 340 nm was recorded.

Incorporation of ^{14}C-labeled SCN^-. The components of the lactoperoxidase system were added to 1% triethanolamine hydrochloride, pH 7.0/20 mM glucose as described above, except that the final thiocyanate concentration was 350 μM (in slight excess over peroxide). The ^{14}C-labeled potassium thiocyanate used in this experiment had a specific activity of 4.8 μCi/μmol. Hexokinase was then added at a final concentration of 0.1 mg/ml. After various incubation times, samples of hexokinase treated in this way were applied to a Sephadex G-25 column to remove unbound $OSCN^-$ and excess SCN^-. Total desalting time was 15 min. Fractions containing hexokinase were pooled and counted in a scintillation counter to determine the quantity of bound ^{14}C.

Measurement of free sulfhydryl groups Free sulfhydryl groups on hexokinase were measured before and after treatment with the lactoperoxidase system. In order to obtain the high hexokinase concentrations necessary for this experiment, the $(NH_4)_2SO_4$ slurry of hexokinase was dialyzed against 1% triethanolamine hydrochloride, pH 7.0, instead of being desalted on the Sephadex G-25 column. The lactoperoxidase system was as described above. Hexokinase was added to the reaction mixture at a final concentration of 4 8 mg/ml and allowed to react for 10 min. The hexokinase was then desalted with 0 1 M Tris, pH 7.0 on Sephadex G-25 to remove $OSCN^-$ which interferes with the measurement of free sulfhydryl groups [7]. Then 2.0 ml of the desalted hexokinase was placed in a cuvette with 0 2 ml of a stock 5,5′-dithiobis(2-nitrobenzoic acid) solution (prepared by adding 13 mg to 50 ml buffer). The final hexokinase concentration was 0.32 mg/ml. The formation of a colored product was monitored at 412 nm as originally described by Ellman [11]. After 1 h, urea was added to the hexokinase samples (0.5 g urea/ml) in order to denature the hexokinase and disclose buried sulfhydryl groups. The absorbance of these solutions was corrected for volume changes due to the addition of urea. Under the conditions of these experiments, it was found that the urea increased the final solution volume by a factor of 1.36, giving a final urea concentration of 6 M The corrected absorbance was used to calculate total free sulfhydryl content.

Lactoperoxidase-free $OSCN^-$. Sepharose 4B with covalently attached lactoperoxidase (Worthington Biochemicals) was packed in a section of 2 ml pipette plugged with sintered glass. The total bed volume of this column was 0.1 ml. The maximum flow rate was 16 ml/h. A solution of peroxide and thiocyanate in the desired buffer was applied to the column in order to obtain fresh $OSCN^-$ in the absence of lactoperoxidase or residual peroxide. The eluate was assayed for the presence of lactoperoxidase, peroxide, thiocyanate and $OSCN^-$

Results

The complete lactoperoxidase system is necessary to achieve substantial inactivation of hexokinase in the presence of phosphate ion. Table I shows the effects of each component of the lactoperoxidase system and of the combinations taken two at a time. The omission of any component eliminates the rapid loss of hexokinase activity. In other experiments, conducted in 1% triethanolamine hydrochloride buffer instead of 0.2 M phosphate, it was found that peroxide and peroxide plus thiocyanate do have small but reproducible effects on hexokinase activity in the absence of lactoperoxidase. Table II demonstrates the lactoperoxidase-dependent and lactoperoxidase-independent inactivation of hexokinase and the effect of glucose on both. Glucose (20 mM) decreases the effect of the complete lactoperoxidase system on hexokinase activity measured after 10 min, and prevents inactivation of hexokinase by peroxide and by peroxide plus thiocyanate. In a similar experiment, it was found that 0.1 M phosphate ion eliminated the effect of peroxide plus thiocyanate on hexokinase.

Effects of pH Fig. 1 shows the pH dependence of the inactivation reaction catalyzed by lactoperoxidase as well as the pH dependence of the lactoperoxidase-independent inactivation caused by peroxide or peroxide plus thiocyanate. In general, hexokinase seems more susceptible to inactivation from all causes at higher pH values. Hexokinase alone (no additions) also demonstrated a pH-dependent loss of activity (data not shown) on standing at 37°C. Below pH 6.0 hexokinase was stable, but at pH 6.5 and above the enzyme activity decreased markedly. This activity loss was completely prevented by 20 mM glucose.

Effects of temperature The inactivation of hexokinase by the lactoperoxidase system progressed rapidly at 37°C, but did not take place at 0°C. In order to rule out the effects of temperature on the production and initial reaction of $OSCN^-$, samples of hexokinase were treated with the lactoperoxidase system at room temperature. Under these conditions, the lactoperoxidase

TABLE I

REACTION OF HEXOKINASE WITH THE LACTOPEROXIDASE SYSTEM

Hexokinase was incubated for 10 min at 37°C in 0 2 M sodium phosphate buffer, pH 6 0 An aliquot was then assayed Percentage inactivation was calculated relative to a control hexokinase preparation containing no additions LPO, lactoperoxidase

Composition of the reaction mixture			Percentage inactivation of hexokinase
H_2O_2	SCN^-	LPO	
+	—	—	4
—	+	—	0
—	—	+	9
—	+	+	6
+	—	+	10
+	+	—	9
+	+	+	51 ± 4 *

* Mean ± S D of three determinations

TABLE II

EFFECTS OF GLUCOSE ON HEXOKINASE INACTIVATION BY THE LACTOPEROXIDASE SYSTEM

Hexokinase was incubated 10 min at 37°C in 1% triethanolamine pH 7 0 containing the components of the lactoperoxidase system indicated Percentage inactivation is the mean of three determinations ± S D Percentage inactivation was calculated relative to a control hexokinase preparation containing either 20 mM glucose or no additions LPO, lactoperoxidase

	Percentage inactivation		
	H_2O_2	$H_2O_2 + SCN^-$	$H_2O_2 + SCN^- + LPO$
20 mM glucose	4 ± 4	4 ± 4	24 ± 2
No glucose	20 ± 6	32 ± 5	53 ± 4

system is active since all peroxide is reacted in less than 15 s. One sample was warmed to 37°C and incubated for 10 min while another was incubated for 10 min at 0°C Hexokinase was inactivated at the higher temperature, but not at 0°C.

Reaction of sulfhydryl groups with $OSCN^-$ Treatment of hexokinase by the lactoperoxidase system was carried out in the presence of 20 mM glucose in order to stabilize the hexokinase against thermal denaturation. The sulfhydryl content of hexokinase was assayed before and after treatment with the lactoperoxidase system. The formation of colored product was followed at 412 nm. Table III shows the results of these measurements. The native (untreated) enzyme contained a total of four sulfhydryl groups per monomer, while the lactoperoxidase-modified hexokinase contained only three or less. Lactoperoxidase-treated hexokinase with one modified sulfhydryl group is fully active or only slightly inactivated, suggesting that the first reactive sulfhydryl group is not essential for enzymatic activity. Hexokinase with an average of only 1.6 sulfhydryl groups remaining per monomer (after 3.5 h in $OSCN^-$) retained only 25% of its initial enzymatic activity.

One sulfhydryl group per hexokinase monomer was 'free' in the native enzyme since it was fast reacting with 5,5′-dithiobis(2-nitrobenzoic acid) (Table III). This is presumably the same highly exposed sulfhydryl group

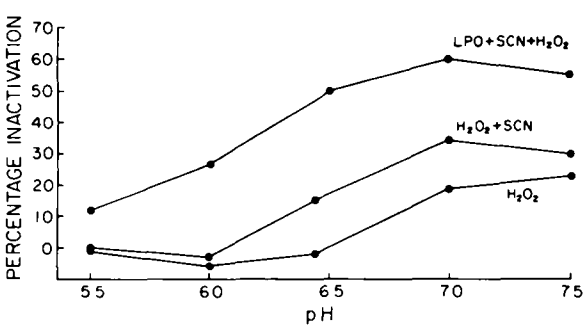

Fig 1 Effect of pH on hexokinase inactivation Hexokinase was incubated in 1% triethanolamine buffer of various pH values with the components of the lactoperoxidase system indicated After 10 min, the activity of the hexokinase was assayed and compared to a control incubated at the same pH with no additions

TABLE III

5,5'-DITHIOBIS(2-NITROBENZOIC ACID) REACTIVE SULFHYDRYL GROUPS PER HEXOKINASE MONOMER

Hexokinase was treated with $OSCN^-$ in the presence of 20 mM glucose and then desalted to remove $OSCN^-$ before 5,5'-dithiobis(2-nitrobenzoic acid) was added Urea (6 M) was added after 5,5'-dithiobis(2-nitrobenzoic acid) as described in the text 'Fast reacting sulfhydryl groups' are those reacting within 10 s

	Native enzyme	Modified enzyme	
		10 min reaction with $OSCN^-$	3 5 h reaction with $OSCN^-$
Total sulfhydryl groups	4 0	2 9	1 6
Fast reacting sulfhydryl groups	1 0	0 8	0 0

which reacts first with $OSCN^-$. However, the sulfhydryl modified form itself contains approx. one (0.8) fast reactive sulfhydryl group which was apparently shielded from $OSCN^-$ reaction by the presence of glucose. When $OSCN^-$ and glucose were removed on the Sephadex G-25 column, this second sulfhydryl group was left exposed and reactive.

Incorporation of ^{14}C SCN^- Hexokinase was treated with the lactoperoxidase system as described in the Materials and Methods section. ^{14}C-labeled SCN^- was covalently bound to hexokinase, and the amount of covalent incorporation increased with time of incubation (Fig. 2). As can be seen from the figure, two phases of incorporation were observed. One type of incorporation was very rapid (about 3000 cpm above background at time zero), occurring before the hexokinase could be applied to the desalting column. This reaction, which occurred without loss of enzyme activity, corresponds to the incorporation of ^{14}C-labeled SCN^- into one sulfenyl thiocyanate group per hexokinase monomer, based on measurement of sulfhydryl content. A second, slow phase of incorporation occurred over the next 5 h and was accompanied by loss of enzymatic activity. This activity loss is slow, due to the presence of 20 mM glucose in the reaction medium.

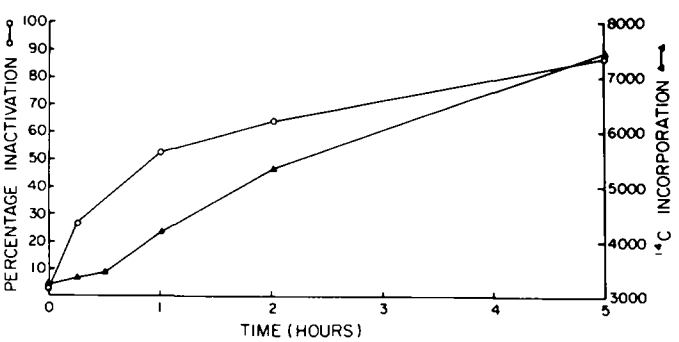

Fig 2 Incorporation of ^{14}C-labeled SCN^- into hexokinase and loss of enzyme activity Hexokinase was incubated with the complete lactoperoxidase system consisting of LPO/H_2O_2/^{14}C-labeled SCN^- At the times indicated, the hexokinase activity was measured to determine percentage inactivation The hexokinase preparation was desalted on Sephadex G-25 to remove excess SCN^- and unbound $OSCN^-$ Fractions containing hexokinase were pooled and counted in a liquid scintillation counter to determine CPM incorporated

Isozymes of hexokinase Both isozymes of yeast hexokinase were inactivated by the lactoperoxidase system. Isozyme A, which was used in all the experiments described above, seemed slightly more susceptible to inactivation than type B, but the differences between the two were small

$OSCN^-$ in the absence of lactoperoxidase $OSCN^-$ was prepared, using Sepharose-bound lactoperoxidase. When a solution containing 750 μM SCN^-/ 200 μM H_2O_2 was applied to the column, the eluate contained no peroxide and 660 μM SCN^-. The concentration of $OSCN^-$ was 80 μM The $OSCN^-$ was converted back to thiocyanate upon addition of mercaptoethanol. Although the peroxide/thiocyanate solution applied to the column had no effect on hexokinase (isozyme A), the column eluate containing $OSCN^-$ inactivated the enzyme rapidly. An assay of the column eluate indicated that no lactoperoxidase had been released from the Sepharose. Therefore, $OSCN^-$ in the absence of lactoperoxidase can inactivate hexokinase

Properties of sulfhydryl-modified hexokinase Hexokinase which had been incubated for 10 min with the lactoperoxidase system in 20 mM glucose (i.e., one sulfhydryl group modified) was desalted on Sephadex G-25 to remove $OSCN^-$ and glucose. This preparation was incubated overnight at 0°C and assayed for activity. Although hexokinase loses only a small fraction of its activity after 10 min incubation with the lactoperoxidase system (in 20 mM glucose), the sulfhydryl-modified preparation incubated overnight at 0°C retained only 25% of the specific activity of the native enzyme. This suggests that it can continue to be inactivated in the absence of $OSCN^-$ even under conditions (0°C) in which the native enzyme is normally stable This preparation was found to be completely stable at 37°C for 30 min in the presence of 20 mM glucose. It was not stable at 37°C in the absence of glucose, but its loss of activity under these conditions was identical to that of the native enzyme.

Discussion

Reaction of sulfhydryl groups of hexokinase with $OSCN^-$ The rapid initial reaction of hexokinase with $OSCN^-$ which involved no loss of enzyme activity (Table III), could also be seen at zero time in radioactive-labeling experiments (Fig. 2). A slower reaction causing loss of activity then occurred with a further increase of incorporation of ^{14}C-labeled SCN^-. These results, which suggest the existence of at least two classes of sulfhydryl groups, are in good agreement with sulfhydryl alkylation experiments conducted on hexokinase B [12], using the affinity reagent *N*-bromoacetyl-1-B-galactosamine. This reagent reacts in the presence of mannose with only one (thiol II in the terminology of Otieno et al. [12]) of the four sulfhydryls per monomer with no loss of enzyme activity. Upon removal of mannose, a second sulfhydryl (thiol I) reacts, causing complete loss of hexokinase activity.

Nature of the covalent bond. In reactions of $OSCN^-$ with proteins (e.g., albumin, lactoglobin), it has been shown [7] that only the sulfhydryl groups are modified. Thiocyanate from $OSCN^-$ is incorporated into protein sulfhydryls as the sulfenyl thiocyanate (R-S-SCN) derivative. This accounts for the unavailability of free sulfhydryl groups for reaction with 5,5′-dithiobis-

(2-nitrobenzoic acid) following reaction with $OSCN^-$, and is consistent with the finding that the ^{14}C-label from SCN^- is covalently bound to hexokinase.

Possible mechanism of hexokinase inactivation. The substrate analog, *N*-bromoacetyl-1-B-galactosamine is only reactive with thiol I above a structural transition temperature of 31°C [12], suggesting that considerable structural flexibility of the hexokinase molecule is required for the substrate binding site to be brought in contact with the essential sulfhydryl. Thus, the essential sulfhydryl may possess a role not directly involving the catalytic site. The results of Lazarus et al. [13] demonstrated that the reaction of two sulfhydryl groups per monomer leads to dissociation of the hexokinase dimer into its monomeric subunits. Considerable controversy has surrounded the question of relative activities of the dimer and monomer of hexokinase with reports of no activity in the monomer [9], partial activity [10] and full activity [14]. The results of Yip and Rudolph [15] strongly suggest that the monomer has less than full activity. If this is true, then the reaction of thiol I with $OSCN^-$ or other sulfhydryl reagents may cause loss of activity by inducing dissociation.

pH Effects The effect of pH on the inactivation of hexokinase by the lactoperoxidase system during 10 min incubations (Fig. 1) cannot be explained by instability of $OSCN^-$ at low pH, since even at the lowest pH tested (5.5) the half-life of $OSCN^-$ exceeds 30 min [5]. The hexokinase monomer-dimer equilibrium is shifted in the direction of monomer by increases in pH [16]. Dissociation of the hexokinase dimer by succinylation of amino groups [17] results in the exposure of a second fast-reacting sulfhydryl. Thus, increasing pH might be expected to make hexokinase more susceptible to reaction with $OSCN^-$.

Glucose effects. The finding that glucose slows lactoperoxidase-dependent inactivation and eliminates lactoperoxidase-independent inactivation during the 10 min incubation period (Table II) is consistent with the finding that substrate protects thiol I from reaction with sulfhydryl reagents [12]. Glucose has also been shown to prevent dissociation of the hexokinase dimer in the absence of phosphate [9]. It has previously been reported that glucose has no effect on hexokinase inactivation by $OSCN^-$ in phosphate buffer [18], but in the absence of phosphate there is a clear effect Glucose offers some protection against lactoperoxidase catalyzed growth inhibition in *Streptococcus mutans* C67-1 [5], suggesting that the protective effects of glucose for thiol I may have physiological significance

Lactoperoxidase-independent inactivation Peroxide is known to react with protein-SH groups [19], thus, the inactivation of hexokinase by peroxide alone could have been anticipated. Peroxide and thiocyanate react at a measurable rate in the absence of enzyme to produce various oxidation products of SCN^- [20]. It is likely that $OSCN^-$ or other sulfhydryl reactive intermediates are among the products of this inorganic reaction.

Temperature effects The effect of temperature on the inhibition of hexokinase was not due to temperature-dependence of the lactoperoxidase reaction itself, since $OSCN^-$ is generated and reacts rapidly, even at room temperature. Since the inactivation of hexokinase at 37°C is much slower than the rate of free sulfhydryl reaction with $OSCN^-$, a likely explanation is that a temperature-dependent structural change in hexokinase exposes the essential thiol

I to reaction with $OSCN^-$. Such a structural change has been reported for hexokinase at temperatures above 30°C [13]. Increases in temperature are also known to contribute to dissociation of the hexokinase dimer [14]. Whether this structural change is a dissociation or a conformational change, it is still likely that it would result in exposure of reactive sulfhydryls.

Physiological significance of hexokinase inactivation. A wide variety of organisms is inhibited by the lactoperoxidase system, including yeast [21], Gram negative and Gram positive bacteria [1,2], mycoplasma [22], virus [3] and mammalian tumor cells [23]. While the mechanism of action may not be the same in all cases, the inactivation of hexokinase would be detrimental to the growth of most of these organisms.

$OSCN^-$ generated on a Sepharose column was still reactive with hexokinase in the absence of lactoperoxidase. This would have to be the case with hexokinase in vivo since the lactoperoxidase molecule would be excluded from the cytosol by the cell membrane. Another site at which the lactoperoxidase system may exert its antimicrobial effects is the membrane-associated phospho*enol*pyruvate-dependent sugar phosphotransferase system [24], which moves glucose into the cell by group translocation at the expense of the high energy phosphate bond in phospho*enol*-pyruvate. Glucose transport in *Streptococcus agalactiae* is inhibited by the lactoperoxidase system [25]. It is possible that $OSCN^-$ has more than one target in the intact cell or that, as suggested by Mickelson [25], the inhibition of glycolysis may deprive the cells of the phospho*enol*pyruvate necessary for glucose transport.

The present results raise questions concerning the nature and kinetics of hexokinase inactivation by $OSCN^-$ in living cells. In our experiments we have not seen total loss of hexokinase activity in vitro. If yeast or bacterial cells retain some hexokinase activity after exposure to $OSCN^-$, it might be expected that growth would be inhibited, even though the cells were not killed. This 'bacteriostatic' effect is exactly what has been seen in experiments with *S mutans* [26].

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