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Rate-determining Step in Phospholipase A₂ Mechanism

¹⁸O ISOTOPE EXCHANGE DETERMINED BY ¹³C NMR*

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H₂¹⁸O isotope exchange into specifically ¹³C-labeled substrate was used to obtain information on the rate-limiting step in the action of the phospholipase A₂ from the venom of the Indian cobra (*Naja naja naja*). Incorporation of ¹⁸O was detected by the effect of ¹⁸O on ¹³C chemical shifts in ¹³C NMR. The enzymatic hydrolysis of a micellar phosphatidylcholine analogue of platelet-activating factor 1-alkyl-2-[1-¹³C]lauroyl-*sn*-glycero-3-phosphorylcholine proceeds by an *O*-acyl cleavage of the *sn*-2 ester bond. The reaction was examined for simultaneous ¹⁸O incorporation into the substrate. No exchange was found, suggesting that the hydrolytic step is not followed by a higher energy transition state and that it or a step before it appears to be rate-limiting. Previous experiments on phosphatidylethanolamine activation indicate that *k*_{cat} is altered but that the *k*_m remains the same upon activation, suggesting that the binding steps occurring before the hydrolytic step are not affected. This strongly suggests that the hydrolytic step is in fact the rate-limiting step under these conditions. The ¹³C, ¹⁸O NMR technique should be generally applicable to mechanistic questions of this type.

is not increased in the presence of certain activators (8-10).

We now wish to report initial studies eventually aimed at examining the merit of the proposal that the product release step is slow in the reaction of phospholipase A₂, but is accelerated when the enzyme shows higher specific activities. In this paper, we have determined the rate-limiting step for the micellar system (e.g. in the presence of certain activator phospholipids and aggregated phospholipids). For this purpose, ¹³C NMR was employed to quantitate the degree of H₂¹⁸O exchange into a suitably ¹³C-labeled substrate and product during reaction with phospholipase A₂. Advantage was taken of the ¹⁸O isotope effect on the ¹³C chemical shifts established by Risley and Van Etten (11, 12). Exchange reactions have been studied using this technique with ¹³C NMR (13, 14) and ³¹P NMR (15, 16). In this initial study, we used a phosphatidylcholine (PC¹) analogue which is a good substrate for the enzyme. It was labeled with ¹³C in the *sn*-2 ester position and contains an alkyl ether instead of an ester in the *sn*-1 position so as to eliminate all ambiguities in the assignment of resonances, especially the possibility of acyl migration (17) which could have complicated the NMR results.

MATERIALS AND METHODS

It is now well established that pancreatic and venom phospholipase A₂ display a marked preference for micellar substrates and act poorly on monomeric substrate (for review, see Ref. 1). Over the years, at least four explanations have been advanced for the requirement of phospholipase A₂ for aggregated substrate which can be summarized as follows: (i) the physical state of the substrate phospholipid is altered in going from monomer to micelle (substrate effect); (ii) the enzyme undergoes a conformational change after it binds micellar phospholipid (enzyme effect); (iii) the higher local concentration of substrate in the micelle brings the enzyme closer to saturation (reaction effect); and (iv) product removal is the rate-limiting step and is facilitated by the micelle (product effect). A detailed consideration of these possibilities can be found elsewhere (1). Our previous studies have focused on the first three possibilities. We have described changes in substrate conformation in going from monomer to micelle (2) and conformational changes in the enzyme upon binding certain phospholipids leading to enzyme aggregation (3-7). We have also shown that substrate saturation on the enzyme

Phospholipase A₂ was purified from cobra venom (*Naja naja naja*) obtained from the Miami Serpenterium as previously described (18, 19). Triton X-100 was provided by Rohm and Haas Co. H₂¹⁸O (>95%) was a Monsanto Research Corp. product. [1-¹³C]Palmitic acid and [1-¹³C]lauric acid (>90%) were from KOR Stable Isotopes. 1-Alkyl-2-[1-¹³C]lauroyl-*sn*-glycero-3-phosphorylcholine (lauroyl-PAF) was synthesized from [1-¹³C]lauric acid and 1-alkyl-2-hydroxy-*sn*-glycero-3-phosphorylcholine, which is the 2-hydroxy derivative of platelet-activator factor prepared from beef heart with mainly C₁₆ and C₁₈ alkyl chains. This compound was synthesized and provided by Dr. Walter Shaw of Avanti Polar Lipids, Inc. Lauroyl-PAF gave a single spot (*R*_F = 0.57) by one-dimensional thin layer chromatography on silica gel plates (Analtech, Inc.) using chloroform/methanol/acetic acid/water (65:15:10:4, v/v/v/v) as developing solvent; iodine vapor was employed for detection. Only one peak was detected by ³¹P NMR.

Tentative ¹H NMR assignments for lauroyl-PAF in CDCl₃ were made using one- and two-dimensional NMR. Spectra were recorded at 360.7 MHz on a homebuilt spectrometer employing a Nicolet 1280 computer. *J*-Correlated spectra (20) were obtained using the following conditions. Spectral width was ±1000 Hz; 1 K memory was used to accumulate 512 spectra (incremented evolution period). The assignment is as follows: 0.90 ppm of ω-CH₃ (acyl + alkyl chain), 1.28 ppm of (CH₂)_n (acyl + alkyl chain), 1.55 ppm of β-CH₂ (alkyl chain), 1.61 ppm of β-CH₂ (acyl chain), 2.34 ppm of α-CH₂ (acyl chain), 3.36 ppm of N(CH₃)₃, 3.40-3.45 ppm of α-CH₂ (alkyl chain), 3.59 ppm of CH₂ (*sn*-1), 3.77 ppm of CH₂N(CH₃)₃, 3.93 ppm of CH₂ (*sn*-3), 4.29 ppm of CH₂CH₂N(CH₃)₃, and 5.15 ppm of CH (*sn*-2).

Hydrolysis of lauroyl-PAF (4.3 mM) by phospholipase A₂ was carried out in H₂¹⁶O, H₂¹⁸O, or a 50:50 mixture at 40 °C in a 50 mM Tris-HCl, pH 7.9, buffer containing 10 mM CaCl₂ and 32 mM Triton

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¹ The abbreviations used are: PC, phosphatidylcholine; lauroyl-PAF, 1-alkyl-2-[1-¹³C]lauroyl-*sn*-glycero-3-phosphorylcholine.

X-100. The activity of the cobra venom phospholipase A₂ toward lauroyl-PAF was about the same as that toward dipalmitoyl-PC (18). The concentration of phospholipase A₂ was adjusted to obtain the desired amount of hydrolysis during the incubation time (generally 12–20 h). The isotope effect on the ¹³C chemical shift is quite small and requires that extremely narrow line widths be quantitated. Although it would be desirable to follow the enzymatic reaction directly in the NMR spectrometer while it is proceeding, the compounds of interest tend to form aggregates in aqueous solution as well as in some organic solvents (resulting in broad lines) and must be extracted first so as to be examined in CHCl₃/CH₃OH. Thus, at the end of the incubation, the reaction was quenched by the addition of EDTA (50 mM final concentration), which, by chelating the Ca²⁺, both inhibits the enzyme and makes the lauric acid completely extractable by the Bligh and Dyer method (21). The residual substrate and fatty acid were extracted with CHCl₃/CH₃OH/acetic acid (2:4:1, v/v/v). After extracting twice, the CHCl₃ phase was dried under nitrogen and *in vacuo* and then dissolved in CDCl₃ or in CDCl₃/CH₃OH (50:50, v/v).

¹³C NMR spectra were obtained at 125.76 MHz on a WM 500-MHz Bruker spectrometer of the Southern California NMR facility of the California Institute of Technology or at 50.31 MHz on a 200-MHz Nicolet spectrometer at the NMR Center of the University of California at San Diego. In both cases, a 16 K memory and broadband proton decoupling were employed. A line broadening of 0–0.2 Hz was applied before the Fourier transformation of the free induction decay. A flip angle between 45 and 70° was used along with a delay time less than *T*₁ necessary to give 90% of the maximal peak intensity (22). *T*₁ values for [1-¹³C]palmitic acid and for the esterified [1-¹³C]lauroyl-PAF were determined by the inversion-recovery method (23) and found equal to 6.4 and 7.1 s, respectively. Chemical shifts are reported relative to the CDCl₃ peak (77.0 ppm).

RESULTS AND DISCUSSION

Assignment of ¹³C Spectra—The phospholipase A₂ hydrolyzes the fatty acid ester bond at the *sn*-2 position of phospholipids leading to free fatty acid (1). Thus, the ¹³C NMR chemical shift of [1-¹³C]lauroyl-PAF was determined along with that of a free fatty acid used as a control ([1-¹³C]palmitic acid), as shown in Fig. 1. The chemical shifts determined in CDCl₃:CH₃OH (50:50, v/v) were 173.30 and 176.17 ppm, respectively, for the substrate and the product of the hydrolysis. These values agree with those reported in the literature (24). It should be noted that the chemical shift of the carboxylic acid carbon atom is very sensitive to many solution parameters, including its concentration (24).

Lauroyl-PAF was incubated with sufficient cobra venom phospholipase A₂ to obtain almost complete hydrolysis in 12 h. One incubation was carried out in the presence of H₂¹⁶O and a second one in the presence of H₂¹⁸O. Both samples were examined after extraction; at 50 MHz, each gave only a single peak (line width about 3 Hz). When mixed together and examined at 126 MHz, two peaks separated by 0.028 ppm (about 3.5 Hz) were found (Fig. 1C). The downfield peak (176.169 ppm) is assigned to the free fatty acid containing ¹⁶O, whereas the upfield peak (176.141 ppm) is assigned to the free fatty acid in which an ¹⁸O is introduced (see below), consistent with the expected isotope effect (14). The magnitude of the upfield shift (0.028 ppm) is characteristic of a carboxylic acid (acetic acid) containing a single ¹⁸O label (13, 25).

Without sufficient EDTA, the Ca²⁺ salt of lauric acid can be extracted leading to a broad peak ($\Delta\nu_{1/2}$ about 50 Hz) at 182.22 ppm, consistent with the resonance of a carboxylic anion (24) and most probably due to the formation of reversed micelles in the apolar solvent. When properly treated with EDTA and extracted, the carboxylic acid peak always appears sharp with the expected chemical shift. In an experiment in which two peaks were observed for the lauric acid after incubation in H₂¹⁶O/H₂¹⁸O mixtures, the sample was checked and the peaks were found to still be present after filtration over Chelex 100. Also some resonances coming from co-

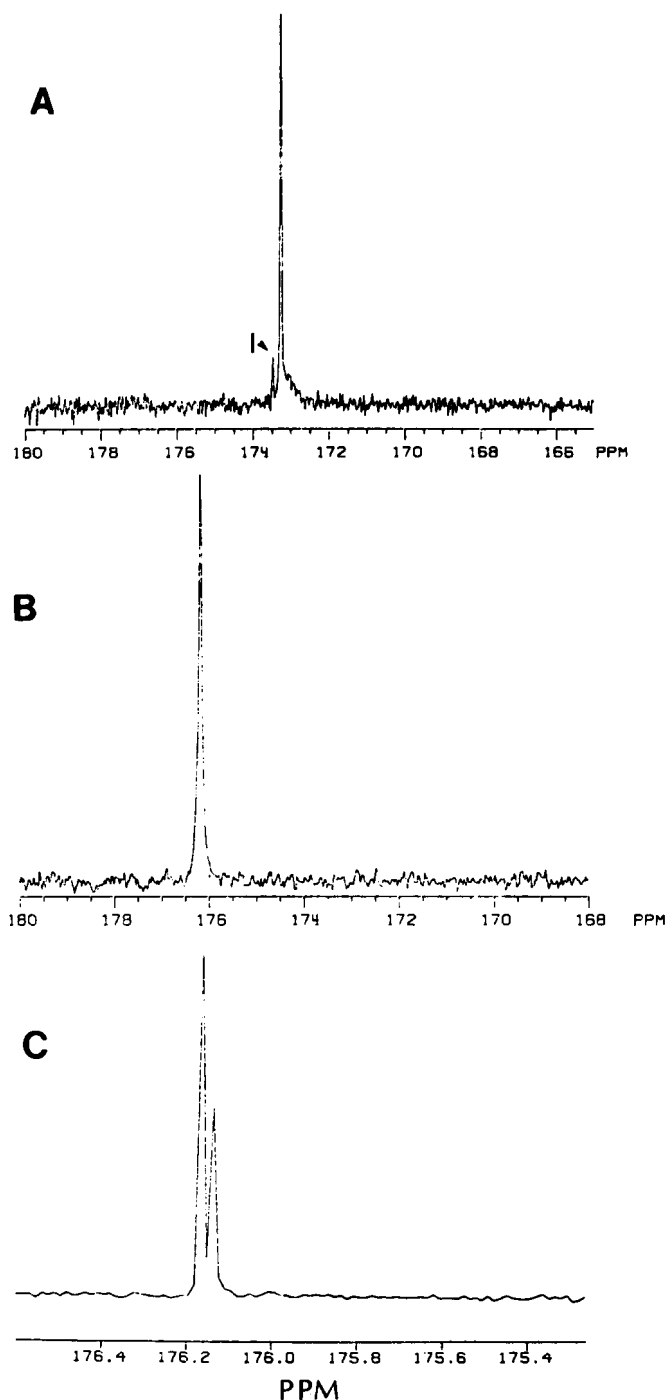
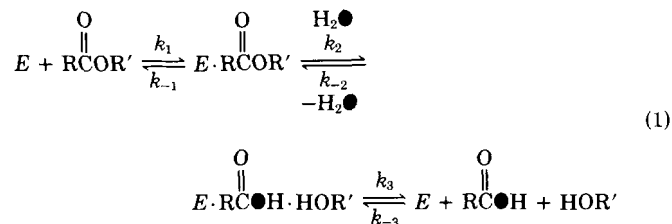


FIG. 1. A, spectrum of [1-¹³C]lauroyl-PAF (4 mM) at 50 MHz in CH₃OH/CDCl₃ (50:50 v/v). 20,000 scans were collected at 25 °C with a spectral width of 10,000 Hz. Peak I indicates a product impurity appearing only in ¹³C NMR. B, spectrum of [1-¹³C]palmitic acid (25 mM) at 50 MHz in CH₃OH/CDCl₃ (50:50, v/v). 1,400 scans were collected under the same conditions as A. C, separate aliquots of [1-¹³C]lauroyl-PAF were hydrolyzed to completion with phospholipase A₂, one in H₂¹⁶O and one in H₂¹⁸O; and the [1-¹³C]lauric acid product of the two samples was combined after extraction. The ¹³C NMR spectrum of the mixed product in CH₃OH/CDCl₃ (50:50, v/v) was recorded at 126 MHz with a spectral width of 30,000 Hz after 3,400 accumulations at ambient temperature.

extracted Triton X-100 could be observed. Nevertheless assignments in Triton X-100 showed that no resonance appears downfield of 160 ppm (26); this demonstrates that the two resonance lines of [1-¹³C]lauric acid cannot be the result of the spectrum of the Triton X-100 present. All of these controls

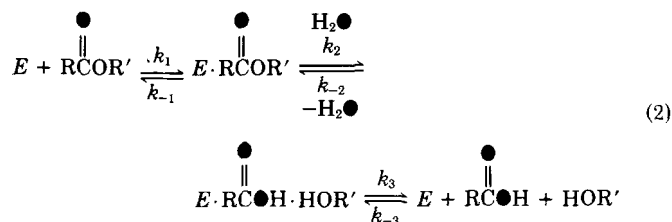
demonstrate that the two peaks for the lauric acid resonance after incubation in the presence of ¹⁸O/¹⁶O water are indeed the result of the isotope effect of ¹⁸O on the ¹³C nuclear shielding.

^{H₂¹⁸O Exchange Studies with Phospholipid}—If the enzymatic hydrolysis of diacyl-PC occurs by an *O*-acyl cleavage, the reaction in the presence of ¹⁸O-labeled water (H₂●, with ● indicating ¹⁸O-label) should occur according to Equation 1.



Thus, the production of ¹⁸O-labeled lauric acid as examined by ¹³C NMR demonstrates that the hydrolysis of diacyl-PC by cobra venom phospholipase A₂ proceeds via the expected *O*-acyl cleavage, as was found with the enzyme from *Crotalus adamanteus* venom by traditional methods employing mass spectroscopy (27).

If exchange of H₂¹⁸O occurs and if the two oxygens on the free fatty acid product are equivalent (28), then at equilibrium the labeling can be viewed as shown in Equation 2.



Exchange of H₂¹⁸O back into the substrate is conceivable for phospholipase A₂ since it is not a serine, sulfhydryl, metallo-, or acid esterase according to all accumulated evidence (reviewed in Ref. 1). Phospholipase A₂ apparently catalyzes a direct attack of water on the substrate ester, presumably by general base catalysis and most likely with histidine (1). The catalytic cycle goes, therefore, through only one tetrahedral intermediate, and the back reaction yields the substrate ester directly without going through an acyl-enzyme intermediate. The enzyme can undoubtedly distinguish the two nonbridging oxygens of the prochiral tetrahedral intermediate, presumably resulting in the loss of the same oxygen that attacked the ester if the tetrahedral intermediate collapses backward. In contrast to the nonenzymatic ester hydrolysis (29), exchange would, therefore, only be expected if a transition state *after* the breakdown of the tetrahedral intermediate (such as a conformation change or product release) was dominant. Exchange also requires the equivalence of the two oxygens of the bound carboxylic acid product. Exchange could also occur by complete resynthesis from dissociated products, but resynthesis would not be expected to be observable because of the extremely exergonic external equilibrium constant.

The hydrolysis of the lauroyl-PAF was carried out to about 50% completion in 50:50 H₂¹⁶O/H₂¹⁸O as solvent. The products of the hydrolysis were extracted, and the spectrum was recorded at 126 MHz. As shown in Fig. 2, the peaks obtained for the free fatty acid resonance at 176.515 ppm are resolved into two peaks separated by 0.024 ppm, whereas a sharp peak is obtained for the residual [1-¹³C]lauroyl-PAF at 173.73 ppm. Small differences in the chemical shifts from Fig. 1 are due

to solvent, temperature, and concentration differences (24, 30).

If a transition state after the breakdown of a tetrahedral intermediate were dominant, the H₂¹⁸O would be incorporated into the substrate (RCOOR') by the reverse reaction, and after prolonged incubation, then an additional ¹⁸O label would also appear in the fatty acid product (RCOOH) due to the cleavage of substrate which was exchanged over time. A third peak in the fatty acid resonance region would then be expected because of the additivity of the nuclear shielding (14, 25, 28). For acetic acid at pH 8, the resonance peak for one ¹⁸O is at 0.027 ppm upfield from the ¹⁶O peak and the peak for two ¹⁸O labels incorporated appears 0.054 ppm upfield (12). For esters, ¹⁸O incorporation into the carbonyl gives a peak 0.038 ppm upfield (14). However, in our case, substrate labeling was not observed during the time of substrate hydrolysis nor was double labeling of the free fatty acid observed.

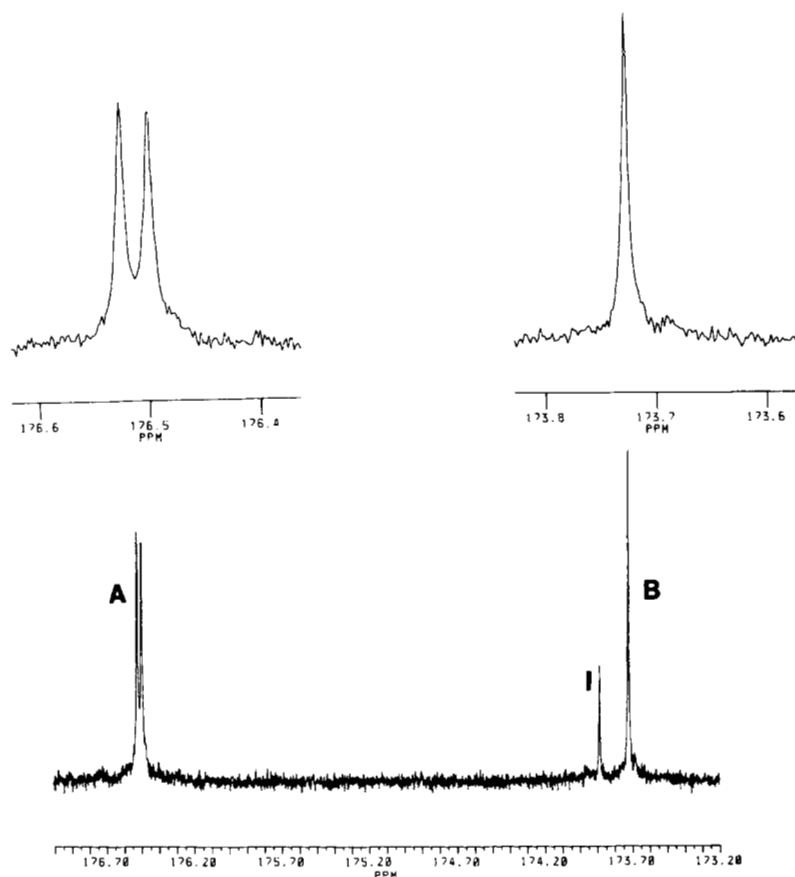
To eliminate nonenzymatic ¹⁸O exchange into the free fatty acid product as a cause of the observed spectra, the following control was carried out. [1-¹³C]Palmitic acid (10 mM) was incubated for 24 h at 40 °C under the conditions described for lauroyl-PAF hydrolysis by phospholipase A₂ except that EDTA was added at the beginning of the incubation to ensure complete solubilization of palmitic acid. At the end of the incubation, [1-¹³C]palmitic acid was extracted and treated as described for the products of the enzymatic catalysis. When examined at 126 MHz, [1-¹³C]palmitic acid gave one sharp peak, indicating that ¹⁸O-labeled [1-¹³C]palmitic acid was not present and that no exchange occurs between the free carboxylic acid liberated by the enzyme and the H₂¹⁸O solvent under assay conditions.

Implications for Rate-determining Step—These experiments show that there is no exchange of H₂¹⁸O back into the substrate ester. This argues against the presence of a transition state (such as a conformational change or product release) after the hydrolytic step of higher energy than that of the tetrahedral transition state for the substrate tested. We would not expect an enzyme that catalyzes a very exergonic reaction such as ester hydrolysis to bind the products so tightly that their release is the most energetically unfavorable process in the overall free energy profile (31); and indeed, such is ruled out by the present experiments.

Cobra venom phospholipase A₂ hydrolysis of poor substrates is activated by phosphorylcholine-containing phospholipids (3-5, 10). Kinetic analysis of the long chain phospholipid mixed micelle system has shown that the activation is due to a change in *k*_{cat}, whereas substrate binding (*K*_m) is unchanged. Whereas these data had to be obtained with thiol analogues of phospholipids for reasons discussed elsewhere (8-10), the analogues behave identically in every respect examined, and we believe that this result most likely holds for natural phospholipids as well. The results presented herein indicate that the step limiting *k*_{cat}/*k*_m occurs at or before the hydrolytic step, and the kinetic results with activators showing no change in *k*_m indicate that the binding steps occurring before the hydrolytic step are not affected. Therefore, the step limiting *k*_{cat} (the rate determining step) is identical to the step limiting *k*_{cat}/*k*_m and thus also occurs at or before hydrolysis. In conclusion, the hydrolytic step appears to be the rate-determining step for cobra venom phospholipase A₂ hydrolysis of micellar phospholipids.

It should be pointed out that the studies reported herein were carried out on a good substrate that behaves similarly to PC. We must now carry out H₂¹⁸O exchange studies on monomeric substrates below the critical micellar concentration and phosphatidylethanolamine substrates in the absence of

FIG. 2. [1-¹³C]Lauroyl-PAF was hydrolyzed to about 50% completion with phospholipase A₂ in a H₂¹⁶O/H₂¹⁸O mixture (50:50, v/v). The remaining substrate and products were extracted, and the spectrum was recorded in CH₃OH/CDCl₃ (50:50, v/v) at 126 MHz with a spectral width of 1831 Hz after 3002 accumulations at 40 °C. Peak A is the [1-¹³C]lauric acid, peak B is the remaining [1-¹³C]lauroyl-PAF, and peak I is as in Fig. 1. Expanded spectra for peaks A and B are shown.



activators to see if the rate-determining step changes upon activation. Such experiments will require more elaborate synthetic approaches. The ¹⁸O isotope effect in ¹³C NMR should also be generally useful in the elucidation of mechanistic questions such as exchange and the position of cleavage in other enzymes.

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