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Probing the Role of Substrate Conformation in Phospholipase A_2 Action on Aggregated Phospholipids Using Constrained Phosphatidylcholine Analogues*

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Phospholipase A_2s hydrolyze aggregated phospholipid substrates much more rapidly than dispersed monomeric ones. Whether this is a consequence of interface-associated conformational changes of the enzyme or of the substrate, or of both, remains a key question in lipid enzymology. This problem is addressed herein using a rationally designed probe of substrate conformation. (1,3/2)-1-*O*-(phosphorylcholine)-2,3-*O*-dihexanoylcyclopentane-1,2,3-triol is a novel short chain phosphatidylcholine analogue in which the glycerol-like backbone is part of a five-membered ring and therefore covalently constrained within a small defined range of conformations. To the extent that the constrained analogue resists aggregation-associated conformational changes, it provides a means for assessing the contribution of such changes to phospholipase A_2 action on aggregated phospholipids. The monomeric (−)-cyclopentanoid analogue is a substrate for phospholipase A_2s from *Naja naja naja* venom. However, when this constrained phospholipid is aggregated, its hydrolysis rate is not enhanced, in contrast to its unconstrained counterpart, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine. This lack of activation was not caused by a failure of the enzyme to bind the micellar, constrained analogue. While the constrained analogue does not show interfacial activation, it does show the activation of phosphatidylethanolamine hydrolysis typical of phosphorylcholine-containing lipids. Hence, these results strongly support the contention that specific packing-induced conformations of aggregated substrate play a substantial role in the large interfacial activations observed with phospholipase A_2.

Phospholipases constitute an excellent model system for studying biological catalysis in the unique physiochemical environment created by the membrane surface. The extracellular phospholipase A_2s (EC 3.1.1.4., henceforth phospholipase A_2s), which catalyze the hydrolysis of the 2-acyl bond of *sn*-glycerol-3-phosphatides (1, 2), are especially suitable in this respect. They are compact, stable, and have been structurally characterized in atomic detail (3, 4), but most importantly, their activity strongly reflects the physical state of the substrate. They hydrolyze phospholipids up to a thousand times faster when the phospholipids are aggregated with their polar regions packed together to form a membrane-like surface (5).[1] Several models, which are by no means mutually exclusive, have been suggested to account for this so called "interfacial activation" phenomenon (review: Ref. 2). These include: an "interface recognition site" model in which an interface-induced conformational change occurs in the protein which results in a more active enzyme species (1); a "dual phospholipid model" in which an activator phospholipid (that is not being cleaved) accelerates, by an enzyme conformational change, the hydrolysis of the phospholipid under catalytic attack (6), and a "substrate effect" model in which packing-induced constraints on the normally flexible substrate molecule yield a higher incidence of productive encounters with the active site (7). In the present paper, we report the use of a novel conformational probe of phospholipase A_2 mechanism to help distinguish between these models.

The conformation of phospholipids in non-crystalline (8) aggregates is not well established. It is not certain whether packing forces within the aggregate induce conformations of the polar region which differ from those preferred by the dispersed monomer. However, such a packing-induced deformation is definitely implied in the substrate effect model. It is not implicit in the interface recognition site model nor in the dual phospholipid model. In order to test these hypotheses, therefore, we designed a "rigid" phospholipid analogue which is a substrate, but which resists steric deformation upon insertion into an aggregate. If a packing-induced substrate conformation does indeed contribute substantially to the aggregate-associated enhancement of phospholipase A_2 activity then, in the case of the rigid analogue, little or no enhancement of rate will occur upon substrate aggregation.

In designing an appropriate analogue for such a study, the following criteria were applied: (i) the analogue must have a built-in conformational constraint which renders a relevant part (the polar region) of the molecule comparatively inflexible, but it should be otherwise chemically similar to naturally occurring phospholipids. (ii) It must be hydrolyzed at the *sn*-2 position by phospholipase A_2 with the same enantiomeric specificity and with the same kinetic mechanism as the naturally occurring substrate. (iii) It must be enantiomerically pure since its enantiomorph might interact differently with the enzyme active site and complicate the kinetic analysis. (Ideally both enantiomorphs should be employed indepen-

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[1] This phrasing of the problem is the traditional one, but it carries a mechanistic bias. It is equally valid to regard the phenomenon as a *decrease* in catalytic activity which accompanies substrate *deaggregation*.

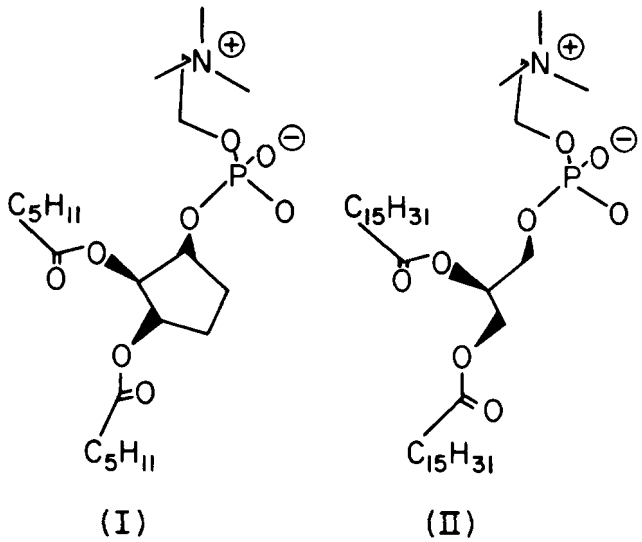

FIG. 1. **The structure of the cyclopentanoid analogue (*I*), fit to the crystal structure for 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (*II*) (8).** The enantiomer of the cyclopentanoid analogue illustrated here is not necessarily the one which is a substrate for phospholipase A_2 (*i.e.* the (−)-enantiomer) but it is the one which can be better fit to the structure of the reference compound using a least-squares method to match the corresponding heteroatoms.

dently). (iv) It must form micelles within the experimental range of the conditions used in phospholipase A_2 assays. Furthermore, the aggregates formed by the constrained analogue should physically resemble those of the unconstrained substrate.

As part of our ongoing study of the molecular mechanism of phospholipase A_2 action and following the pioneering studies of Sable, Hancock, and their co-workers (9–11), we (12) recently reported the chemical synthesis of a short acyl-chain phosphatidylcholine analogue (together with its enantiomorph) in which the glycerol backbone is replaced by all-*trans* cyclopentane-1,2,3-triol; (−)-(1,3/2)-1-*O*-(phosphorylcholine)-2,3-*O*-dihexanoylcyclopentane-1,2,3-triol (henceforth (−)-cyclopentanoid analogue, Fig. 1).[2,3] The relatively rigid cyclopentane ring confers a severe constraint on the flexibility of the glycerol-like backbone. Apart from the steric bulk of the "ethylene bridge," this conformational constraint is the only way in which the cyclopentanoid analogue differs from short acyl-chain derivatives of phosphatidylcholine (PC).[4] We have demonstrated that this constrained analogue was hydrolyzed at the 2-acyl position and in an enantiomerically specific manner by phospholipase A_2 (12).[3]

In this study, we present the results of a kinetic analysis of the action of a snake venom phospholipase A_2 on the dihexanoylcyclopentanoid analogue compared to its action on the unconstrained 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine (L-dihexanoyl-PC). We have shown that the (−)-cyclopentanoid analogue (absolute stereochemical configuration unestablished) meets the criteria listed above and we have proceeded to use it (in conjunction with its enantiomorph) to probe the molecular basis of phospholipase A_2 activity towards aggregated substrates.

MATERIALS AND METHODS

Materials—1,2-Dihexanoyl-*sn*-glycero-3-phosphorylcholine purchased from Avanti Polar Lipids Inc. was pure by proton magnetic resonance and thin layer chromatographic (TLC) analysis and was used without purification. Egg phosphatidylethanolamine (PE), prepared by transesterification of egg phosphatidylcholine (13), was also obtained from Avanti Polar Lipids, Inc. The (±)-(1,3/2)-1-*O*-(phosphorylcholine)-2,3-*O*-dihexanoylcyclopentane-1,2,3-triol was synthesized in five steps from (±)-(1,3/2)-1-*O*-benzoyl-cyclopentane-1,2,3-triol (10) as is described in detail elsewhere (12). Resolution of the enantiomers was achieved by treating the racemic mixture with *Crotalus atrox* phospholipase A_2 which was demonstrated by several methods to hydrolyze specifically the (−)-cyclopentanoid analogue at the *sn*-2 position (12). After enzymatic hydrolysis, the (+)-enantiomer and the lyso derivative were separated by chromatography on a DEAE-cellulose column, and the (−)-enantiomer was obtained by reacylation followed by purification (12). All other reagents were of the highest grade available.

Phospholipase A_2—Phospholipase A_2 from *N. n. naja* venom was purified as described by Dennis and co-workers (14). Protein concentrations were calculated from the extinction coefficient $\epsilon_{280}^{1\%} = 22.7$ (15). Hydrolysis was followed by the pH-stat technique as described in detail elsewhere (16) at pH 8.0 and 40 °C in the presence of 10 mM Ca^{2+}.

Other Analytical Methods—Critical micellar concentrations (cmcs) were determined by the drop-weight (stalagmometric) method as described by Tausk *et al.* (17) and by the spectral shift induced upon the incorporation of rhodamine 6-G into micelles (18). Proton magnetic resonance spectra were obtained on a Bruker 270 MHz FT-NMR instrument in D_2O at sample concentrations below and above the cmc (7 and 26 mM). Specific rotations were measured on a Pye-Unicam spectropolarimeter (in H_2O); $[\alpha]_{546}^{22} = +$ or $-$ 7.4 ± 0.6 ° for the (+) and (−) enantiomers of the constrained analogue respectively ($[\alpha]_{546}^{22} = +$ 9.2 ° for L-dihexanoyl-PC); since the cyclopentanoid analogue contains two additional chiral centers, the sign and magnitude of the optical rotatory power of the constrained and unconstrained compounds cannot be readily used to compare their stereochemistry. Thin layer chromatographic analyses were performed on 40 × 80-mm plates precoated with a layer (0.25 mm thick) of silica gel (Polygram SIL G/UV_{254}, Macherey Nagel) using chloroform/methanol/water (65:25:4, by volume) as mobile phase. The phosphorus-containing components were detected by the spray of Dittmer and Lester (19). R_F values were as follows: (± or −)-dihexanoylcyclopentanoid analogue, 0.35; its lysoderivative, 0.25. Phospholipid concentrations were measured by the procedure for phosphorus determination described by Rouser and Fleischer (20).

RESULTS

Physical Studies—The values obtained for the cmc (10 mM Tris·HCl, pH 8.0, 25 °C) by the two procedures described under "Materials and Methods" were in close agreement. The cmc procedures yielded a value of 10.0 ± 0.2 mM for L-dihexanoyl-PC and 11.2 ± 0.5 mM for the cyclopentanoid analogue. At the cmc, the area per molecule (A_0) at the air-water interface may be obtained from the relationship: $A_0 = 1/\Gamma_i \cdot 1/N$ where Γ_i is the surface excess of phospholipid (calculated from the slope of the surface tension *versus* concentration plot (not shown)) according to the Gibbs adsorption isotherm (17), and N is Avogadro's number. The value obtained for the cyclopentanoid analogue (68 ± 4 Å^2/molecule) was not significantly different from that obtained for L-dihexanoyl-PC (66 ± 2 Å^2/molecule).

Table I shows that a similar increase in magnetic nonequivalence between the α-methylene protons of the acyl-chain at

[2] The cyclopentanoid analogue is named in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature (1968) *Arch. Biochem. Biophys.* **128**, 269–279.

[3] It has been demonstrated (Lister, M. D., and Hancock, A. J. (1981) 17th Midwest Regional Meeting American Chemical Society, Columbia, MO, Abstr. 207 and M. D. Lister and E. A. Dennis, unpublished observation) that in the case of dipalmitoyl-cyclopentanoid analogues, only the analogue based on the all-*trans* diastereoisomer of cyclopentane-1,2,3-triol, and not those based on the all-*cis* or the *cis-trans* diastereoisomers, were susceptible to significant hydrolysis by phospholipase A_2.

[4] The abbreviations used are: PC, phosphatidylcholine; cyclopentanoid analogue, (1,3/2)-1-*O*-(phosphorylcholine)-2,3-*O*-dihexanoylcyclopentane-1,2,3-triol; L-dihexanoyl-PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; PE, phosphatidylethanolamine; TLC, thin layer chromatographic; cmc, critical micellar concentration.

TABLE I

Chemical shifts of the hydrocarbon chain signals for L-*dihexanoyl-PC compared to the cyclopentanoid PC-analogue*

Signal	Chemical shifts			
	Below cmc (7 mM)[a]		Above cmc (26 mM)	
	Dihexanoyl-PC	Analogue	Dihexanoyl-PC	Analogue
	ppm		*ppm*	
$-CH_3$	0.87	0.87	0.87[b]	0.87
$-(CH_2)_2-$	1.30	1.31[b]	1.30	1.30
$-CH_2-CCO-$	1.01[b]	1.01	1.01[b]	1.01[b]
$-CH_2(c)-CO-$	2.40, 2.43	2.39, 2.43	2.37, 2.41	2.36, 2.42
Δ[d]	0.028	0.038	0.046	0.065

[a] Concentration of the sample in D_2O; the cmc is 10.0 mM for the dihexanoyl-PC and 11.2 mM for the analogue.

[b] Complex splitting patterns.

[c] The α-methylene protons produce a pair of overlapping triplets; the chemical shifts entered in this line of the table correspond to the center peak of each triplet.

[d] Nonequivalence (±0.001 ppm) calculated by subtracting the signal (center peak of the triplet) due to one pair of α-methylene protons from that due to the other.

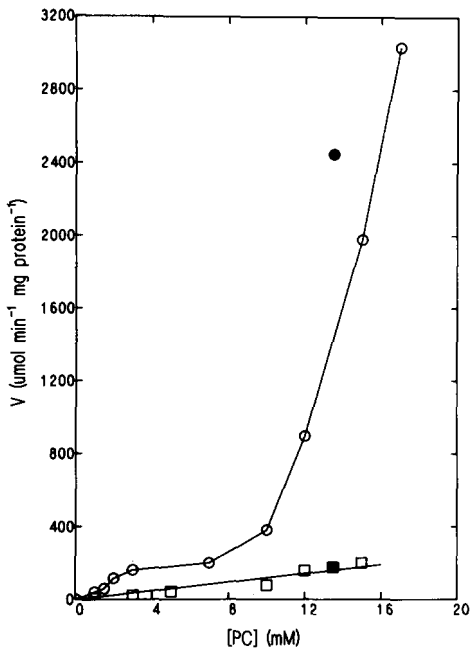

FIG. 2. **Velocity *versus* substrate concentration curves for *N. n. naja* phospholipase A_2 using** L-**dihexanoyl-PC (○) and the (−)-cyclopentanoid analogue (□) as substrate.** *Filled symbols* (●, ■) represents data points obtained using a 2:1 molar ratio of Triton X-100 to phospholipid.

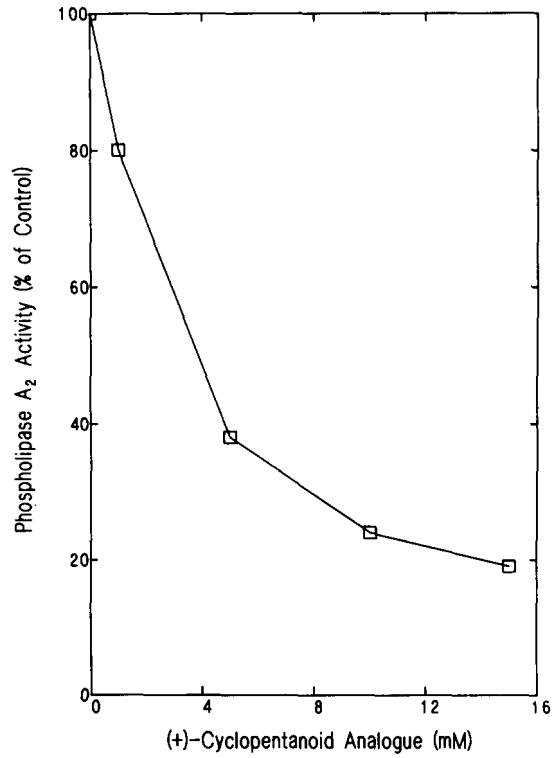

FIG. 3. **Dose-response curve showing the effects of the (+)-cyclopentanoid analogue on phospholipase A_2 activity toward** L-**dihexanoyl-PC.** The assays were performed under standard assay conditions with L-dihexanoyl-PC above its cmc (15 mM).

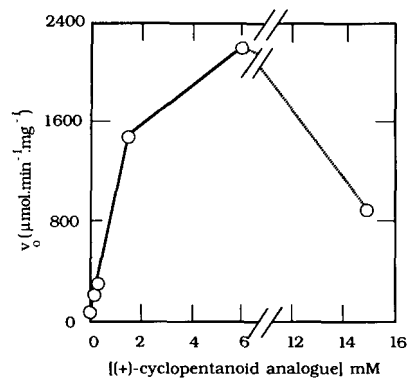

FIG. 4. Effect of the (+)-cyclopentanoid analogue on the rate of hydrolysis of egg phosphatidylethanolamine (5 mM) in mixed micelles with Triton X-100 (40 mM).

the 1-position and the α-methylene protons on the acyl-chain at the 2-position accompanies the monomer to micelle transition for both the cyclic analogue and the unconstrained substrate. Such an increase in magnetic nonequivalence is typical for the aggregation of naturally occurring phospholipids (21, 22) and is ascribed to the difference in hydrophobicity of the environments experienced by the two sets of protons in the aggregate.

Enzymatic Studies on Micelles—Fig. 2 shows typical velocity *versus* substrate concentration curves for *N. n. naja* phospholipase A_2 acting upon L-dihexanoyl-PC or the (−)-cyclopentanoid analogue. In the case of L-dihexanoyl-PC, an apparent saturation curve is observed up to about 8 mM followed by an abrupt, steep increase in velocity above the cmc. This increase in the rate of hydrolysis, which accompanies substrate aggregation, represents a dramatic activation by the aggregate. In contrast to the situation with the unconstrained compound, there is no enhanced catalytic activity displayed toward the (−)-cyclopentanoid analogue upon its aggregation. Furthermore, when the analogue was codispersed in mixed micelles with Triton X-100 (at a molar ratio of detergent to lipid of 2:1, Fig. 2), there was still no enhanced enzymatic activity in contrast to the case with the L-dihexanoyl-PC. Therefore, the enhancement of catalytic activity associated with substrate aggregation, the most characteristic functional feature of the phospholipase A_2 employed in this study, does not occur for the constrained analogue.

The Effect of the (+)-Cyclopentanoid Analogue—The monomeric (+)-cyclopentanoid analogue, which is not hydrolyzed by the *N. n. naja* phospholipase A_2, appears to behave as a strong inhibitor of the hydrolysis of L-dihexanoyl-PC, at least when the substrate is above its cmc. Fig. 3 shows the dose-response effect of the analogue on the enzyme acting on 15

mM L-dihexanoyl-PC. The plot shows that as little as 3 mM of the nonhydrolyzable analogue is sufficient to cause 50% inhibition.

Since the *N. n. naja* enzyme undergoes an activation of its hydrolysis of PE, it was of interest to determine whether the constrained analogue could function as an activator (16, 23–25) of PE hydrolysis as do other phosphorylcholine-containing compounds. Fig. 4 illustrates that the normally inhibitory (+)-cyclopentanoid analogue does indeed stimulate the hydrolysis of long acyl-chain PE incorporated into mixed micelles with Triton X-100. Stimulation appears to approach a maximum (about 22-fold) at a concentration of the analogue just below its cmc. At higher concentrations of the analogue, the magnitude of the stimulation declines, presumably as a result of inhibition and/or surface dilution as has been observed with other activators.

DISCUSSION

The cyclopentanoid analogues are chemically similar to PC itself, but they have a built-in conformational constraint which confines the geometry of the glycerol-like backbone within a small range defined by the puckering limits of an all-*trans* 1,2,3-substituted cyclopentane. Apart from this, the only way in which they differ from naturally occurring phosphatidylcholine is the steric bulk of the ethylene moiety required to tie back the glycerol part of the analogue into the five-membered ring.

We have shown that the cmc of the six-carbon acyl-chain cyclopentanoid analogues is close to 11 mM, facilitating their use as substrates or inhibitors of phospholipase A_2s both below and above their cmc. This value is within 10% of the cmc of the reference compound, L-dihexanoyl-PC. The monomer to micelle transition of the analogue is accompanied by much the same changes in magnetic nonequivalence of the α-methylene protons as is seen for the normal substrate (21). Hence, we have no evidence to suggest that the micelles of the analogue and normal substrate differ greatly from one another in their physical properties.

Phospholipase A_2 from the venoms of the elapid, *N. n. naja*, and the crotalid, *C. atrox* hydrolyze the (−)-cyclopentanoid analogue at the 2-position with strict enantiomeric specificity (12).[3] This implies that the stereochemistry of the functional groups involved in the catalytic steps for both the constrained analogue and the substrate is similar. Hence, the (−)-cyclopentanoid analogue meets all the requirements listed in the Introduction for an effective probe for the substrate conformation model for aggregation-dependent changes in phospholipase A_2 activity. Either above the cmc or when incorporated into mixed micelles with Triton X-100, it does not undergo enhanced catalytic attack. It appears, therefore, that it is the substrate's susceptibility to deformation that determines the degree to which aggregation promotes enhanced catalytic activity. The covalently constrained analogue cannot assume some critical aspect of the conformation imposed on normal flexible phospholipid molecules by packing forces within the aggregate. Hence, this lack of activation is consistent with the substrate effect model. Can these observations also be accommodated within the other models for aggregation-associated enhancement of activity?

If an interface-induced conformational change of the enzyme were to be the only event in the phenomenon, as is proposed in the interface recognition site model (1), it is difficult to explain why the introduction of a conformational constraint in the substrate should prevent activation. It could be argued that the constrained analogue forms a micelle to which the enzyme cannot absorb or that the "interfacial quality" provided by the micelle is inadequate to produce activation. However, the enzymatic rate increased above the cyclopentanoid's cmc in a substrate-dependent manner. If the enzyme could not absorb to the micelle, one would have expected the rate to have maximized at the cmc. Moreover, the micellar physical properties for both the constrained and unconstrained compound are the same. While an interface-induced conformational change of the enzyme cannot explain these results, it is still possible that a conformational change of both the enzyme and the phospholipid are required.

According to the dual phospholipid model, the phospholipase A_2 molecule has two binding sites, a PE activator site which requires a phosphorylcholine-containing lipid and a catalytic site which is headgroup nonspecific (24). Occupancy of the activator site by PC, together with the presence of a substrate in a lipid-water interface, produces a more catalytically active enzyme (25). The (+)-cyclopentanoid analogue can fully fulfill the role of "activator" as illustrated by the stimulation of the hydrolysis of long acyl chain PE. Whatever constraints the cyclopentane ring imposes on the phospholipid, they do not affect the ability of the phosphorylcholine moiety to bind to the activator site and function efficiently. While consistent with the dual phospholipid model, the reason for the lack of interfacial activation in the case of the constrained analogue must reside at the level of the substrate's structure and its ability to productively interact at the catalytic site.

One other possible explanation for why the constrained molecule is not activated above the cmc is that the ethylene bridge sterically interfers with some portion of the activated enzyme, but does not interact in the nonactivated enzyme. This possibility cannot be totally ruled out at the present time. However, the more straightforward explanation is that the lack of activation is a consequence of the lack of flexibility of the glycerol-like backbone.

In summary, by employing enantiomerically pure, constrained PC analogues, we have been able to test some of the models which purport to explain why phospholipase A_2s hydrolyze aggregated substrates with a much greater catalytic efficiency than monomeric ones. We have shown that the interfacial recognition site model cannot explain interfacial activation without also requiring a conformational change in the substrate. On the other hand, the results presented herein are quite consistent with models for activation in which specific packing-induced conformation of the phospholipid molecule is involved with interfacial activation. This would occur in the binding of the phospholipid molecule to the catalytic site of the enzyme in the dual phospholipid model.

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