Pyrimidine nucleotidases from human erythrocyte possess phosphotransferase activities specific for pyrimidine nucleotides

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Abstract Two cytoplasmic forms of pyrimidine nucleotidase (PN-I and PN-II) have been purified from human erythrocytes to apparent homogeneity and partially characterized. They preferentially hydrolyse pyrimidine 5′-monophosphates and 3′-monophosphates respectively. PN-I and PN-II operate as interconverting activities, capable of transferring the phosphate from the pyrimidine nucleoside monophosphate donor(s) to various nucleoside acceptors, including important drugs like 3′-azido-3′-deoxy-thymidine (AZT), cytosine-β-D-arabinofuranoside (AraC) and 5-fluoro-2′-deoxy-uridine (5FdUrd), pyrimidine analogues widely used in chemotherapy. Kinetic analysis showed linear behaviour for both PN-I and PN-II. PN-I phosphotransferase activity revealed higher affinity for oxy-nucleosides with respect to deoxy-nucleosides, whereas the contrary seems to be true for PN-II. These results show for the first time that soluble pyrimidine nucleotidases are endowed with pyrimidine-specific phosphotransferase activity.

Key words: Human erythrocyte pyrimidine-specific phosphotransferase; Pyrimidine nucleoside analogue; Soluble pyrimidine nucleotidase

1. Introduction

Two pyrimidine-specific nucleotidases (PN-I and PN-II; EC 3.1.3.5), are present in the soluble fraction of human erythrocytes [1,2]. Unlike nucleotidases from other sources, PN-I and PN-II are essentially inactive toward purine nucleotides. This unique substrate restriction appears to match with the needs of the red cell metabolism, which critically depends upon a limited reservoir of ATP, whose base cannot be synthesized de novo in the erythrocyte. Since the components of adenine nucleotide pool, in erythrocytes, are in rapid equilibrium through the mediation of adenylate kinase, the presence of a nucleotidase acting on AMP and producing diffusible adenosine should impose their inexorable drain [3]. Indeed a purine-specific nucleotidase, shown to be present in yeast, is almost inactive toward adenylic nucleoside monophosphates. It is well established that hereditary deficiency of one of these enzymes (PN-I) results in non-spherocytic haemolytic anemia. Such a condition determines a marked erythrocyte accumulation of pyrimidine nucleotides, detectable both by spectrophotometrically [1] and by a HPLC-based assay [4], and is accompanied by a distinctive basophilic stippling of erythrocytes upon Wright staining of blood smears [1]. Furthermore it has been shown [3,5,6] that affected patients, although defective in erythrocyte 5′-nucleotidase activity towards UMP, CMP and dCMP, conserved normal activity toward dUMP and dTMP. These findings were explained by the presence of PN-II activity [2,7]. Both enzymes have been purified to apparent homogeneity in our laboratory [8,9]. The two nucleotidase activities exhibited different enzymatic and molecular properties, supporting the hypothesis on the existence of two different genes coding for the two enzymatic proteins [7]. Additional interest on the study of the erythrocyte nucleotide metabolism derives from the use of pyrimidine analogues encapsulated as non-diffusible monophosphates in autologous erythrocytes, which have been used as carriers and bioreactors for the conversion of encapsulated pyrimidine analogues into their diffusible and pharmacologically active form [10]. The same authors reported on the involvement of the erythrocyte dephosphorylating machinery in the conversion of 5′-FdUMP to diffusible 5FdUrd, a potent antineoplastic agent. Furthermore it has been shown that purine nucleoside analogues can be phosphorylated through the action of 5′-nucleotidases, endowed with phosphotransferase activity [11]. This phosphorylating activity has drawn the attention of several research groups because nucleotidase might represent an alternative route to phosphorylate nucleoside analogues, which are not substrate of known cellular nucleoside kinases [12–14]. Thus, the study of the properties of the cytoplasmic pyrimidine nucleotidases, which may play both a catabolic and an interconverting role, might be of help for the understanding of the metabolic function of these two enzymes and for their possible utilization for the targeted activation of pyrimidine analogues. Knowledge of such enzyme system involved in the metabolism of pyrimidine nucleosides is therefore important in designing new drugs and innovative therapeutic approaches.

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Abbreviations: PN-I, pyrimidine 5′-nucleotidase type I; PN-II, pyrimidine nucleotidase type II; 5′-AZT-MP, 3′-azido-3′-deoxy-thymidine-5′-monophosphate; 5′-Ara-CMP, cytosine-β-D-arabinofuranoside-5′-monophosphate; 5′-FdUMP, 5-fluoro-deoxy-uridine-5′-monophosphate; AZT, 3′-azido-3′-deoxy-thymidine; AraC, cytosine-*R*-D-arabinofuranoside; 5FdUrd, 5-fluoro-2′-deoxy-uridine; DTT, dithiothreitol; p-CMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(2′-nitrobenzoic acid)

2. Material and methods

2.1. Materials

Nucleosides, nucleotides, imidazole, Tris were purchased from Sigma (St. Louis, MN, USA). Potassium dihydrogen phosphate, DTT, EDTA and chromatography grade methanol were from Merck (Darmstadt, Germany). Other reagents were of analytical grade.

2.2. Nucleotidase assay

Nucleotidase activity was measured by HPLC-based assay. The standard reaction mixture contained: 50 mM Tris/HCl pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM substrate, and the appropriate amount of enzyme sample, in a final volume of 500 μl. Incubations were carried out at 37°C for 5–120 min. The reaction was stopped by

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adding 100 μl of assay mixture to 50 μl of ice-cold 1.2 M $HClO_4$ in an Eppendorf tube. After 10 min at 0°C protein pellet was removed by 1 min centrifugation on microfuge. 130 μl of supernatant were neutralized by the addition of 35 μl of 1 M K_2CO_3 and formed $KClO_4$ was removed by centrifugation. An appropriate aliquot of the neutralized supernatant was injected into a HPCL apparatus to evaluate the amount of produced nucleoside. The elution was performed by the isocratic separation described by Amici et al. [15]. The HPLC was equipped with a 20 mm×4.6 mm I.D. C-18 guard column, 5 μm particle size, equilibrated and eluted with 100 mM potassium phosphate buffer, pH 6.0, at 2 ml/min flow rate. In an alternative assay procedure, the enzyme activity was tested by measuring the amount of phosphate released, according to the method described by Ames [16], which uses the ammonium molybdate-ascorbic acid complex.

2.3. Phosphotransferase assay

Phosphotransferase activity was measured by a HPLC-based assay of formed nucleoside monophosphate. The reaction mixture conditions were the same of nucleotidase assay, with the addition of an appropriate amount of nucleoside acceptor during the incubation at 37°C. An appropriate aliquot of the neutralized supernatant was injected into a HPLC apparatus to evaluate the amount of produced nucleoside. The HPLC was equipped with a 250 mm×4.6 mm I.D. C-18 column, 5 μm particle size, equilibrated with 100 mM potassium phosphate buffer pH 6.0 (buffer A) at a flow rate of 1.3 ml/min. The elution of nucleosides and nucleoside monophosphates of the mixture was achieved by the following gradient of methanol in buffer A; 9 min at no methanol, 6 min at up to 2.4% of methanol, 2.5 min at up to 9% of methanol and 2.5 min at up to 20% of methanol. The column was then flushed for 5 min at 20% of methanol and equilibrated with buffer A for 5 min prior to the next run. Replicate determinations at different incubation times were used to calculate each enzymatic activity point. One unit of enzyme activity (U) is defined as the amount of enzyme producing 1 μmol of phosphate, nucleoside or nucleoside monophosphate per minute under the standard assay conditions.

2.4. Purification procedures of PN-I and PN-II

In order to obtain enzyme preparations of suitable purity for kinetic experiments, the purification scheme described earlier by us [9] was applied both for PN-I and PN-II. Outdated bank blood was used as enzyme source. The two activities copurified from lysate through Phenyl-Sepharose CL4B (step 1 to step 4) and separated by the hydroxyapatite chromatography (step 5), as reported in [9]. The enzymes were further purified by parallel identical procedures performed in a FPLC system, consisting of step 6 and step 7, described in [9]. The resulting preparations were subjected to SDS-PAGE [17] and gel filtration chromatography to check for their homogeneity.

2.5. Other procedures

Protein concentration was determined as described by Bradford [18]. SDS-PAGE was conducted essentially as described by Schagger and von Jagow [17] with Tris/Tricine buffer, 10% acrylamide/bisacrylamide (T = 3%) running gel and 4% stacking gel. After electrophoresis, gel was Silver-stained [19]. Native Mr determination according to Andrews [20] was performed on a Superose 12 HR 10/30 column (Pharmacia) connected to a FPLC system. The column was equilibrated and eluted by using 50 mM Bis-Tris, pH 6.5, and 0.5 M NaCl, at a flow rate of 0.5 ml/min. The FPLC system (LKB) consisted of a single pump (model 2150), a gradient controller (model 2152), a fixed wavelength detector (Uvicord SD, model 2158) with a 279 nm filter, a two channel recorder (model 2210), and an automatic fraction collector (Elirac, model 2212). Samples were injected through a Valco valve equipped with a 1.0 ml loop. Isoelectric point determination was carried out by isoelectrofocusing on a 110 LKB 8100 ampholine column using a linear gradient of glycerol (0–65% w/v) and 1% (w/v) pH 3.5–10 ampholine carrier ampholytes.

3. Results and discussion

3.1. Enzyme purification

The present study was carried out with apparently homogeneous preparations of PN-I and PN-II purified from human erythrocytes. The purification and the characterization of PN-II have been previously reported by us [9]. PN-I and PN-II copurified until the fourth step of the purification procedure and then were separated during the hydroxyapatite chromatography step. The PN-I fraction, obtained in the fourth step, was further purified by two FPLC steps identical to those described for PN-II, yielding a chromatographically and electrophoretically homogeneous preparation. The purification of both enzymes is summarized in Table 1.

3.2. Molecular and enzymatic properties

The final preparation of PN-I had a specific activity of 28 U/mg. SDS-PAGE showed a single band corresponding to a molecular weight of 34 000 in agreement with the value of 36 000 as determined by gel filtration, suggesting a monomeric structure for PN-I (not shown). The isoelectric point was found to be 5.1. The pH optimum was 7.5 and Mg^{2+} ions were required for PN-I catalytic activity. The presence of DTT was absolutely required during the early, common steps of the purification procedure since PN-I rapidly inactivates in the absence of sulphydryl protective reagents. Furthermore, the enzyme was totally inactivated by both p-CMB and DTNB at 1 mM concentration, thus suggesting the involvement of cysteine residues in the enzymatic catalysis. The properties of PN-II have been previously reported in [9]. The specific activity of the preparation utilized in the present work was 73.9 U/mg.

3.3. Substrate specificity

PN-I preferentially hydrolyzed, in the order 5′-UMP, 5′-CMP, 5′-AZTMP, 5′-Ara-CMP, 5′-dCMP, 5′-dTMP, 5′-dUMP, while it was totally inactive toward purine nucleoside monophosphates, regardless the position of the phosphate moiety. PN-II, as it has been reported in [9], shows a high specificity toward pyrimidine nucleotides, being also able to dephosphorylate inosine and guanosine monophosphates, even though to a lesser extent. Since both PN-I and PN-II

Table 1
Purification of human erythrocyte PN enzymes

Step	PN-I			PN-II		
	Activity[a] (units)	Spec. act. (U/mg)	Yield (%)	Activity[a] (units)	Spec. act. (U/mg)	Yield (%)
Lysate	112	0.00016	100	259	0.000371	100
DEAE-cellulose	54	0.0129	48	237	0.0564	91.5
Ammonium sulfate	46.5	0.0155	42	245	0.0817	94.6
Phe-Seph. CL4B	19.3	0.0425	17	147	0.323	56.8
Hydroxyapatite	10.6	0.960	9.5	102	9.27	39.4
TSK-DEAE 5PW	4.9	8.00	4.3	14.5	28.3	5.6
TSK-Phe 5PW	2.9	28.2	2.6	13.6	73.9	5.3

[a]Activity was assayed by using 5′-CMP (PN-I) and 3′-UMP (PN-II) as the substrates.

hydrolyzed known antineoplastic agents, like 5′-AZTMP, 5′-Ara-CMP, 5′-FdUMP, they might be expected to play a relevant role in the metabolism of such pyrimidine analogues. Therefore the identification and/or rational design of effectors influencing PN-I and PN-II activity could be useful for the optimization of possible antineoplastic therapeutic protocols. Among a broad variety of compounds tested as hypothetical effectors of the nucleotidase activity, including inorganic phosphate, purine and pyrimidine bases, oxy- and deoxy-nucleosides, mono-, di-, and triphosphates, dinucleoside polyphosphates, tiazofurin, theophylline, methylated uric acids, theobromine and caffeine, only the reaction products, i.e. the nucleoside and phosphate, resulted inhibitory. Furthermore, unlike other previously described soluble purine nucleotidases [22], PN-I and PN-II are not stimulated by ATP, ADP and 2,3-bisphosphoglycerate, thus ruling out a possible contamination of our preparation by the mentioned purine nucleotidase activities. Product inhibition studies on PN-II were carried out by using substrate 3′-UMP concentrations ranging from 0.1 to 1 mM, at different fixed concentrations of either phosphate (1–2 mM) or uridine (10–20 mM). Double reciprocal plots show that the inhibition exerted by phosphate appears to be competitive, with a K_i value of 3.5 mM, as calculated from the slopes replot. The inhibition exerted by uridine is non-competitive of the mixed type and the intercepts replot exhibits a non-linear pattern approaching to a plateau at high uridine concentration. Since the intercept represented the reciprocal of apparent maximal nucleotidase activity, the plateau at high inhibitor concentration represents the residual activity of the inhibited enzyme. The data obtained from the product inhibition experiments were consistent with an ordered reaction, involving the release of nucleoside as the first product and of phosphate as the second [23].

3.4. PN-I and PN-II associated phosphotransferase activity

3.4.1. Kinetic measurements. During the study of the inhibition exerted by different nucleosides on both PN-I and PN-II by means of the HPLC-based assay, the appearance of the monophosphates of the nucleoside inhibitor was observed in the elution pattern. In particular, when dThd was used as the inhibitor of PN-II, by using 3′-dUMP as the substrate, 3′-dTMP was formed. The production of 3′-dTMP was depending upon the presence of 3′-dUMP in the incubation mixture and it was linearly time dependent (not

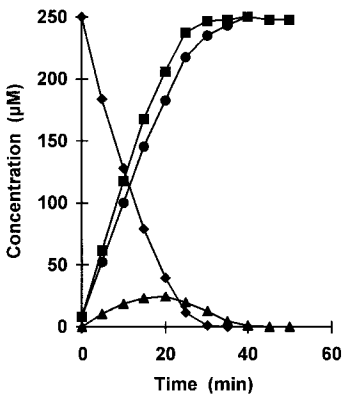

Fig. 1. Time course of PN-I activities in the presence of 5′-CMP (250 μM), Urd (20 mM) and 10 mU/ml of pure PN-I under the assay conditions described in Section 2. (♦), 5′-CMP; (■), Cyd; (●), phosphate and (▲), UMP were determined.

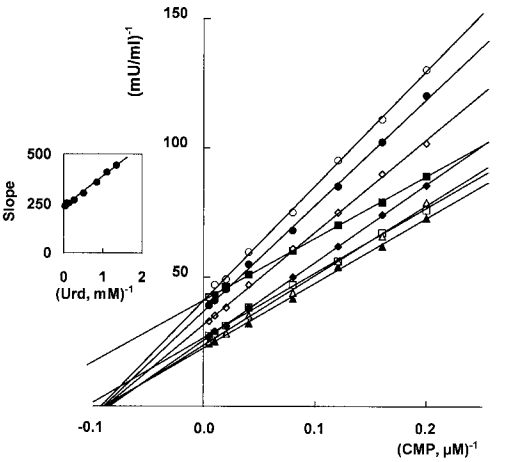

Fig. 2. Lineweaver-Burk plot of phosphotransferase activity of PN-I. 5′-CMP and Urd were used as substrates. Urd phosphorylation was measured as described in Section 2 at different fixed concentrations of Urd (mM): (○), 0.75; (●), 0.9; (◇), 1.2; (♦), 2.0; (△), 3.75; (▲), 7.5; (□), 15; (■), 30. In the inset, slopes of interpolation lines are plotted against 1/(Urd).

shown) in the initial velocity conditions. This finding was consistent with a phosphotransferase activity associated with the PN-II enzyme protein, whereby the phosphate from the nucleoside monophosphate substrate was enzymatically transferred to the inhibitor nucleoside acceptor. Furthermore, an identical behaviour was observed for PN-I when it was incubated with 5′-CMP and Urd, leading to the production of 5′-UMP. To our knowledge the existence of nucleotidases endowed with pyrimidine-specific phosphotransferase activity has never been described before. This observation appears of particular interest in view of the fact that several chemotherapeutic strategies are based on the use of pyrimidine analogues, whose metabolic activation pathways may be profoundly affected by the existence of such phosphotransferase activities. This prompted us to undertake an extensive study of the kinetic properties of the individual PN-I and PN-II enzyme activities, whose results could be exploited in the designing of novel chemotherapeutic approaches as well as in the modulation of the existing ones. Fig. 1 shows the time course observed by incubating PN-I with 5′-CMP as the substrate and Urd as the inhibitor/phosphate acceptor, leading to the formation of 5′-UMP. The same figure shows that upon prolonged incubation time, the newly formed 5′-UMP was hydrolyzed to Urd and inorganic phosphate after 40 min, whereas 5′-CMP was completely consumed after 30 min. As evidenced in Fig. 1, this resulted in a transient accumulation of 5′-UMP, the product of the phosphotransferase activity. Phosphotransferase activity was also detected by using stroma-free hemolysate of fresh erythrocytes, as well as by using enzyme preparations at different degrees of purity. The fraction of the transferred phosphate over the hydrolysed one was constant throughout all steps of the purification procedure (10% for PN-I, 7% for PN-II), thus suggesting that in human erythrocytes the pyrimidine nucleotidase and the pyrimidine phosphotransferase activities reside in the same protein. The examination of the Lineweaver-Burk plots of phosphotransferase activity showed converging patterns with respect to both donor and acceptor substrates (Figs. 2 and 3). The evaluation of the kinetic mechanism of phosphotransferase is not straightforward, since the enzymatic protein simultaneously

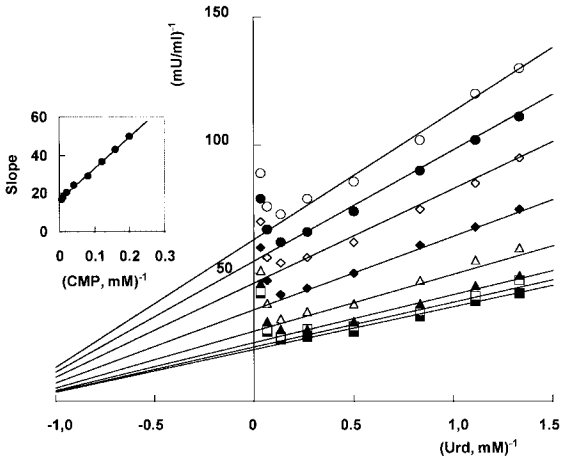

Fig. 3. Lineweaver-Burk plot of phosphotransferase activity of PN-I. 5′-CMP and Urd were used as substrates. Urd phosphorylation was measured as described in Section 2 at different fixed concentrations of 5′-CMP (μM): (○), 5.0; (●), 6.25; (◇), 8.3; (◆), 12.5; (△), 25; (▲), 50; (□), 100; (■), 200. In the inset, slopes of interpolation lines are plotted against 1/(5′-CMP).

catalyzes both the hydrolytic and phosphotransferasic reaction. Indeed, due to the action of the intrinsic nucleotidase activity, this behaviour might be consistent both with an Ordered bi bi and a Ping-Pong kinetic mechanism, like it has been previously described for nucleoside phosphotransferase from barley [21]. In order to distinguish between the two mechanisms further experiments need to be done. In particular, experiments are in progress for the purpose of isolating a putative phosphoenzyme intermediate. Furthermore, from Figs. 2 and 3 plots, substrate inhibition was evidenced at high concentrations of the nucleoside acceptor, which was not abolished by increasing nucleotide concentrations (Fig. 3). From the same plots the kinetic constants of PN-I were calculated to be: K_m(5′-CMP) = 9.3×10^{-6} M, K_m(Urd) = 6.4×10^{-4} M, K_i(5′-CMP) = 8.7×10^{-6} M, K_i(Urd) = 3.0×10^{-3} M.

3.4.2. Substrate specificity. In order to study the apparent specificity of PN-I and PN-II phosphotransferase activities, several nucleoside monophosphates were tested as donors towards various nucleoside acceptors. The relative activity was calculated as the ratio of the nucleoside monophosphate formed by the phosphotransferase activity over the nucleoside liberated via the nucleotidase activity. The results are summarized in Table 2. For PN-I the data show that the apparent specificity is principally influenced by the nucleoside acceptor, as evidenced by comparing the results obtained with a single acceptor (e.g. Cyd) with several donors, to those obtained with a single donor (e.g. 5′-CMP) with different acceptors; in this latter case a larger spreading of the apparent specificities is observed by inspection of the data in Table 2. PN-I shows also a relatively low, but significant phosphotransferase activity toward AZT and AraC as the acceptors. Such an activity could play a relevant role both in the metabolism and in the pharmacokinetics of these important drugs. The phosphorylated product could be rapidly converted to the corresponding diphosphate by intracellular nucleotide kinase, widely distributed in human tissues, thus protecting it from the nucleotidase activity. PN-II phosphotransferase activity was most effective towards pyrimidine nucleoside 3′-monophosphates leading to the production of nucleotides with the phosphate in the same position. In Table 2, the K_m values for phosphotransferase activity possessed by PN-I and PN-II, towards several substrates, are summarized. PN-I exhibited K_m values for deoxy-nucleosides one order of magnitude higher than those for oxy-nucleosides, whereas the contrary appears to hold for PN-II. Furthermore, unlike other previously described soluble purine nucleotidase/phosphotransferases [22], PN-I and PN-II phosphotransferase associated activities are not affected by ATP, ADP and 2,3-bisphosphoglycerate, thus ruling out the possibility that our pyrimidine nucleotidase preparations are contaminated by erythrocyte purine-specific nucleotidase/phosphotransferase activity. In addition, similarly to the behaviour of the hydrolytic activities, the phosphotransferase activities possessed by PN-I and PN-II are not influenced by other metabolites tested, including purine and pyrimidine bases, oxy- and deoxy-nucleoside mono-, di- and triphosphates, dinucleoside polyphosphates, tiazofurin, theophylline, methylated uric acids, theobromine and caffeine. In our laboratory the search for an efficient modulator of both phosphotransferases is actively in progress. The interest on the possibility to modulate such metabolic pathways resides in their possible exploitation for the development of metabolic

Table 2
Phosphotransferase activity of PN-I and PN-II

PN-I				PN-II			
Donor (1 mM)	Acceptor (10 mM)	Act. (%)[a]	K_m[b]	Donor (1 mM)	Acceptor (10 mM)	Act. (%)[a]	K_m[b]
CMP	Urd	25	0.64	3′-UMP	dUrd	29	6
CMP	dUrd	1.5	–[c]	3′-dUMP	dThd	16.8	12.3
CMP	dCyd	16	7.4	3′-dUMP	Urd	2	>100
CMP	dThd	0.5	5.3	3′-dUMP	Cyd	n.d.[d]	
CMP	Ado	n.d.[d]		3′-dUMP	dCyd	n.d.	
CMP	AZT	1	–	3′-dUMP	Ado	n.d.	
CMP	AraC	4	–	3′-dUMP	dAdo	n.d.	
CMP	5FdUrd	n.d.		3′-dUMP	Ino	n.d.	
UMP	Cyd	36	0.79	3′-dUMP	sIno	n.d.	
dCMP	Cyd	43	–	3′-dUMP	AZT	n.d.	
dUMP	Cyd	15	–	3′-dUMP	AraC	n.d.	
dTMP	Cyd	30	–	3′-dUMP	5FdUrd	19.7	7.6

[a]Percentage of phosphate transferred from nucleoside monophosphate donor to nucleoside acceptor.
[b]K_m of acceptor, values in mM.
[c]Not determined.
[d]Not detectable.

engineering-based antineoplastic and antiviral therapeutic strategies.

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