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A New Mechanism of Acyl Group Transfer in the Reaction Catalyzed by D-Glyceraldehyde-3-Phosphate Dehydrogenase

Elena V. Schmalhausen, Vladimir I. Muronetz,* and Natalya K. Nagradova

A. N. Belozersky Institute of Physico-Chemical Boilogy, Moscow State University, Moscow 119899, Russia, E-mail: muronets@biochem.msu.ru

Abstract

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) catalyzes the oxidative phosphorylation of its substrate in a two-step reaction. As a result of the first, oxidative step, the covalent intermediate wherein 3-phosphoglyceroyl moiety is bound to Cys149 of the active center is subjected to nucleophilic attack by inorganic phosphate, but remains resistant to hydrolytic decomposition. This ensures tight coupling of oxidation with phosphorylation in glycolysis. In this article, we present the experimental evidence for the conversion of GAPDH into a form capable of performing the reaction in the absence of inorganic phosphate. The structural basis for this conversion is the oxidation of a cysteine residue (probably Cys 153) into a sulfenic acid derivative under mild conditions to affect the integrity of the essential Cys 149. As a result, an intramolecular transfer of 3-phosphoglyceroyl group from the active center Cys 149 to Cys 153 becomes possible with subsequent hydrolysis of the sulfenyl carboxylate intermediate.

Index Entries: D-glyceraldehyde-3-phosphate dehydrogenase; catalytic mechanism; acyl transfer; phosphorolysis; hydrolysis; sulfenic acid; hydrogen peroxide.

Introduction

D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPDH) catalyzes a three-step reaction:

The oxidative step (1) produces a covalent acyl-thioester intermediate in complex with NADH. This complex is resistant to deacylating agents, and its conversion is only possible after replacing NADH by NAD^+ (2). The third step (3), further conversion of the intermediate, requires the presence

*Author to whom all correspondence and reprint requests should be addressed.

of inorganic phosphate: hydrolytic decomposition of the acyl-enzyme (4) at pH 8.5 is 50,000 times slower than phosphorolysis *(1)*. Despite the large body of structural information accumulated so far, the molecular basis of the enzyme functioning is not yet sufficiently understood *(2)*. In particular, the factors that determine the enzyme selectivity to the structure of the acyl acceptor and hence a coupling of substrate oxidation with phosphorylation have to be clarified. Little is known about the mechanism of deacylation that can take place in the absence of inorganic phosphate.

In our studies it was observed that the rate of hydrolytic decomposition of 3-phosphoglyceroyl-enzyme progressively increased on storage of the crystalline enzyme preparations at pH 7.5–8.0 in the absence of reducing agents. Because the reaction was shown to result in the accumulation of 3-phosphoglycerate, it was attributed to a nonphosphorylating dehydrogenase activity. Low-molecular-weight thiols effectively inhibited the activity without influencing the rate of oxidative phosphorylation reaction catalyzed by the enzyme *(3,4)*. This led us to propose that hydrolytic activity could depend on spontaneous oxidation of some functional groups of the enzyme molecule, other than the essential Cys 149 residues. In this work, the mechanism of GAPDH conversion into the form exhibiting the nonphosphorylating dehydrogenase activity was investigated. Experimental evidence supports the hypothesis on intramolecular transfer of 3-phosphoglyceroyl group from Cys 149 to a sulfenic acid derivative of another cysteine residue (probably Cys 153) with the subsequent hydrolysis of the sulfenyl-carboxylate intermediate. The existence of two forms of GAPDH differing in the mechanism of acyl transfer is postulated.

Materials and Methods

1,3-Bisphosphoglycerate was prepared and purified as described in ref. *5*. GAPDH was isolated from rabbit muscle by the method of Scopes *(6)* followed by gel filtration on a Sephadex G-100 column. The phosphorylating GAPDH activity was followed at 340 nm. The reaction was carried out at pH 8.9 and was initiated by the addition of 5 μg of the enzyme to a mixture containing 50 m*M* glycine, 50 m*M* potassium phosphate, 5 m*M* EDTA, 0.5 m*M* NAD^+, and 0.5 m*M* of D-glyceraldehyde-3-phosphate.

The nonphosphorylating GAPDH activity was followed at 340 nm by accumulation of NADH owing to oxidation of D-glyceraldehyde-3-phosphate to yield 3-phosphoglycerate in the absence of inorganic phosphate. The reaction was carried out at pH 7.6 in a mixture containing 50 m*M* Hepes, 5 m*M* EDTA, 0.5 m*M* NAD^+, 0.5 m*M* D-glyceraldehyde-3-phosphate, and 7 μ*M* GAPDH, and was initiated by the addition of the substrate. Two stages were observed in the reaction: a fast initial burst of NADH accumulation corresponding to the formation of the 3-phosphoglyceroyl-enzyme·NADH complex (the presteady state) was followed by a slower accumulation of NADH owing to the acyl enzyme hydrolysis (the steady state). The steady-state rate of NADH formation was used to calculate the

activity. The NADH burst value corresponding to the number of the acylated active centers was calculated by subtraction of the steady-state rate from the total rate of NADH accumulation.

The acylation of the enzyme by 1,3-bisphosphoglycerate and the hydrolytic decomposition of 3-phosphoglyceroyl-enzyme were followed at 360 nm by the drop in absorbance in the Racker band region *(7)* and its reappearance. The extinction coefficient for the charge transfer complex was determined by titration of GAPDH excess with NAD^+ and was found to be 1000 M^{-1} cm^{-1} at pH 7.6.

To obtain the "reduced" GAPDH form (i.e., to minimize the nonphosphorylating activity usually present in GAPDH preparations), the enzyme was incubated with 2 m*M* mercaptoethanol for 30 min. The excess of mercaptoethanol was then removed on a Sephadex G-50 column.

Results and Discussion

It was shown in our previous studies that some GAPDH preparations stored in the absence of reducing reagents exhibited nonphosphorylating dehydrogenase activity, which could be completely blocked by low concentrations of cysteine, reduced glutathione, and other thiol-containing compounds *(2,3)*. Figure 1 demonstrates the pH-dependence of the effect of reduced glutathione on the rate of hydrolytic decomposition of the acyl-enzyme. It is seen that the effect was maximal within the pH range corresponding to the protonated form of sulfhydryl groups of the reagent. This suggests that the inhibitory effect should involve reduction of some groups in the protein. The most probable one seems to be an oxidized cysteine residue.

A reduction of the active center Cys 149 was thought to be unlikely, since SH-containing compounds blocking hydrolysis exhibited no detectable effect on the catalytic reaction performed in the presence of inorganic phosphate, i.e., on the process depending on the integrity of the essential Cys 149. It was then inferred that the effect of the reducing agents was directed to some other SH-group. To clarify the situation, we investigated the oxidation of the enzyme's SH groups under strictly controlled conditions, using very low concentrations of hydrogen peroxide *(8)*.

Figure 2 shows the effect of hydrogen peroxide on the two activities, the phosphorylating one carried out in the presence of inorganic phosphate and the nonphosphorylating activity performed in its absence. It is seen that the nonphosphorylating (hydrolytic) activity is highly activated in the presence of one to two equivalents of hydrogen peroxide per tetramer, whereas the phosphorylating dehydrogenase activity is slightly changed. At higher hydrogen peroxide concentrations, both the nonphosphorylating and phosphorylating dehydrogenase activities are proportionally reduced, which can be explained by the oxidation of the essential catalytic residue Cys 149 under these conditions. The time-course of the effect of hydrogen peroxide added in the beginning of the experiment

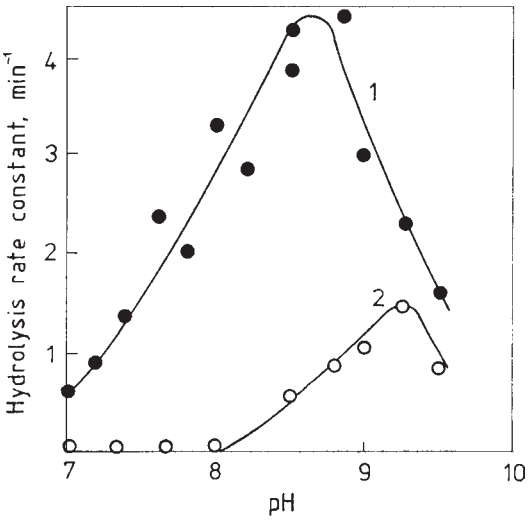

Fig. 1. The pH-dependencies of the rate constants of 3-phosphoglyceroyl-enzyme hydrolysis performed in the absence (1) or in the presence (2) of 10 μ*M* reduced glutathione. The reaction was carried out in 50 m*M* HEPES-NaOH (pH 7.0–8.5) or in 50 m*M* CHES-NaOH (pH 8.5–9.5), 5 m*M* EDTA, 0.2 m*M* NAD^+, 0.2 m*M* D-glyceraldehyde-3-phosphate, 8.5 μ*M* GAPDH, and initiated by the addition of the substrate.

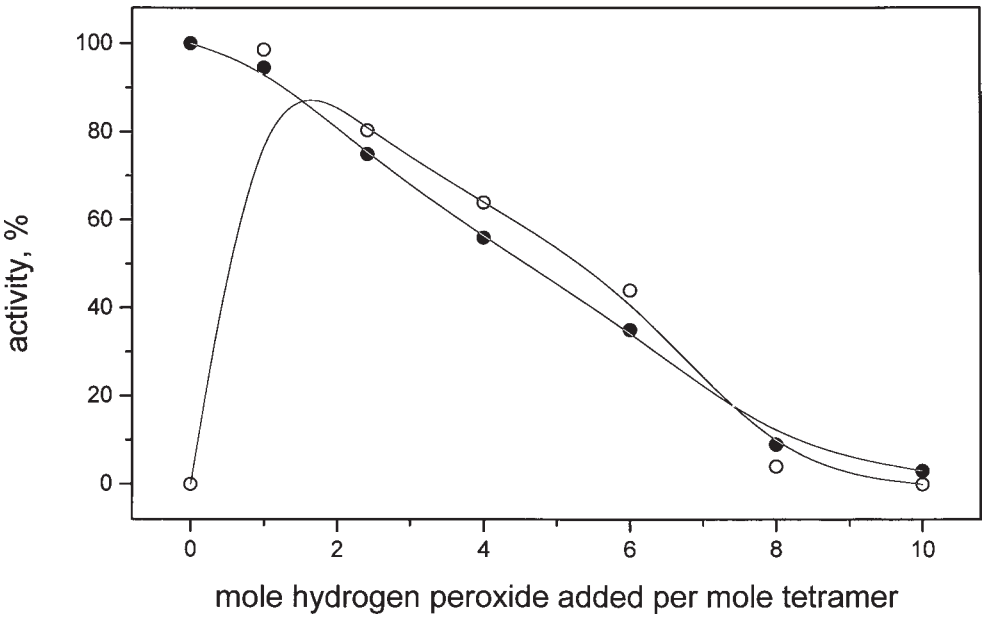

Fig. 2. The effect of hydrogen peroxide on the GAPDH activity measured in the presence (●, phosphorylating activity) and in the absence (○, nonphosphorylating activity) of inorganic phosphate. 6.7 μ*M* GAPDH was incubated for 1 h at 20°C and pH 7.6 in 50 m*M* HEPES containing 5 m*M* EDTA, 0.5 m*M* NAD^+, and hydrogen peroxide in the indicated concentrations; then both activities were measured.

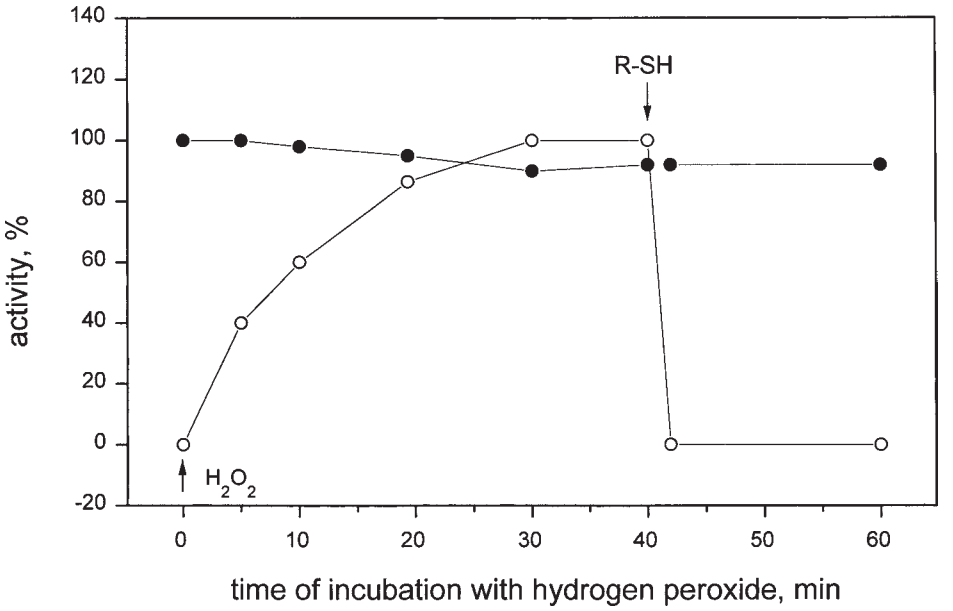

Fig. 3. The effect of hydrogen peroxide on two activities of GAPDH. ●, phosphorylating activity; ○, nonphosphorylating activity. The enzyme (7 μ*M*) was incubated at 20°C and pH 7.6 in 50 m*M* HEPES, 5 m*M* EDTA, and 0.5 m*M* NAD^+ in the presence of 10 μ*M* H_2O_2. The reaction was initiated by the addition of hydrogen peroxide at the moment indicated by the first arrow. At the fixed time intervals, samples were taken to measure the activities. The second arrow indicates the addition of 0.1 m*M* dithiothreitol.

is shown in Fig. 3. One can see that a considerable increase in the hydrolytic enzyme activity was observed with insignificant change of the phosphorylating activity under the conditions employed in this experiment. It is also seen that the addition of a reducing agent immediately blocked the hydrolytic activity.

Taken together, these results indicate that one can choose the experimental conditions with the selective induction of the nonphosphorylating activity but a minimum alteration of the phosphorylating one. They also suggest that the rise of the hydrolytic activity exhibited by the enzyme after treatment with micromolar concentrations of hydrogen peroxide had no relation to the oxidation of the essential Cys 149. Pronounced oxidation of this residue can only be detected at higher concentrations of H_2O_2 (not shown), and it is accompanied by the lowering of the dehydrogenase activity measured in the presence of inorganic phosphate. At the same time, the integrity of Cys 149 proved to be obviously necessary for both enzyme activities, the phosphorylating and the nonphosphorylating ones.

To reconcile these facts, we suggest the following mechanism of the hydrogen peroxide-induced alterations of the GAPDH functional properties. Very low H_2O_2 concentrations oxidize a functional group adjacent to the active center of the enzyme molecule (other than Cys 149) yielding a

modified group that readily accepts the acyl transferred from Cys 149. The next step of the reaction is hydrolysis of the intermediate and 3-phosphoglycerate release. A good candidate for the role of the acyl acceptor can be the oxygen of a sulfenic acid, the product of mild oxidation of cysteine residues in proteins. A sulfenic acid derivative of Cys 149 in GAPDH has been shown to exhibit a high acyl phosphatase activity, especially with 1,3-bisphosphoglycerate as a substrate *(9–11)*.

The evidence supporting the above hypothesis was obtained in additional experiments. First, the proposal on sulfenic acid formation as a product of mild oxidation of GAPDH by hydrogen peroxide was supported by the reversibility of oxidation. The characteristic property of sulfenic acids is their susceptibility to reduction even by very mild reductants, such as sodium arsenite *(12)*. The investigation of the effect of this agent on the properties of the oxidized GAPDH showed that the hydrolytic activity induced by hydrogen peroxide completely disappeared on incubation of the enzyme with 50 m*M* sodium arsenite.

Sulfenic acids also readily react with a number of amino compounds with the formation of the corresponding sulfenamides *(13)*. The incubation of the oxidized GAPDH in the presence of hydroxylamine resulted in the disappearance of the hydrolytic activity. Complete inhibition of the hydrolytic activity was also observed in the presence of 50 m*M* glycylglycine. Some α-amino acids (glycine, alanine, phenylalanine) were also shown to inhibit the reaction, whereas β-alanine exhibited no effect. We speculate that a reaction between sulfenic acid and an α-amino acid may occur yielding a sulfenamide. The effect of α-amino acids appeared to be reversible (the hydrolytic activity was restored after the removal of the reagents by gel filtration).

Another piece of evidence supporting our suggestion about the transfer of 3-phosphoglyceroyl group from Cys 149 to a sulfenic acid derivative of another cysteine residue produced under mild GAPDH oxidation was gained from the study of a direct acylation of the enzyme by 1,3-bisphosphoglycerate. The free SH-group of the active center Cys 149 residue is known to participate in the formation of a charge transfer complex with NAD^+ detected by absorption at 360 nm *(14)*. Acylation of the holoenzyme is accompanied by the disappearance of the so called Racker band.

As shown in Fig. 4, the almost complete disappearance of the Racker band was observed upon addition of 4 mol of 1,3-bisphosphoglycerate per mol of enzyme tetramer with the reduced enzyme form. This corresponds to acylation of 3.7 active sites per tetramer. It is seen that the formed 3-phosphoglyceroyl-enzyme is subject to a very slow hydrolysis. In the case of the enzyme treated with H_2O_2, the number of acylated sites was considerably lowered and increased again upon treatment of the oxidized enzyme with 100 μ*M* mercaptoethanol (not shown). Thus, oxidation of some groups was probably responsible for the difference in the stoichiometry of acylation. The possibility of the oxidation of Cys 149 SH groups under these conditions could be excluded on the basis of the determination of their number using the absorption of the charge transfer complex.

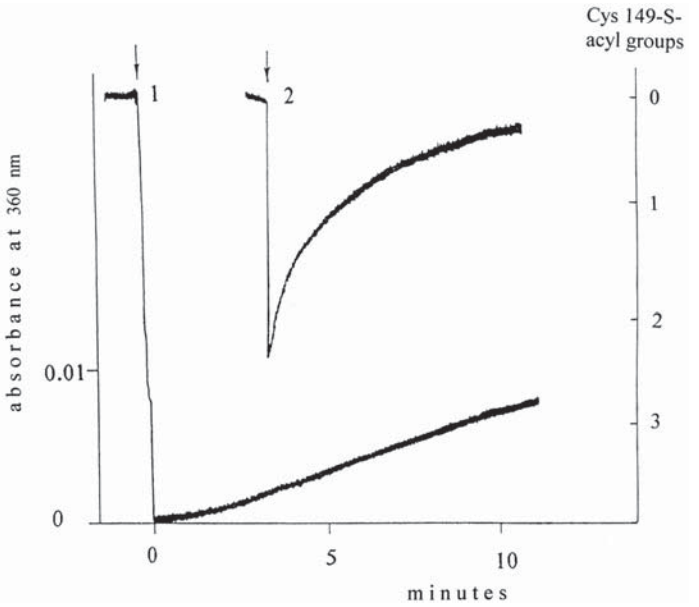

Fig. 4. Acylation of GAPDH by 1,3-bisphosphoglycerate and hydrolysis of the acyl-enzyme. The reaction mixture contained 50 m*M* HEPES pH 7.6, 5 m*M* EDTA, 0.5 m*M* NAD^+, 7 μ*M* GAPDH (calculated per tetramer). At the moment indicated by the arrow, 1,3-bisphosphoglycerate was added to a final concentration of 28 μ*M*. The drop in absorbance is due to the enzyme acylation; the slow reappearance of the Racker band is related to the acyl-enzyme hydrolysis. 1, "reduced" enzyme; 2, the same as 1, but after 30 min incubation with 10 μ*M* H_2O_2. Left ordinate, absorbance in the Racker band region. Right ordinate, the number of acylated Cys 149 residues per tetramer. See text for details.

Another possibility is the oxidation of some other groups resulting in the appearance of additional acyl acceptor sites. In this case, initial acylation of all four active site Cys 149 residues can be followed by partial transfer of 3-phosphoglyceroyl groups to the additional sites. Assuming that the process is sufficiently rapid, one can expect that the number of acylated Cys 149 residues measured under the experimental conditions will be lowered. This is in agreement with the results obtained. The acceleration of the rate of deacylation observed upon incubation of the enzyme with H_2O_2 (Fig. 4, curve 1) is probably due to the appearance of the sites that accept 3-phosphoglyceroyl groups from Cys 149 residues and are able to transfer them to water.

These putative sites still remain unidentified. Nevertheless, the existing structural information suggests Cys 153 oxidation to a sulfenic acid derivative under mild conditions. Not being directly involved in the formation of the active center, this residue is, however, located in its near proximity, sited in a conformationally flexible region of the enzyme molecule. It is easily accessible to modifying reagents *(15)*. Cys 153 possesses much greater reactivity than the remaining cysteine residues in the GAPDH

A
149 SH
153 SOH
+ R–C–H
O
NAD+
①
149 S–C–R
O
153 SOH
NADH
NAD+ NADH
②
149 S–C–R
O
153 SOH
HO-PO3 2-
③
149 SH
153 SOH
+ C–R
O
O–PO3 2-
④
⑤
149 SH
153 S–O–C–R
R
O
HOH
⑥
149 SH
153 SOH
O=C–R
OH
B
149 SH
153 SH
H2O2
R–SH
149 SH
153 SOH
Oxidation of R–C=O
H
is tightly coupled with phosphorylation
Oxidation of R–C=O
H
and accumulation of NADH are possible in the absence of Pi.

Fig. 5

molecule *(16)*. Several lines of evidence indicate a close interrelationship between the conformation of the catalytic center and the microenvironment of Cys 153. An intramolecular disulfide bond is formed between Cys 149 and Cys 153 on modification of the enzyme with 5,5'-dithiobis-(2-nitrobenzoate) *(17)*, and this indicates that the two residues can be brought into proximity.

Figure 5 summarizes our present view concerning the mechanism of alteration of the catalytic properties of GAPDH by means of covalent modification with an oxidizing agent. According to our hypothesis, GAPDH can exist in two easily interconvertible forms, which differ in their properties related to acyl transfer. (Fig. 5B). The first, "reduced," form is practically devoid of the ability to transfer the 3-phosphoglyceroyl group to water, whereas the "oxidized" form can catalyze acyl transfer to both inorganic phosphate and water. The very mild conditions of GAPDH transformation into the form exhibiting the nonphosphorylating activity lend support for the hypothesis that this process can be physiologically important *(18)*.

The mechanism of acyl transfer to inorganic phosphate must be identical with both enzyme forms, as they catalyze the phosphorylating dehydrogenase reaction at similar rates. At the same time, the "mildly oxidized" form acquires an ability to transfer the acyl group by a new mechanism, which includes an intramolecular transfer of 3-phosphoglyceroyl group resulting in the formation of a sulfenyl-carboxylate intermediate, subjected to subsequent hydrolysis.

Acknowledgments

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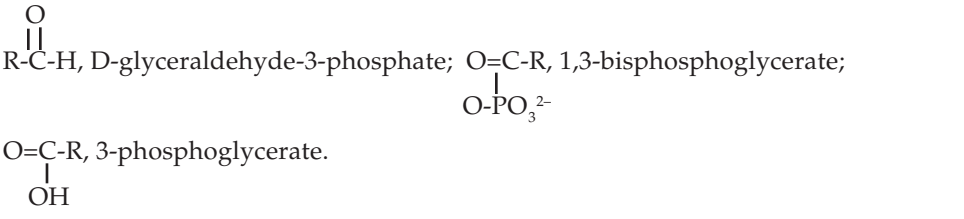
Fig. 5. *(previous page)* Hypothetical mechanisms of acyl transfer in the reaction catalyzed by GAPDH. **(A)** The steps of the reaction catalyzed by the "mildly oxidized" enzyme form: 1, formation of 3-phosphoglyceroyl-enzyme · NADH complex; 2, replacement of NADH by NAD^+; 3, phosphorolysis producing 1,3-bisphosphoglycerate; 4 and 5, intramolecular transfer of the acyl group; 6, hydrolysis of the sulfenyl-carboxylate intermediate producing 3-phosphoglycerate. **(B)** The interconversion of the reduced and "mildly oxidized" enzyme forms. 149-SH and 153-SH , the reduced forms of Cys 149 and Cys 153, respectively. 153-SOH, a sulfenic acid derivative of Cys 153.



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