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Forum Review

Thiol–Disulfide Balance: From the Concept of Oxidative Stress to that of Redox Regulation

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ABSTRACT

Originally, small thiols, including glutathione, were viewed as protective antioxidants, acting as free radical scavengers in the context of oxidative damage. Recently, there is a growing literature showing that protein glutathionylation (formation of protein-glutathione mixed disulfides) and other forms of cysteine oxidation may be a means of redox regulation under physiological conditions. This review discusses the importance of protein oxidation in redox regulation in view of the recent data originating from the application of redox proteomics to identify redox-sensitive targets. *Antioxid. Redox Signal.* 7, 964–972.

HISTORICAL PERSPECTIVES: FROM OXIDATIVE STRESS TO REDOX REGULATION

THERE is a growing interest in the study of reversible oxidation of protein cysteines as a means of redox regulation. This is, in part, a result of the evolution of the concept of redox regulation from that of oxidative stress, with the exception of the formation of protein–protein disulfide bonds, which belongs mainly to the field of protein folding.

Oxidative stress

Several data appeared in the 1960s on the biological effects of peroxidation of membrane lipids. The first occurrence in Pubmed is a 1960 *Nature* article by Tappel and Zalkin (78) on the protective effects of antioxidants, including glutathione and vitamin E. With all the biases of doing a simple Pubmed search, the first occurrence of the term "oxidative stress" is in 1970 in an article on red blood cells by Paniker *et al.* (63), and from then on several articles were published in the 1970s on oxidative stress-induced erythrocyte lysis and membrane peroxidation and damage, and the protective effects of reduced glutathione (GSH) in the system. The field of excellence of these studies has long been that of toxicology of compounds that, by various mechanisms, generate oxygen radicals or other free radicals: adriamycin, carbon tetrachloride, iron, and, obviously, oxygen and ionizing radiation. But soon it was clear that oxidative stress, triggered by reactive oxygen species (ROS), was also present in many disparate diseases, from cancer to inflammation and ageing, although it is still questionable whether it has any pathogenic role, despite the popularity of the use of antioxidant molecules as nutritional supplements.

Redox regulation

The mid 1980s saw the identification, purification, and cloning of several molecules that would later be shown to be key ones in the inflammatory response, including the cytokines tumor necrosis factor (TNF) (6, 65) and interleukin-1 (3, 55), and the transcription factor nuclear factor-κB (NF-κB) (73). In the next years, several articles appeared reporting the following: that oxidants participate in the activation of NF-κB and thiol antioxidants, including GSH and *N*-acetylcysteine (NAC), inhibit it (72, 75); that antioxidants, including GSH and NAC, inhibit the production and the action of TNF (66, 82); that TNF augments, and GSH or NAC inhibit, HIV proliferation (41, 60, 71, 75).

The articles reporting these findings, including those from our group, were still focused on the "simple" concept that oxidative stress was implicated in inflammatory and infective diseases, and that it could participate in triggering cytokine production and NF-κB activation or in augmenting the sensitivity to their action. However, this introduced the notion, in the title of a seminal article from the group of Baeuerle, that

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ROS could serve as "messengers" (72). Although the interpretation was often the simple one that ROS are bad and antioxidants are good, buried in some of those works are some observations that may have anticipated some of the current concepts in the role of protein thiol modification in redox regulation. In particular, Dröge and associates provided evidence that oxidized glutathione (glutathione disulfide, GSSG) is required for optimal activation of NF-κB in T cells (23). This aspect is not yet fully understood, but it was hypothesized that GSSG could stabilize phosphotyrosine by inhibiting protein phosphatases that have a cysteine in the active site that must be reduced for them to be active.

In any case, the observation that thiols inhibit cytokine production and NF-κB activation induced by stimuli, such as lipopolysaccharide, not necessarily inducing overproduction of ROS, at least *in vitro*, is, in our opinion, what makes it unlikely that these thiols, including NAC and GSH, act only as free radical scavengers.

It was also shown that ROS are not only produced under pathological conditions. For instance, epidermal growth factor induces production of hydrogen peroxide (4). The fact that ROS do not act only as pathogenic mediators, but also as intracellular messengers, is now established in the literature.

Our current hypothesis, far less established, is that, under these circumstances and/or under physiological conditions, these molecules may act as reducing agents rather than only as free radical scavengers.

PROTEIN CYSTEINE OXIDATION

If one looks at a classical textbook of biochemistry, the structure of many proteins is univocal, or highly stable, with some cysteines as free SH and others engaged in disulfide bonds. A typical example is serum albumin, with 17 cysteines, of which 16 are engaged in eight disulfide bonds and one is free. However, in some proteins, thiols and disulfides can undergo oxidoreduction under physiological conditions, or at least under conditions not associated with cellular dysfunctions or death. This is, in our opinion, what really identifies protein undergoing redox regulation, whether they are the ultimate target or the "redox sensor" upstream in the signaling cascade.

Thus, the challenge is to identify proteins whose thiol–disulfides can be oxidoreduced, a more complicate problem than the identification of "structural" disulfide bonds.

Several oxidation states of cysteines have been described. These include formation of intra- and interchain disulfide bonds, but also oxidation to sulfinic and sulfenic acids, and formation of mixed disulfides with small-molecular-weight thiols, including cysteine (cysteinylation) and glutathione (glutathionylation). The latter modification has gained particular attention as a means of redox regulation because they are readily reversible by thiol–disulfide exchange and by protein disulfide oxidoreductases, particularly glutaredoxin (35, 36).

If we consider glutathionylation as a regulatory posttranslational modification analogous to phosphorylation, then the point made above, that thiol antioxidants do not just act as free radical scavengers, is strengthened. In this perspective, not only is GSH a free radical scavenger and antioxidant, but glutathione (GSH and GSSG) is a signaling molecule. Although GSH oxidation to GSSG will favor protein glutathionylation by thiol–disulfide exchange, depletion of total glutathione levels, such as that achieved with the widely used inhibitor of protein synthesis buthionine sulfoximine, will remove an essential signaling molecule (somewhat like depleting cells of phosphate would not only deplete energy and ATP levels, but also prevent signaling mediated by protein kinases).

COMPARTMENTALIZATION OF PROTEIN THIOLS

One important concept in the biochemistry of protein thiol–disulfides is their subcellular localization. In fact, the cytoplasmic environment is a reducing one, mainly because of the high concentration of glutathione. Under normal conditions, the GSH/GSSG ratio in the cytosol is 30–100 (37). For this reason, it is normally accepted that most cysteines in cytosolic proteins are present as free thiols, and few proteins have disulfide bonds. This holds true for the cytosol, as in the endoplasmic reticulum the environment is more oxidizing, with a GSH/GSSG ratio of 1–3 (37). On the other hand, extracellular proteins exist in an oxidizing environment and thus show few free SH (26, 27, 70), which is evident in globular secreted proteins, as in the case of serum albumin mentioned above (79).

The plasma membrane, at the interface between these two extremes, may represent an interesting site both for "redox sensors," as extracellular, circulating and xenobiotic, oxidants will likely encounter the plasma membrane first, and for targets of redox regulation, as the membrane is rich with receptors, ion channels, etc. Interestingly, whereas the reducing environment in the cell is maintained by GSH, exofacial protein thiols are maintained by enzymatic systems that include protein disulfide isomerase (PDI) (40).

This compartmentalization probably explains why most known proteins undergoing redox regulation through oxidoreduction of cysteines are intracellular proteins whose thiols can be oxidized to disulfides. The opposite reaction is seldom considered, although it is clear that thiol oxidation is reversible. This could be due to the fact that redox regulation processes are often (still) viewed in the perspective of oxidative stress. But another reason is that most protein cysteines in the cytoplasm are in the reduced state, as mentioned above, and thus sensitive to oxidation when the redox balance is shifted toward a more oxidative condition (when the GSH/GSSG ratio decreases, or when hydrogen peroxide is added to or generated by the cells). Having very few disulfide bonds, their cysteines cannot be reduced further when the GSH/GSSG ratio increases or basal peroxide production decreases.

However, it should be noted that the statement that intracellular proteins have few disulfide bonds regards protein–protein disulfides, either intra- or interchain. It is possible that, under the normal redox conditions of the cytosol, cysteines may be present as mixed disulfides with glutathione. In fact, early publications by the group of Helmuth Sies has estimated that, in normal liver, up to 30 nmol of GSH in 1 g of normal liver is present as mixed disulfide with proteins (8, 9).

The percentage of protein-bound glutathione over total glutathione (including GSH and GSSG) in the liver is ~20% and can reach up to 50% under starvation, which is associated with a decrease in GSH levels [see review by Gilbert (33)]. Whereas these calculations were made based on chemical analyses, western blot analysis using anti-GSH antibodies have confirmed glutathionylation under basal conditions for actin in fibroblasts (64), γ-crystallin from human lens (19), and hemoglobin in erythrocytes (59). The formation and reduction of these disulfides may therefore act to sense not only oxidative stress, but also changes toward a more reduced redox state. Although most of the identified proteins are cytosolic, a study using immunocytochemical staining of cells and confocal microscopy has suggested that glutathionylated proteins are mainly associated with membrane blebs, the nucleus, and the perinuclear region (74).

As mentioned above, cytosolic proteins have few disulfide bonds, with the exception of redox enzymes with reactive cysteines in their active site, that form intramolecular disulfide bonds during their redox cycle, a phenomenon typical of all protein disulfide oxidoreductases, including PDI, thioredoxin, and glutaredoxin, where a disulfide bond is formed between the two vicinal cysteines of the CXXC active site (35, 36). However, in a recent study using proteomics techniques, almost 60 cytosolic proteins were identified to form disulfide bonds during oxidative stress (20).

This is even more evident for membrane proteins. Exofacial proteins are exposed to a highly oxidizing extracellular environment, and thus are mostly oxidized. We could show that exofacial protein thiols are very sensitive to reduction by thiols such as NAC. Using redox proteomics techniques to identify these proteins, we found that integrin VLA-4 is readily reduced by NAC and this augments the adhesive capacity of the cells (50). Right now, many membrane proteins, including ion channels, receptors, and adhesion molecules, have been shown to be regulated by thiol–disulfide oxidoreduction (see 50 for citations). This suggests that many of the biological effects of this and other thiol antioxidants might well be due to their reducing action on proteins on the cell membrane, rather than to an effect on intracellular molecules and most popular targets of redox regulation, such as NF-κB or activator protein-1.

OXIDATIVE MODIFICATIONS OF PROTEINS IDENTIFIED BY PROTEOMICS STUDIES: PROTEIN GLUTATHIONYLATION

Needless to say, most of the techniques to study cysteine oxidoreduction rely on methodologies developed to detect, identify, or quantitate free thiols, after steps of alkylation and reduction. More recently, antibodies have become available that recognize GSH present on proteins or *S*-nitrosylated proteins. To date, these have not been used extensively in proteomic studies, but clearly they will allow not only detection of oxidatively modified cysteines on gels, but also immunoaffinity purification of oxidized proteins.

The term proteomics is often used to describe techniques that allow identification of as many proteins as possible in a biological sample, and rely on the use of bidimensional electrophoresis followed by identification of the spots by triptic digestion and peptide mass fingerprinting using mass spectrometry. We originally used the term redox proteomics for a study to identify proteins undergoing glutathionylation in lymphocytes (11, 29, 32), and later in other cell types (30). In these experiments, we radiolabeled the intracellular GSH/GSSG pool using ^{35}S, then identified proteins that incorporate radioactivity using bidimensional electrophoresis and autoradiography. A very similar method has been described that makes use of biotin-labeled GSH (25, 77). The latter method has the advantage of allowing purification of glutathionylated proteins on avidin affinity column. A different approach based on the affinity of glutathionylated proteins for glutaredoxin was used by the group of Jan Cotgreave (53).

However, these approaches, with the exception of the latter one, may not allow the identification of all the proteins that are glutathionylated. They rather pick up the proteins that can undergo glutathionylation, normally under oxidative stress [we, for instance, used diamide, hydrogen peroxide, or menadione (11, 29, 30)]. It is likely that those proteins whose cysteines are already heavily glutathionylated will not incorporate further GSH. As mentioned above, the use of anti-glutathione antibodies might lead us to identify the glutathionylation status of proteins as well as we can do for protein phosphorylation. Nevertheless, a large number of glutathionylated proteins have been identified, and a not exhaustive list is given in Table 1.

OTHER FORMS OF PROTEIN OXIDATION

Although our work has been focused on protein glutathionylation, other forms of protein oxidation are described. *S*-Nitrosylation to form *S*-nitrosothiols is, in some way, similar to glutathionylation, although obviously it does not represent a mixed disulfide. Other oxidative modifications include methionine oxidation, tyrosine nitration (39), and carbonylation. Tyrosine nitration occurs by a reaction of protein cysteines with peroxynitrite or nitrosothiols, including *S*-nitrosoglutathione. Carbonylation is often referred to as "oxidative damage," a term that clearly denotes an orientation toward the concept of oxidative stress rather than redox regulation. Protein carbonyls result from ROS reacting with side chains of several amino acids (lysine, arginine, proline, and threonine) and can be easily measured using western blot following derivatization with dinitrophenylhydrazine (52, 76).

When we consider glutathionylation a means of redox regulation, we do so because it fulfills the main criteria for being a regulatory mechanism and not just one kind of protein damage: reversibility by glutaredoxin. In this sense, the other forms of oxidation are generally considered irreversible modifications. Protein carbonylation is thought to be irreversible and to lead to degradation of the oxidized proteins by the proteasome (24, 34).

In fact, other forms of oxidation can be reduced, including methionine sulfoxide, cysteine sulfinic, and sulfenic acid (58).

Table 1. Proteins Undergoing Glutathionylation

Actin capping protein	Glycogen phosphorylase	Peroxiredoxin 6
Actin-β	Heat shock cognate 70	Phosphoglycerate kinase
Adenylate kinase 2	HSP10	6-Phosphogluconolactonase
Aldolase A	HSP60	Profilin
Aldose reductase	HSP70 al	Prohibitin
Annexin A2	HSP90	Prolyl 4-hydroxylase
Ash protein	Hemoglobin	Phospholipase Cγ
Aspartyl-tRNA synthetase	Hepatoma-derived growth factor	Protein tyrosine phosphatase 1B
c-Jun	Histidine triad nucleotide-binding protein 2	Pyruvate kinase. M2 isozyme 57
Caspase 3	Ig λ chain	Ran-specific GTPase-activating protein
Cofilin	Inosine 5′-monophosphate dehydrogenase 2	h-Ras
Creatine kinase	Laminin (p40)	RNA-binding protein regulatory subunit
CRK-like protein	L-Lactate dehydrogenase	SFR1 splicing factor
Cytochrome *c* oxidase Va	Lymphocyte-specific protein 1	Stress-induced phosphoprotein 1
Cytochrome *c* oxidase Vb	Malate dehydrogenase	T complex protein 1
dUTP pyrophosphatase	My032 protein	Thioredoxin
Endoplasmic reticulum protein	Myosin	Transgelin, SM22 homologue calponin-like
ERP60*	Neuropolypeptide h3	Translation elongation factor
ERP72*	Nicotinamide *N*-methyltransferase	Triosephosphate isomerase
Glutaredoxin	Nucleophosmin	Tubulin
GRP94	Nucleoside diphosphate kinase A	Tropomyosin
Enolase 1, α	Nudix-type motif 6	Ubiquitin carboxyl-terminal hydrolase L3
Enoyl CoA hydratase	NF-κB p50 subunit	Ubiquitin conjugating enzyme E2N
Eukaryotic translation initiation factor 6	PDI	Vimentin
Fatty acid binding protein	Peptidylprolyl isomerase (cyclophilin A)	14–3-3 protein σ
β-Galactoside soluble lectin (galectin 1)	Peroxiredoxin 1	14–3-3 protein ζ
Glucosidase II precursor	Peroxiredoxin 2	20S proteasome subunit
Glutathione *S*-transferase	Peroxiredoxin 4	40S ribosomal protein S12
GAPDH	Peroxiredoxin 5	

ERP, endoplasmic reticulum protein; GRP, glucose-regulated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat-shock protein. The list of proteins is largely based on a review by Fratelli *et al.* (31), plus other references (21, 46, 56, 62).

Enzymatic denitration of nitrotyrosine was also suggested recently (38, 42).

The successful identification of posttranslationally modified proteins using redox proteomics suffers the classical problems of proteomics, *i.e.,* proteins that are present in higher concentration, or that are easily extractable and separated by electrophoresis, are favored. Nevertheless, in the literature we have noticed that some proteins recurred in studies identifying proteins susceptible to protein oxidation. In Table 2 we give a list, obtained from a partial survey of the literature, of those proteins that were found to undergo more than one form of oxidation.

We listed glutathionylated proteins (based on those listed in Table 1), as well as proteins that can form carbonyls or nitrotyrosine, as detected immunologically. We also considered those cytosolic proteins that can form disulfide bonds under oxidative stress, based on two interesting articles that appeared recently (7, 20). Although many regulatory proteins have been described to be redox-regulated, forming disulfides, we decided not to list bacterial proteins, which are reviewed elsewhere (54).

It can be seen from Table 2 that many proteins are particularly sensitive to oxidation. The classes of proteins that are most represented are glycolytic enzymes (aldolase, enolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and triosephosphate isomerase), heat shock proteins, chaperonin and proteins in the family of the PDI that assist in the formation of disulfide bonds and protein folding, and peroxiredoxins. Several cytoskeletal proteins are also represented. Although it can be easily predicted that proteins with reactive cysteines (due either to the accessibility in the three-dimensional structure or to its chemical reactivity) can equally form mixed disulfides with glutathione, protein disulfides, or other cysteine oxidation product, it is important to note that carbonylation and nitrosylation target other amino acids and with totally different chemical reactions. We cannot say, at present, whether these proteins represent preferential targets for oxidation or whether they are just casually found in the identification of oxidized proteins due to their favorable susceptibility to identification.

CONCLUSIONS

From Tables 1 and 2, it is clear that many glycolytic enzymes are easily oxidized. This is generally associated with

Table 2. Proteins Susceptible to More than One Type of Oxidation

	Glutathionylated	*Carbonylated*	*Nitrated*	*Disulfides*
Actin	+	+		+
Aconitase		+	+	+
Aldolase	+	+	+	
Annexin II	+		+	
Aldose reductase	+		+	
Calreticulin		+		+
Creatine kinase	+	+	+	+
Dihydropyrimidase-related		+	+	
Enolase	+	+	+	+
Fructose biphosphatase		+	+	
GAPDH	+	+	+	+
Glutathione *S*-transferase	+		+	
Hemoglobin	+	+		
HSP cognate 70	+			+
HSP60	+	+		
HSP70	+	+	+	+
HSP90	+			+
Ketoacyl CoA thiolase			+	+
Laminin	+		+	
L-Lactate dehydrogenase	+	+		+
Malate dehydrogenase	+		+	+
Myosin	+			+
NADPH-ubiquinone reductase			+	+
Neuropolypeptide h3	+	+		+
Nucleoside diphosphate kinase A	+			+
Peroxiredoxin 1	+			+
Peroxiredoxin 2	+	+		+
Peroxiredoxin 3	+		+	+
Prohibitin	+		+	+
PDI	+		+	
Pyruvate kinase	+		+	+
SOD1		+		+
SOD2			+	+
T-complex protein 1	+			+
Triosephosphate isomerase	+	+	+	+
Tropomyosin	+	+		+
Tubulin	+	+	+	+
Ubiquitin carboxyl-terminal hydrolase	+	+		
Vimentin	+	+		

SOD, superoxide dismutase; for other abbreviations, see Table 1. References for glutathionylated proteins are as in Table 1. Additional references on other forms of oxidation were obtained elsewhere (1, 2, 7, 10, 12–18, 20, 22, 28, 43–45, 47, 49, 57, 61, 67–69, 80).

inactivation of the enzyme activity. Importantly, this is in agreement with, and provides a molecular mechanism for, previous reports by Gilbert (33), who showed that the GSH/GSSG ratio regulates several enzymes of glycolysis/gluconeogenesis. Figure 1 shows the glycolytic pathway with those enzyme targets of protein oxidation marked in bold. It will be important, when the list of oxidatively modified proteins is longer, to identify other clusters of proteins. But it is already evident from the tables in this review how many stress proteins, including heat shock proteins and proteins of the endoplasmic reticulum, are among those.

One cautionary word to make clear what we have not discussed in the present review. In Table 2 and Fig. 1, we have shown common targets of different oxidative modifications. It should be noted, however, that, unlike the other forms of protein oxidation, protein glutathionylation may have roles other than regulating enzymes' activity: it can protect protein cysteines from irreversible forms of oxidation, and it can serve as a means to maintain glutathione inside the cell, whereas GSH oxidized to GSSG is rapidly exported.

Protein oxidation can thus be viewed as a major means by which the redox state of the cells regulates the various metabolic pathways. This picture is far from being complete and is a complex one. The redox state is defined by various parameters, the thiol/disulfide ratio, including the GSH/GSSG ratio, but also that of other redox couples with different redox potential or subcellular localization. It is also defined by the intracellular levels of various ROS and reactive nitrogen intermediates. In this redox regulation network, several enzymes will play a regulatory role in analogy with the various protein

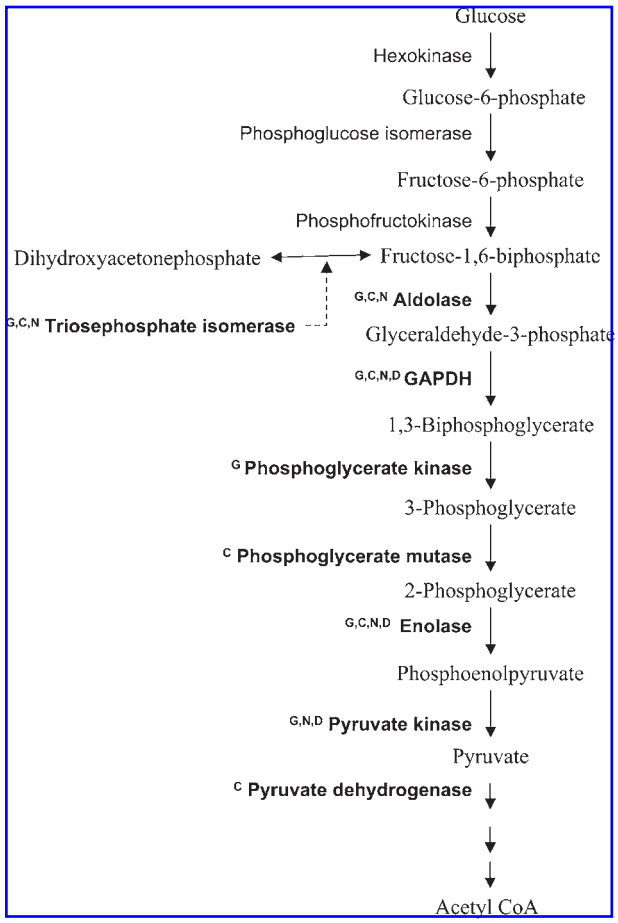

FIG. 1. Glycolytic enzymes undergoing protein oxidation. Enzymes in bold denote those that can undergo oxidative modifications, indicated by superscript as follows: [G]glutathionylated; [C]carbonylated; [N]nitrotyrosine; [D]disulfides (see text).

kinases and phosphatases. These include the enzyme that generate ROS and reactive nitrogen intermediates (*e.g.,* glucose oxidase, xanthine oxidase, nitric oxide synthase) and those antioxidant enzymes that can degrade them, specifically for the thiol–disulfide systems, the enzymes that can reduce or oxidize protein thiols. These proteins are characterized by a redox-active center represented by a CXXC motif, and include glutaredoxins, thioredoxins, PDIs, and the recently identified (in prokaryotes) sulfhydryl oxidases (81). The identification of the substrate specificity of these proteins, not only in terms of sequence specificity but in terms of redox potential, will be necessary to have a clearer picture. The recent studies to identify the substrates and targets for thioredoxin and peroxiredoxin (5, 48, 51) are a first step in this direction.

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ABBREVIATIONS

GSH, glutathione; GSSG, glutathione disulfide; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; PDI, protein disulfide isomerase; ROS, reactive oxygen species; TNF, tumor necrosis factor.

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