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SCIENTIFIC REVIEW

Kinetic Mechanism and Molecular Properties of Glutathione Reductase

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Summary

Glutathione reductase (GR) is a crucial enzyme (EC 1.6.4.2) that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH by the NADPH-dependent mechanism, an important cellular antioxidant system. Due to its significance, the enzyme has been purified from a number of animals, plants and microbial sources and studied in an effort to identify and explain its structure, kinetic mechanism and molecular properties since 1935. The kinetic mechanism of GR has been studied by several investigators and several models have been proposed for this enzyme. The kinetic mechanism is known to be a ping-pong/sequential ordered hybrid model. GR is a homodimeric flavoprotein and contains two FAD molecules as a prosthetic group, which is reducible by NADPH. The estimated molecular weight of the dimeric enzyme ranges from 100 to 120 kDa. Stability of GR has been tested in various organisms in a variety of ways and it was found that GR is one of the thermostable enzymes. Bovine liver and kidney cortex GR activity are relatively stable at high temperatures. GR belongs to the defense system protecting the organism against chemical and oxidative stress. Deficiency of the enzyme is characterized by hemolysis due to increased sensitivity of erythrocyte membranes to H_2O_2 and contributes to oxidative stress, which plays a key role in the pathogenesis of many diseases. Many studies have been carried out to explain the interaction of GR with drugs or chemicals and diseases. This review focuses on the molecular properties and kinetic mechanism of GR.

Key Words: *Glutathione reductase, oxidative stress, purification, kinetic mechanism, molecular properties.*
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Glutatyon Redüktazın Moleküler Özellikleri ve Kinetik Mekanizması
Özet

Glutatyon redüktaz (GR) glutatyon disülfidini sülfidril formuna (GSH) NADPH-bağımlı mekanizma ile indirgeyen hücresel antioksidan sistemin çok önemli bir enzimidir. Enzim 1935 yılından itibaren birçok hayvan, bitki ve mikrobiyal kaynaktan saflaştırılmış; yapısı, kinetik mekanizması ve moleküler özellikleri tanımlanmaya çalışılmıştır. Glutatyon redüktazın kinetik mekanizması birçok araştırmacı tarafından incelenmiş ve çeşitli modeller önerilmiştir. Kinetik mekanizması ping-pong/sıralı hibrid modele uygundur. GR prostetik grup olarak iki FAD molekülü taşıyan homodimerik flavoproteindir ve FAD grupları NADPH ile indirgenir. Dimerik enzimin yaklaşık moleküler ağırlığı 100–120 kDa arasında değişir. GR' nin stabilitesi çeşitli organizmalarda incelenmiştir ve ısıya dayanıklı bir enzim olduğu bulunmuştur. Sığır karaciğeri ve böbrek korteks glutatyon redüktazı yüksek sıcaklıklara karşı dayanıklıdır. GR organizmayı kimyasallara ve oksidatif strese karşı koruyan savunma sisteminin bir parçasıdır. Enzim eksikliğinin en önemli belirtisi eritrosit membranının aşırı duyarlılığının sonucu oluşan hemolizdir, ayrıca birçok hastalığın oluşumunda etkili rol oynayan oksidatif stres artmaktadır. GR' nin kimyasallar, ilaçlar ve hastalıklarla ilişkisini açıklamak için birçok araştırma yapılmaktadır. Bu derleme glutatyon redüktazın moleküler özellikleri ve kinetik mekanizması ile ilgili bilgiler içermektedir.

Anahtar Kelimeler: *Glutatyon redüktaz, oksidatif stres, saflaştırma, kinetik mekanizma, moleküler özellikler*

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INTRODUCTION

Glutathione reductase (GR, NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2) is a ubiquitous enzyme required for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) concomitantly oxidizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction essential for the stability and integrity of red cells (1). The historical progress of GR began in 1935 by Meldrum and Tarr (2), who found that GSSG was reduced by rat blood and demonstrated the function of TPNH (NADPH) as a cofactor in this system. In 1955, GR was partially purified from *Escherichia coli* by Asnis (3). GR has been purified from a variety of sources, from bacteria to mammalian cells, with different purification folds and yields, including the bovine liver, anoxia-tolerant turtle, *Trachemys scripta elegans, Escherichia coli,* chicken liver, rat liver, bovine brain, calf liver and pea leaves (4-11). In the purification of GR, DEAE-Sephadex, Sephadex G-100, hydroxyapatite (10), Sephadex G-75, CM-cellulose, Sephacryl S-200 (8), and DEAE-Sepharose (4) columns were used frequently. The enzyme has been purified very rapidly in high yield by employing 2',5'-ADP-Sepharose 4B (4,10,11) as affinity column. Reactive Red-120-Agarose, Sephacryl S–300 (12), fast protein liquid chromatography (FPLC)-anion exchange, and FPLC-hydrophobic interaction chromatography (11) are also used for the purification of GR.

The homodimeric FAD-containing GR belongs to the family of NADPH-dependent oxidoreductases and is present in many pro- and eukaryotic organisms (13). The enzyme is a homodimeric protein constituted by subunits with molecular weight of about 55 kDa. It has been mentioned that in the absence of thiols, GR shows a tendency to form tetramers and larger forms. Although these large forms are catalytically active, under cellular conditions the presence of its product, GSH, should maintain the enzyme in its dimeric form (14).

Free radicals are generated from regular biochemical and physiological reactions in the cell and are involved in the etiology of many diseases such as cancer and cardiovascular and pulmonary disorders. The human body is protected against damaging effects of these radicals by a broad variety of systems (15). The enzyme of the glutathione redox cycle, GR is involved in the defense mechanism of the cell and protects it from the harmful effects of endogenous and exogenous hydroperoxides (16). Alterations in the activities of antioxidant enzymes GR, glutathione peroxidase (GPx), glutathione. S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) (Fig. 1) may affect the cellular defense system.

The Molecular Structure of Glutathione Reductase

GR is classified under the name of disulfide oxidoreductases, and in these enzymes shows high sequence and structural homology and catalyzes the pyridine-nucleotide-dependent reduction of a variety of substrates, including disulfide-bonded substrates (lipoamide dehydrogenase, GR, thioredoxin reductase, and alkylhydroperoxide reductase), mercuric ion (mercuric ion reductase), hydrogen peroxide (NADH peroxidase), and molecular oxygen (NADH oxidase) (17). Both mechanistically and structurally, lipoamide dehydrogenase and GR have been proven more closely related than either is to thioredoxin reductase (18).

All GR family members share a similar three-dimensional structure in their FAD binding domain as well as at least one conserved sequence motif (19). The topology of the GR family consists of a central five-stranded parallel β sheet (β 1, β 2, β 3, β 7, and β 8) surrounded by α helices (α 1 and α 2) and an additional crossover connection composed of a three-stranded antiparallel β sheet (β 4–6) (20). In every FAD-binding family, the pyrophosphate moiety binds to the most strongly conserved sequence motif, suggesting that pyrophosphate binding is a significant component of molecular recognition, and sequence motifs can identify proteins that bind phosphate-containing ligands (19).

GR is a homodimeric enzyme of which each subunit contains four well-defined domains (18,19). The dimeric nature of the enzyme is critical for its function,

because both subunits contribute with essential residues to the constitution of the active site (21). The catalytic cycle of GR has two phases: a reductive half-reaction and an oxidative half-reaction. During the reductive half-reaction, FAD, the prosthetic group of GR, is reduced by NADPH and reducing equivalents are transferred to a redox-active disulfide. In the oxidative half-reaction, the resulting dithiol reacts with the glutathione disulfide and the final electron acceptor GSSG is reduced to two GSH at the active site of GR. The enzyme contains an acid catalyst, His-456, having a pK(a) of 9.2 that protonates the first glutathione (22). His-439 and Tyr-99 are proton donor / acceptor in the glutathione-binding pocket and have an important role in the catalytic mechanism (23). The Cys-2 moiety of GR is involved in the aggregation of the enzyme. Cys-58 and Cys-63 (formerly Cys-41 and Cys-46) are the enzyme's redox-active dithiol. Cys-90 is located at the twofold axis and forms a disulfide bridge with Cys-90 of the other peptide chain of the enzyme (24).

Tyr-114 and Tyr-197 are located in the GSSG binding site and NADPH binding site, respectively, and are directly involved in catalysis. Mutation of either residue has important effects on the enzymatic mechanism. For example, mutation of Tyr-197 leads to a decrease in Km for GSSG, and mutation of Tyr-114 leads to a decrease in Km for NADPH. This behavior is predicted for enzymes operating by a ping-pong mechanism (25). The second glycine residue (Gly-176) of the conserved GXGXXA "fingerprint" motif occurs at the N-terminus of the alpha-helix that characterizes the dinucleotide-binding domain, in close proximity to the pyrophosphate bridge of the bound coenzyme (26). GR deficiency is very rare, but mutations in the GR gene and nutritional deficiency of riboflavin affect the normal activity of the enzyme. It has been found that Gly-330 is a highly conserved residue in the superfamily of NADPH-dependent disulfide reductases. G330 A is the first mutation identified in the GR gene causing clinical GR deficiency, and this mutation affects the thermostability of the protein. There are also other studies on enzyme deficiency that was identified with different mutations (27).

The Kinetic Mechanism of Glutathione Reductase

The enzyme catalyzes the reduction of glutathione disulfide by NADPH, which is shown below (21,28).

$$\mathbf{NADPH + GSSG \xleftrightarrow{GR} NADP^{+} + 2GSH}$$

GR has high substrate specificity. NADPH is the only nucleotide coenzyme in the cell that gives any significant activity under physiological conditions (29), even though NADH can function as an electron donor at low ionic strength in vitro (30).

The enzymatic mechanism of GR has been thoroughly investigated by spectroscopic, kinetic and genetic approaches from a variety of sources, from bacteria to mammalian cells. In the studies of the reaction mechanism of yeast and *Phycomyces blakesleeanus* GR, at low concentrations of GSSG, the ping-pong mechanism prevails, whereas at high concentrations, the ordered mechanism appears to dominate (31,32). Mannervik (33) suggested that GR is acting according to both ping-pong and sequential mechanism, a type of mechanism termed as branched mechanism. Branched mechanism is favored by the nonlinear inhibition pattern produced by $NADP^+$. However, at low GSSG concentrations, the rate of the equation can be approximated by that of a simple ping-pong mechanism (8).

To determine whether the mechanism is ping-pong or sequential, product inhibition studies were performed and $NADP^+$ was found as a competitive inhibitor with respect to NADPH (4). Competitive inhibition by a product against the corresponding substrate could be expected in various sequential mechanisms (Theorell-Chance, ordered, or rapid equilibrium random), but not in a ping-pong mechanism (34). In ping-pong mechanism, on the other hand, $NADP^+$ is a noncompetitive inhibitor. GSH is expected to be noncompetitive with both substrates for either of the two mechanisms considered (35). In our study, we have reported that the kinetic mechanism of bovine liver GR is consistent with a branched mechanism (4); the kinetic mechanism of GR was also found as branched mechanism in *Cyanobacterium*

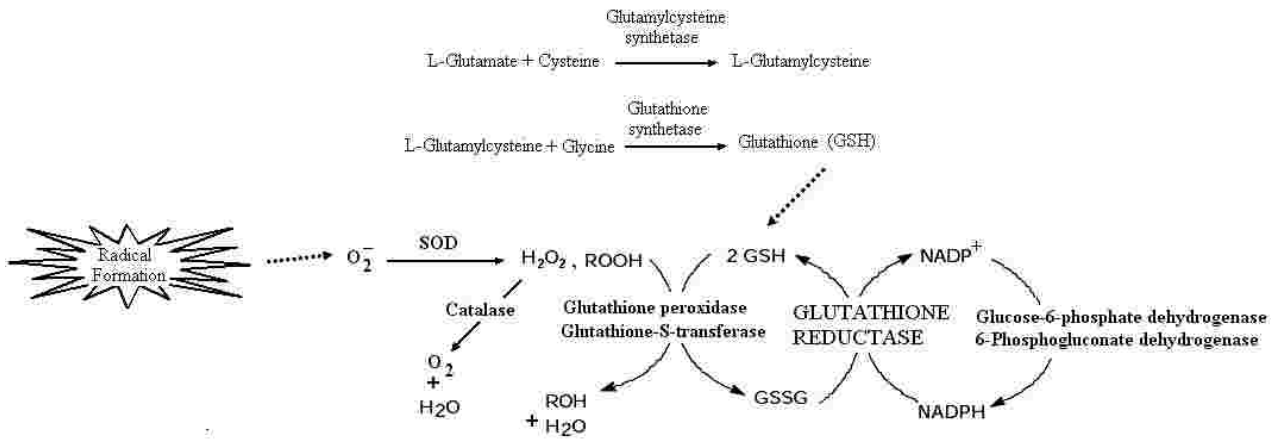

Figure 1. Glutathione reductase has a key role in the antioxidant system.

anabaena and the mouse liver (5,36). The kinetic mechanism of human erythrocyte GR follows the ping-pong / sequential ordered hybrid model (37).

The Stability of Glutathione Reductase

GR is one of the thermostable enzymes. The mouse liver enzyme was stabilized against thermal inactivation at 80°C by GSSG and less markedly by $NADP^+$ and GSH, but not by NADPH or FAD (36). GR from wheat grain was very resistant to high and low temperatures (38). The human lens GR maintains nearly 100% of its original activity up to 65°C (39). Serum GR activity decreases if left at room temperature. It has been shown that in some clinical disorders, dilution of the sample slows this decay, but reverses the inactivation in sera from patients with liver or biliary tract disease. The inactivation may be the result of conformational folding with "burying" of active sites, or of molecular aggregation brought about by hydrogen bonding or disulfide bond formation (40). In another study, GR from *Saccharomyces cerevisiae* and *Escherichia coli* were rapidly inactivated following aerobic incubation with NADPH, NADH and several other reductants, in a time- and temperature-dependent process (41).

We tested the thermostability of GR in two bovine tissues: liver and kidney cortex. All the procedures were carried out at +4°C. GR activity was determined according to modified Stall method (42). The stability of the bovine kidney and liver GR was tested in 105 000 x g one hour supernatants, which wer e homogenized with 10 mM Tris / HCI buffer, pH 7.6, containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The liver and kidney enzyme was stable at 4°C for two weeks, but after seven weeks the kidney enzyme has lost 66% of its activity. When the enzyme was stored at -20°C, kidney and liver enzyme became more stable and activity loss was only 14% and 26%, respectively. No preserver or reductant was added to the buffer to affect the stability of the enzyme during this period of time. Bovine liver GR is more stable than kidney enzyme according to time and temperature. The stability of the enzymes change over time and at 80°C, the enzymes are rapidly inactivated. These results are shown in Table 1.

Table 1. Thermostability of the bovine liver and kidney glutathione reductases

Temperature & Time	Tissue	% Remaining Protein	% Remaining GR Activity
45°C 110'	Kidney cortex	69.9	76.1
	Liver	58.1	94.4
50°C 110'	Kidney cortex	53	71.1
	Liver	53.9	90.9
55°C 110'	Kidney cortex	44.2	70
	Liver	53.4	90.3
60°C 110'	Kidney cortex	36.2	64
	Liver	16.1	78.8
70°C 110'	Kidney cortex	20.9	58.9
	Liver	9.2	61.8
80°C 60'	Kidney cortex	14.5	20.8
	Liver	7.6	45.3

CONCLUSION

Glutathione reductase is a key enzyme of the antioxidative system that protects cells against free radicals. The enzyme catalyzes the reduction of GSSG to GSH by the NADPH-dependent mechanism. Decreased GSH / GSSG ratio contributes to oxidative stress and there is increasing evidence indicating that oxidative stress plays an important role in the pathogenesis of many diseases. However, there is a need to continue to investigate the mechanisms of diseases by which increased oxidative stress accelerates. Due to its important role, this enzyme is more stable than the other cytosolic enzymes and can protect its activity at high temperatures. This kind of stability can be seen in thermophylic species. In the inhibition of GR and disturbance in the cellular prooxidant-antioxidant balance, intracellular GSSG accumulates, and the loss of thiol redox balance may cause loss of cellular homeostasis and numerous diseases. Chemicals or drugs significantly induce activities of detoxification and antioxidant enzymes such as GR. Although there have been many structural, physiological and clinical studies to explain all properties of GR, it seems that additional studies about this enzyme are necessary in the future. The function of GR and balance of the GSH / GSSG ratio are the key factors of various diseases and are awaiting resolution in future researches. During oxidative stress and deficiency of GR, intracellular GSSG accumulates, and the loss of thiol redox balance may cause deleterious consequences for metabolic regulation, cellular integrity, and organ homeostasis. GR inhibition disturbs cellular prooxidant-antioxidant balance and may contribute to the genesis of many diseases.

The reduced form of GSH is very important for cell viability, proliferation and protection from oxidative damage. Thus, clarifying the molecular structure and kinetic mechanism of the enzyme is gaining in importance for developing novel drugs for cancer therapy and other diseases.

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