Glutathione Reductase

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Glutathione reductase (GR) has been detected in bacteria[1], yeast[2], peas[3], wheat germ[4], plants[5], in nearly all animal tissues[6], in erythrocytes, platelets[7], and in human serum[6] and that of other animal species[8-11]. In the rat the activity decreases in the following order: kidney > liver > spleen > heart > brain > skeletal muscle > erythrocytes > serum[6]. The enzyme is located in all fractions of the cell. In rat brain homogenates 11.9% of the total activity was found in the cell nuclei, 35.4% in the mitochondria, 9.5% in the microsomes and 43.9% in the soluble supernatant after high speed centrifuging[12].

Glutathione reductase is not absolutely TPN-specific (equation 1). The crystalline enzyme from yeast[2] and highly purified preparations from, for example, ox liver[2], rat and rabbit liver[13] can catalyse the reduction of oxidized glutathione (GSSG) by DPNH. An enzyme purified more than 300-fold from rat liver was only active with TPNH; this finding has still to be confirmed.

Whether glutathione reductase is an enzyme with two active sites or whether it is a mixture of two different enzymes is still not clear.

The glutathione reductase from *E. coli*[1] and plants[5] are flavoproteins. The flavin can be split off by mild acid hydrolysis and after addition of flavine adenine dinucleotide to the apoenzyme the full activity is restored. Flavine mononucleotide and riboflavin are inactive and riboflavin may even inhibit[5].

The activity of the enzyme can be measured by several different physical or chemical methods[15,16]. The measurements described here are spectrophotometric[8].

For the role of glutathione reductase in the differential diagnosis of liver diseases, see, for example[17].

Principle

Glutathione reductase (GR) catalyses the reactions:

(1) $$GSSG + TPNH + H^+ \longrightarrow 2\,GSH + TPN^+$$

(2) $$GSSG + DPNH + H^+ \longrightarrow 2\,GSH + DPN^+$$

The reactions proceed to completion; they are irreversible. The measure of the activity of the enzyme is the rate of decrease of optical density due to the oxidation of TPNH or DPNH.

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A. TPNH-specific Glutathione Reductase

Optimum Conditions for Measurements

For the enzyme in human serum the pH optimum in phosphate and tris-maleate buffer is between 6.4 and 6.7. At pH 6.5 the enzyme is saturated with 10^{-3} M GSSG and 4×10^{-4} M TPNH. The Michaelis constant for TPNH with GSSG saturation is 2.5×10^{-5} M, and for GSSG with TPNH saturation is 7×10^{-5} M. A GSSG concentration of $>5\times10^{-3}$ M inhibits. The reaction is linear with time up to $\Delta E_{366} = 0.100$.

Reagents

1. Potassium dihydrogen phosphate, KH_2PO_4
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\,H_2O$
3. Sodium hydroxide, 1 N
4. Sodium hydrogen carbonate, 1% (w/v)
5. Reduced triphosphopyridine nucleotide, TPNH
   tetrasodium salt, TPNH-Na_4. Commercial preparation, see p. 1030.
6. Oxidized glutathione, GSSG
   commercial preparation, see p. 1019.

Preparation of Solutions

I. Phosphate buffer (0.067 M; pH 6.6):
   a) Dissolve 9.087 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
   b) Dissolve 11.876 g. $Na_2HPO_4 \cdot 2\,H_2O$ in doubly distilled water and make up to 1000 ml.
   Dilute 360 ml. solution b) to 1000 ml. with solution a).

II. Reduced triphosphopyridine nucleotide (*ca.* 6×10^{-3} M β-TPNH):
   Dissolve 6 mg. TPNH-Na_4 in 1% $NaHCO_3$ solution and make up to 10.0 ml.

III. Oxidized glutathione (7.5×10^{-3} M GSSG):
   Dissolve 230 mg. GSSG in *ca.* 20 ml. doubly distilled water, adjust to pH 6.6 with 1 N NaOH and dilute to 50 ml. with doubly distilled water.

Stability of the solutions

The GSSG solution keeps for at least 4 weeks in the frozen state. The TPNH solution loses activity after 1 week in the frozen state. The GR activity in serum does not decrease within 5–6 days if the sample is kept in a deep-freeze.

Procedure

Spectrophotometric measurements

Preferably use fresh serum free from haemolysis.
Wavelength: 340 or 366 mμ light path: 1 cm.; final volume: 3 ml.; temperature: 25°C (constant!). For each series of measurements prepare a control containing water instead of GSSG solution.

Measure against air or water.

Pipette successively into the cuvette:

2.4 ml. phosphate buffer (solution I)
0.2 ml. TPNH solution (II)
0.2 ml. GSSG solution (III)
0.2 ml. serum.

Mix and read the optical density E_0. After exactly 1, 2 and 3 min. read the optical densities E_1, E_2 and E_3. Proceed in the same way with the control. Average the values of ΔE/min.

Under these conditions the TPNH-dependent glutathione reduction is linear with time. With very high activity or with non-linear reaction curves dilute the serum.

Calculations

A unit is the amount of enzyme which reduces 1 μmole GSSG in 1 hour at 25° C.

Calculate the difference of the average values (ΔE/min.) for the sample and control: ΔE_{GR}/min. For measurements at 340 mμ, with 0.2 ml. serum in an assay volume of 3 ml.:

$$\frac{(\Delta E_{GR}/\text{min.}) \times 3 \times 60}{6.22 \times 0.2} = (\Delta E_{GR}/\text{min.}) \times 144 = \text{units/ml. serum}$$

For measurements at 366 mμ:

$$\frac{(\Delta E_{GR}/\text{min.}) \times 3 \times 60}{3.3 \times 0.2} = (\Delta E_{GR}/\text{min.}) \times 272 = \text{units/ml. serum}$$

where

6.22 and 3.3 [cm.^{2}/μmole] are the extinction coefficients for TPNH (DPNH) at 340 and 366 mμ respectively.

3 = volume of the assay mixture
60 = conversion from min. to hr.
0.2 = ml. serum in the assay mixture

B. DPNH-specific Glutathione Reductase

Optimum Conditions for Measurements

The pH optimum for the enzyme in human serum is at pH 6.2. It has been reported that phosphate ions activate the reaction [2], but this has never been observed with serum. Tris-maleate inhibits the enzyme (maleate is a sulphydryl reagent and the enzyme has functional SH groups, see below). Phosphate also inhibits the enzyme and the inhibition is proportional to the concentration; 0.15 M inhibits 30% [8]. In 0.067 M phosphate buffer (pH 6.2) the enzyme is saturated with 5×10^{-4} M GSSG; when it is half saturated with DPNH, the Michaelis constant for GSSG is 2.5×10^{-5} M. That for DPNH is 5×10^{-4} M when the enzyme is saturated with GSSG.

The enzyme cannot be saturated with DPNH in this method, because 20 times more DPNH is required for saturation than can be taken for reliable optical measurements. The conditions of measurements are therefore not optimum.

Reagents

As on p. 876, but instead of TPNH

5. Reduced diphosphopyridine nucleotide, DPNH
disodium salt, DPNH-Na_2. Commercial preparation, see p. 1011.

Preparation of Solutions

I. Phosphate buffer (0.067 M; pH 6.2):
   a) Dissolve 9.087 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
   b) Dissolve 11.876 g. $Na_2HPO_4 \cdot 2\,H_2O$ in doubly distilled water and make up to 1000 ml. Dilute 185 ml. solution b) to 1000 ml. with solution a).

II. Reduced diphosphopyridine nucleotide (*ca.* 1.2×10^{-2} M β-DPNH):
Dissolve 100 mg. DPNH-Na_2 in 1% $NaHCO_3$ solution and make up to 10 ml.

III. Oxidized glutathione (7.5×10^{-3} M GSSG):
See p. 876.

Stability of the solutions

The DPNH solution can be stored frozen for several weeks without loss of activity. GSSG solution, see p. 876.

Procedure

Spectrophotometric measurements

Preferably use fresh serum free from haemolysis.

Wavelength: 340 or 366 mμ; light path: 1 cm.; final volume: 3 ml.; temperature: 25°C (constant!). For each sample prepare a control containing water instead of GSSG solution. Measure against air or water.

Pipette successively into the cuvette:

2.2 ml. phosphate buffer solution (I)
0.1 ml. DPNH solution (II)
0.2 ml. GSSG solution (III)
0.5 ml. serum.

Mix and read optical density E_0. Pour the cuvette contents into a test tube and incubate at 25°C in water bath. Pour back into the cuvette punctually so that the optical density E_{20} can be read after exactly 20 min. incubation (because of the low activity of the DPNH-specific GR in serum a second reading after 20 min. is sufficient). Proceed in the same way with the control. The $E_0 - E_{20}$ for the sample and control are used for the calculations.

The reaction is linear with time up to an optical density difference of 0.1. With very high activity in serum read at shorter intervals, for example, 5 or 10 min.

Calculations

See calculations for the TPNH assay, p. 877. For measurements at 340 mμ, with 0.5 ml. serum in a 3 ml. assay volume:

$$\frac{(\Delta E_{GR}/20\text{ min.}) \times 3 \times 60}{6.22 \times 0.5 \times 20} = (\Delta E_{GR}/20\text{ min.}) \times 2.9 = \text{units/ml. serum}$$

for measurements at 366 mμ:

$$\frac{(\Delta E_{GR}/20\text{ min.}) \times 3 \times 60}{3.3 \times 0.5 \times 20} = (\Delta E_{GR}/20\text{ min.}) \times 5.4 = \text{units/ml. serum}$$

If the time of measurements is shorter than 20 min., this must be allowed for in the calculations.

Values for Human Serum

We found in 10 normal sera an average of 0.77 units/ml. for the TPN-specific enzyme and 0.42 units/ml. for the DPN-specific enzyme. The corresponding values for serum in untreated pernicious anaemia: 9.17 and 4.7; liver metastases (carcinoma of the stomach): 2.34 and 1.32; acute attack of porphyria: 6.53 and 3.05. In all cases the ratio of the activity of the two enzymes is about 1.9.

Effect of Therapeutic Agents

Glutathione reductase requires functional SH groups for its activity. The enzyme is therefore completely inhibited by salyrgan, but can be reactivated by excess cysteine. Divalent cations also inhibit[18], zinc causes more than a 50% inhibition at 10^{-6} M[14]. Insulin with a high zinc content inhibits strongly[14]. Pre-incubation of the enzyme with TPNH and GSSG can protect the enzyme from the action of zinc or insulin. It is not possible to reactivate the enzyme with EDTA after heavy metal inhibition. Heparin inhibits (pH-dependent), especially the DPNH reaction. Under the optimum conditions for the reaction (pH 6.2) the enzyme is completely inhibited by 2×10^{-6} M heparin[8].

In certain pathological conditions the serum enzyme is completely inhibited so that no activity can be detected[9]. The reason for this is not known.

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[18] *L. C. T. Young* and *E. E. Conn*, ref.[5], p. 108.