OXIDASE ACTIVITY OF GLUTATHIONE REDUCTASE EFFECTED BY 2,4,6-TRINITROBENZENESULFONATE

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1. Introduction

Glutathione reductase (EC 1.6.4.2) is a flavoprotein dependent for its catalytic function on an FAD prosthetic group and a cystine disulfide in each of its two subunits [1]. During catalytic turnover the enzyme is considered to cycle between a fully oxidized form and a 2 e^--reduced form [1]. The natural donor and acceptor of reducing equivalents in the reaction catalyzed are NADPH and GSSG, respectively. The electrophilic reagent 2,4,6-trinitrobenzenesulfonate (TNBS) has been found to be a strong reversible inhibitor of the GSSG reduction, probably by interacting with a dithiol in the GSSG binding site (G-site) of the reduced enzyme [2]. These inhibition studies were extended to lipoamide dehydrogenase [3]. In addition to TNBS another electrophilic reagent 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was used. In contrast to glutathione reductase, lipoamide dehydrogenase was shown not to be significantly inhibited by the electrophilic reagents [3]. However, for both enzymes evidence was obtained that the electrophilic reagents induce oxidase activity, indicating an effect of the reagents that is different from mere inhibition of electron transport. This report gives definite evidence for a TNBS-dependent oxidase activity of glutathione reductase and shows that the effect is very specific for the reagent used.

2. Experimental

Glutathione reductase was purified from human erythrocytes, rat liver, and calf liver essentially by the methods in [2,4,5]. All enzyme preparations had spec. act. 200–250 units/mg in the assay system of [5]. Glutathione reductase from yeast was obtained from Boehringer Mannheim and was used without further purification. All chemicals were of the highest purity available from commercial sources.

Oxidation of NADPH was measured spectrophotometrically as in [2] except for substitution of 0.1 M Hepes/OH (pH 7.4) for phosphate buffer. Oxygen consumption was measured polarographically under similar conditions at 30°C in a 3 ml vessel fitted with a Clark electrode.

3. Results and discussion

Native glutathione reductase has an oxidase activity that under normal assay conditions is negligible in comparison with the rate of GSSG reduction. However, in the presence of 1 mM TNBS an oxidase activity of 1840 μmol . min^{-1} . μmol FAD^{-1} was demonstrated. This oxidase activity is ~20% of the activity obtained with 1 mM GSSG as electron acceptor in the absence of TNBS. The oxidase activity could be monitored by measuring the oxygen consumption (cf. fig.1) or the NADPH oxidation. If limiting amounts of NADPH were present in the assay system, the oxygen reduction ceased when an amount of oxygen about equimolar to that of NADPH had been consumed. After introduction of additional NADPH, the oxidase activity was regained as long as oxygen was available. When the NADPH oxidation was followed spectrophotometrically it was shown that a limited amount of TNBS could support the oxidation of repeated additions of NADPH which were made (together with oxygen) to the same cuvette after completion of each reaction cycle. TNBS concentrations in the μM range, found to give strong inhibition of the GSSG reduction [2], gave no detectable NADPH oxidation or oxygen consumption at ~1 nM glutathione reductase. There-

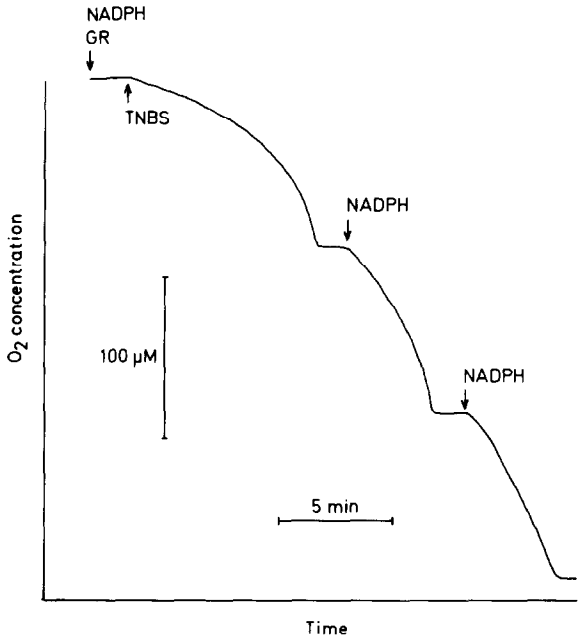

Fig.1. TNBS-induced oxidase activity of glutathione reductase. Oxygen tension was monitored polarographically with a Clark electrode. The reaction vessel contained in 3 ml: 0.1 M Hepes/OH buffer (pH 7.4); 1 mM EDTA; 100 μM NADPH; and 2.5 μM glutathione reductase (5 μM FAD) from human erythrocytes. All solutions were air-saturated at 30°C. The reaction was initiated by addition of TNBS to 1 μM in the reaction vessel. When NADPH had been consumed completely, new additions were made as indicated by arrows.

fore the oxidase activity was not detected in [2]. However, by using 2.5 μM glutathione reductase (5 μM FAD) and 1 μM TNBS the oxidase activity was clearly demonstrable (fig.1). This low concentration of TNBS supported the oxidation of repeated additions of NADPH (each addition equivalent to 100 μM in the reaction medium). These findings show clearly that TNBS serves as a catalytic cofactor and oxygen as the ultimate electron acceptor of the reaction. This conclusion is also supported by the observed stoichiometry between oxygen consumption and NADPH oxidation which was close to 1:1 (fig.2). The nonlinear curves of oxygen consumption shown in fig.1 are not representative of the reaction traces recorded at higher TNBS concentrations. Typical traces at high TNBS concentrations are linear for 80–90% of the course of reaction. The rate of oxygen consumption was proportional to the concentration of glutathione reductase (fig.3).

The electron donor NADPH could not be substituted by sodium borohydride, which reduces the

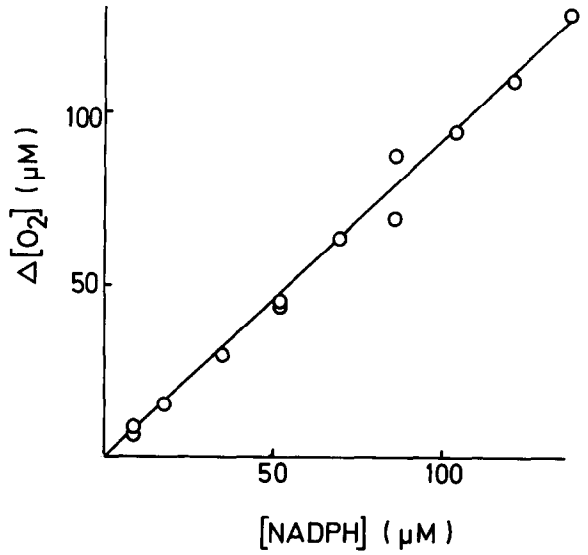

Fig.2. Ratio between oxygen consumption and NADPH oxidation in the TNBS-dependent oxidase activity of glutathione reductase. The changes in oxygen concentration were determined polarographically as a function of the total concentration of NADPH added.

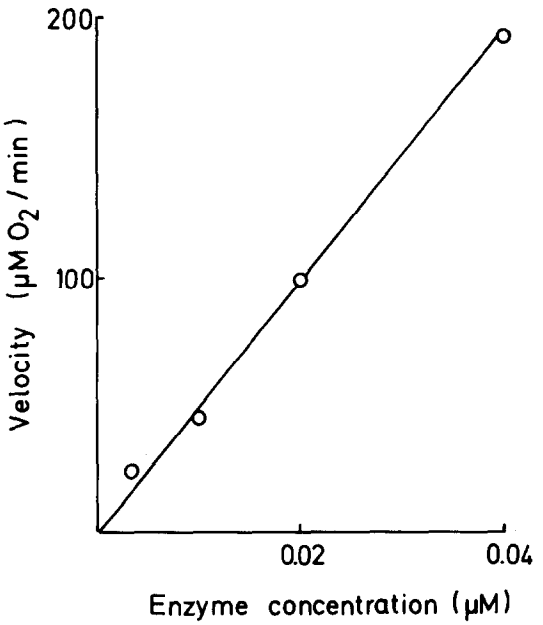

Fig.3. Dependence of the steady-state velocity of oxygen consumption induced by TNBS on the concentration of glutathione reductase. The conditions were the same as for the reaction in fig.1, with the exceptions that TNBS was 1 mM and the enzyme concentrations were >50-fold lower than in fig.1. The reaction was monitored polarographically.

Table 1
Reagents tested as possible inducers of NADPH oxidase activity in glutathione reductase

Reagent	Conc. (mM)	Oxidase activity[a] (μmol O_2 . min^{-1} . μmol FAD^{-1})
Iodoacetate	1	<2
N-Ethylmaleimide	1	<2
p-Mercuribenzoate	1	<2
Arsenite	1	<2
Cd^{2+}	1	<2
Hg^{2+}	2	<2
5,5'-Dithiobis(2-nitrobenzoate)	0.5	5
2-Chloro-4-nitroaniline	1	<2
1-Chloro-2,4-dinitrobenzene	1	5
2,5-Dinitrobenzoate	1	33
3,5-Dinitrobenzoate	1	5
Nitrofurantoin	1	7.5
Benzofuroxan	1	<2
4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole	1	45
Sulfite	1	<2
Nitrite	3	<2
2,4,6-Trinitrobenzenesulfonate	1	1840

[a] Oxygen consumption was measured polarographically at 30°C in a 3 ml vessel containing 0.1 M Hepes/OH (pH 7.4), 1 mM EDTA, 0.1 mM NADPH, and 0.8 nmol (80 μg) glutathione reductase from human erythrocytes. (When TNBS was tested only 2 μg of enzyme was used)

The values given as <2 μmol . min^{-1} . μmol FAD^{-1} represent activities which cannot be detected under the experimental conditions used

enzyme to a redox state implied to be the same as that of the 2 e^--reduced intermediate in the catalytic mechanism [1]. NADH, on the other hand, gave 10–15% of the activity obtained with NADPH. The ratio between the oxidase activities observed with NADH and NADPH, respectively, is similar to the ratio of the GSSG-reducing activities observed with these cofactors under the same conditions.

Previous studies indicate strongly that TNBS binds to the G-site of glutathione reductase. It was assumed that the inhibition was caused by interaction of the dithiol of the reduced form of the enzyme [2]. Therefore, various sulfhydryl-group blocking reagents, including the dithiol-directed agents arsenite and Cd^{2+}, were tested as possible inducers of oxidase activity. Inorganic nitrite and sulfite, possible degradation products of TNBS, were also used. Table 1 shows that none of these compounds gives rise to any significant oxidase activity. Neither did various organic nitro compounds cause any activity which was comparable to that obtained with TNBS. Nitrobenzene and nitrofuran derivatives have previously been shown to be powerful inhibitors of the GSSG reduction [6]. The only compound so far discovered to induce a substantial oxidase activity is TNBS. In the case of lipoamide dehydrogenase, it has been found that inorganic salts, such as KI, give rise to oxidase activity [7], but no significant effect of salts has been demonstrated with glutathione reductase in the present investigation.

Most of the above experiments were done with glutathione reductase from human erythrocytes, but the TNBS-induced oxidase activity has been demonstrated also with enzyme from calf liver, rat liver, and yeast (*Saccharomyces cerevisiae*).

A new activity of glutathione reductase, i.e., a TNBS- and pyridine nucleotide-dependent oxidase activity, has been discovered. The effect of TNBS is specific and requires only catalytic quantities of the reagent. By utilization of this finding, novel parameters are obtained for the study of interactions between the distinct binding sites for NADPH and GSSG as well as the chain of events in the transfer of reducing equivalents in the catalytic process.

Acknowledgement

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