INHIBITION OF GLUTATHIONE REDUCTASE BY INTERACTION OF 2,4,6-TRINITROBENZENESULFONATE WITH THE ACTIVE-SITE DITHIOL

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1. Introduction

Glutathione reductase (EC 1.6.4.2) is one of the few flavoenzymes for which the three-dimensional structure is beginning to be unravelled [1,2]. Like the pyridine nucleotide-dependent dehydrogenases studied [3], the glutathione reductase molecule contains nucleotide-binding domains. The arrangement of supersecondary structures is such that an active site of the enzyme is formed by the union of two substrate-binding cavities, one on each side of the molecule. One cavity binds the pyridine nucleotide, the other binds glutathione disulfide (GSSG), and the bottoms of the two cavities are separated by the isoalloxazine ring of FAD and a redox-active disulfide/dithiol. The catalytic process involves transfer of reducing equivalents from NADPH to GSSG via FAD and the protein disulfide, and for an understanding of the action of the enzyme it is important to find out how the two binding sites communicate. In order to formulate a catalytic mechanism it is furthermore necessary to define the chemical groups involved. Thus, it was found that a histidine residue was essential for enzymatic activity [4] in addition to FAD and the disulfide group recognized [5]. It is also desirable to find out if there are inhibitors, which selectively block the catalytic action of one of the two different substrate-binding sites. The use of such inhibitors may reveal to what extent the partial reactions of the catalysis are coupled. We report here that 2,4,6-trinitrobenzenesulfonate (TNBS) selectively blocks the reduction of GSSG, but leaves unchanged the transhydrogenase activity of the pyridine nucleotide-binding site.

2. Materials and methods

Glutathione reductase from calf liver was purified by a method involving chromatography on DEAE–Sephadex, gel filtration, and chromatography on 2′,5′-ADP–Sepharose 4-B (in preparation). The enzyme was essentially homogeneous as judged from specific activity (~200 units/mg), polyacrylamide gel electrophoresis and flavin spectrum. All chemicals used were commercial products of high purity. Glutathione reductase activity was measured at 30°C as the decrease in A_{340} in a system containing 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA, 0.1 mM NADPH, 1 mM GSSG and a suitable amount of enzyme. Transhydrogenase activity was measured as increase in A_{395} due to reduction of thionicotinamide adenine dinucleotide phosphate by NADPH in the same buffer system [6].

3. Results

Glutathione reductase was strongly inhibited by low concentrations of TNBS; 0.05 μM reagent gave 60% inhibition in the standard assay system. The results presented here were obtained with enzyme from calf liver, but glutathione reductase from rat liver, porcine erythrocytes, human erythrocytes, yeast, *Rhodospirillum rubrum* and spinach was also found to be inhibited by TNBS, and we believe that the results reported here apply to the enzyme in general. The inhibition showed no time-dependence and was completely reversible as demonstrated by dilution or by dialysis. The effect on the enzymatic

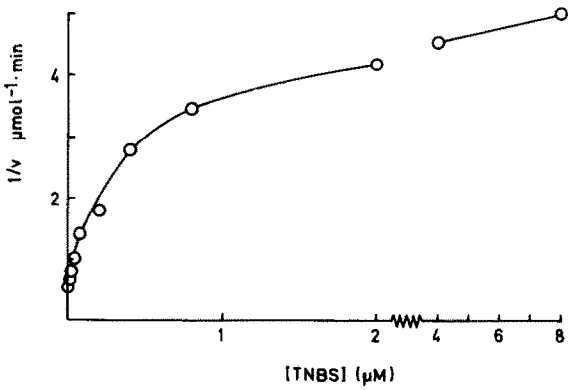

Fig.1. Dixon plot of the effect of TNBS on the initial velocity of glutathione reductase. The substrate concentrations were 1.0 mM and 0.1 mM for GSSG and NADPH, respectively.

activity, including the reversibility, was the same irrespective of whether TNBS was added to oxidized enzyme or to borohydride-reduced enzyme.

The inhibition was nonlinear with TNBS concentration (fig.1). The rate equation appeared to be a 1:2 function in inhibitor concentration as judged by nonlinear regression analysis (cf. [7]). Under the conditions investigated the inhibition was never >90%. The steady-state kinetics were also investigated using variable substrate concentrations at fixed TNBS levels. Figure 2 shows that the effect of TNBS was apparently uncompetitive with NADPH according to the generalized definitions of inhibition patterns [8]. The inhibition was generalized competitive

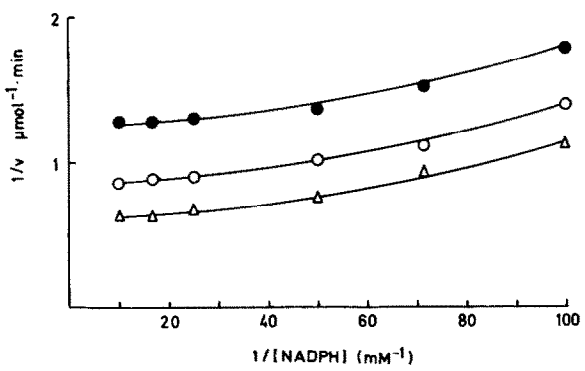

Fig.2. Effect of TNBS on the initial velocity of glutathione reductase using NADPH as the varied substrate. GSSG was 0.5 mM and TNBS was zero (△): 0.02 μM (○): 0.05 μM (●).

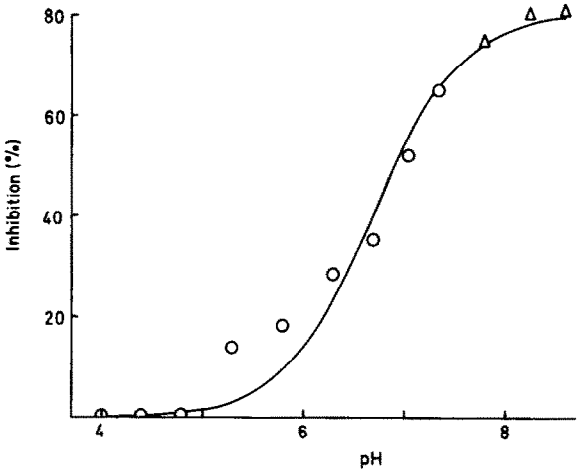

Fig.3. pH dependence of the inhibition of glutathione reductase by TNBS. The activity was monitored at 366 nm using 2.0 mM GSSG and 0.2 mM NADPH as substrates. The buffers used were: 50 mM sodium citrate/phosphate (○): 0.1 M Tris/HCl (△).

against GSSG. The possibility that the competitive effect against GSSG resulted from destruction of TNBS, caused by reaction of TNBS with free amino groups of GSSG, seemed to be excluded by the failure of added glutamine (in twice the highest concentration of GSSG) to counteract the inhibition. These results lead to the conclusion that TNBS competes with GSSG for the reduced form of the enzyme and that an increasing NADPH concentration promotes the inhibition.

The inhibitory effect of TNBS increased with pH (fig.3). The best fit of a titration curve for a single dissociation was obtained with a pK at pH 6.9.

The visible spectrum of the oxidized enzyme was not affected by TNBS. However, the spectrum of the $2e^-$-reduced form was drastically changed (fig.4). Treatment of reduced enzyme with an amount of TNBS, which was stoichiometric with enzyme-bound flavin, immediately changed the spectrum into that of the oxidized enzyme. The result was the same irrespective of whether the enzyme had been reduced by borohydride or by NADPH.

The transhydrogenase activity of glutathione reductase [9,10] was not affected by TNBS; 2 μM TNBS did not inhibit the transhydrogenase activity measured with 50 μM NADPH and 0.5 mM thionicoti-

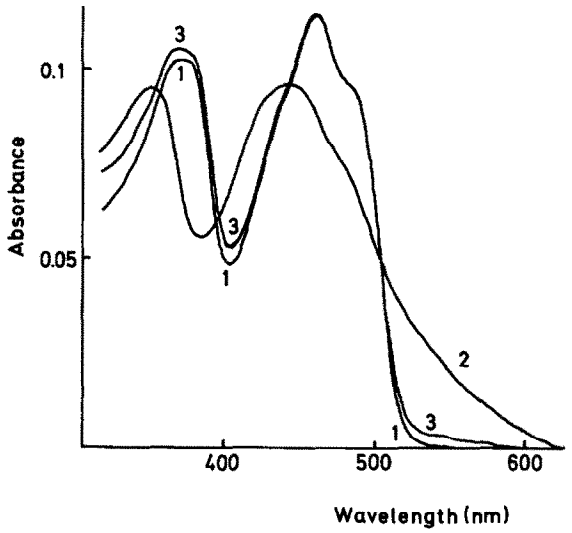

Fig.4. Effect of TNBS on the spectrum of borohydride-reduced glutathione reductase. The spectra were recorded aerobically using enzyme (10.2 μM flavin) in 0.1 M potassium phosphate, 1 mM EDTA (pH 7.5). Spectrum 1, oxidized enzyme; spectrum 2 was recorded after addition of sodium borohydride (0.25 mM final conc.); spectrum 3 was recorded after addition of TNBS (7.5 μM final conc.) to the reduced enzyme.

namide adenine dinucleotide phosphate. The same concentration of TNBS caused 87% inhibition of the reaction involving GSSG and NADPH as substrates.

Glutathione reductase from calf liver is, like the enzyme from other sources, inhibited by iodoacetamide. This inhibition preferentially affects the 'distal' sulfur atom [2] of the dithiol in the active center of the reduced enzyme [11]. Incubation of borohydride-reduced enzyme (10 μM flavin) from calf liver with 0.4 mM iodoacetamide for 60 min (pH 7.0, 30°C) caused 88% inactivation of GSSG-reducing activity. Inclusion of 0.4 mM TNBS in the incubation mixture completely protected the enzyme against inactivation by iodoacetamide.

4. Discussion

The results show that TNBS is a strong and specific inhibitor of glutathione reductase. The effect is very selective, as it does not affect the transhydrogenase activity but only the GSSG-reduction. The enzyme has one NADP(H)- and one GSSG-binding cavity per subunit [1,2], and the selective inactivation indicates that TNBS does not affect the pyridine nucleotide-binding site. This interpretation is supported by the competitive interaction between GSSG and TNBS and the uncompetitive inhibition versus NADPH. The inhibition pattern is explainable in terms of an increased concentration of reduced enzyme obtained by raising the NADPH concentration; GSSG and TNBS apparently compete for reduced enzyme. The localization of the TNBS binding to the GSSG-binding cleft is also supported by the finding that the closely-related picric acid binds preferentially to this site [12].

The chemical nature of the interaction of TNBS with glutathione reductase is evidently of importance for the understanding of the catalytic apparatus of the enzyme. The reversibility of the inhibition excludes modification of an amino group. Direct interaction with the (reduced) flavin appears to be ruled out by the lack of effect on the transhydrogenase activity, which may be expected to involve the flavin in the catalysis. The effect on the spectrum of reduced enzyme is explainable by assuming that TNBS interacts with the dithiol of the active site which mediates reducing equivalents from the flavin to GSSG. Evidently, TNBS breaks the interaction between dithiol and flavin, which has been ascribed to charge transfer. In contrast, alkylation of one of the sulfhydryl groups of the yeast enzyme does not eliminate the charge transfer band of the spectrum [11]. The binding of TNBS to the enzyme may be noncovalent, but a bond between the trinitrophenyl group and a sulfur atom (e.g., in the form of a Meisenheimer complex) is not expected to be very stable; both alternatives may explain the reversibility of the inhibition. If TNBS would bind covalently to a sulfhydryl group in the active center of the enzyme, it might be possible for TNBS to act as a substrate, giving trinitrobenzene and HSO_3^- at the expense of NADPH. However, no reduction of TNBS could be detected. Neither could any enzymatic hydrolysis of TNBS to picric acid be observed.

The protection afforded by TNBS against inactivation of reduced enzyme by iodoacetamide indicates that the 'distal' sulfur (Cys-41 in the human enzyme [2]) interacts with TNBS. The apparent pH 6.9 for the inhibition may be that of a thiolate–imidazolium

ion pair involving a cysteine ('Cys-41') and the catalytic histidine [4], tentatively identified as residue 450 in the human enzyme [2]. Such an ion pair has been discussed as an intermediate in the catalysis [4,11,13].

In conclusion, the results show that TNBS is a strong and specific reversible inhibitor of glutathione reductase. The inhibition is apparently restricted to the GSSG-binding site and probably affects the 'distal' sulfhydryl group ('Cys-41') of the active-site dithiol of 2-electron-reduced enzyme. The finding that the transhydrogenase activity of the pyridine-nucleotide-binding site is unaffected by TNBS implies that reduction of the enzyme by NADPH is not coupled directly to the subsequent GSSG-reduction. The reagent TNBS should be a valuable tool in the further investigation of the catalytic mechanism of the enzyme.

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