Interaction of 2,4,6-Trinitrobenzenesulfonate and 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole with the Active Sites of Glutathione Reductase and Lipoamide Dehydrogenase *

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Glutathione reductase and lipoamide dehydrogenase are two flavoproteins displaying extensive similarities. Both enzymes catalyze the reduction of disulfide bonds by use of reduced pyridine nucleotides as reductants. Flavin adenine dinucleotide and a cystine disulfide of the polypeptide chain mediate the transfer of reducing equivalents from reduced to oxidized substrates, and both enzymes have as a catalytic intermediate a two-electron reduced form characterized by absorbance at 530 nm, which is ascribed to charge-transfer interaction between a thiolate and oxidized flavin.[1] Furthermore, for lipoamide dehydrogenase there is indirect [2] and for glutathione reductase direct evidence [3] that a histidine residue is essential in the catalytic mechanism. Besides the similarities, several differences between the two enzymes exist, a functional one being that the physiological role of lipoamide dehydrogenase is to oxidize the dithiol of dihydrolipoamide, whereas the role of glutathione reductase is to reduce the disulfide of glutathione. For a more intimate understanding of the functions of the two enzymes it is necessary to explore further the differences and similarities. It was recently found that 2,4,6-trinitrobenzenesulfonate (TNBS **) is a potent inhibitor of the glutathione reductase-catalyzed reduction of glutathione disulfide (GSSG); the inhibition apparently being due to interaction with the dithiol of the two-electron-reduced enzyme.[4] The present study was undertaken to extend these studies to lipoamide dehydrogenase and, in addition, to evaluate the effect of another aromatic nitro compound, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), which was expected to be capable of reacting with the active site dithiol.

In spite of the finding that 0.05 μM TNBS gave about 50 % inhibition of glutathione reductase,[4] no significant effect on lipoamide dehydrogenase activity could be detected by use of TNBS concentrations in the range of 0.05 μM to 1 mM. The effect on glutathione reductase was competitive with GSSG,[4] but even using a fivefold lower lipoamide concentration than that in the standard assay system no inhibition of lipoamide dehydrogenase was observed.

The possibility that TNBS might serve as a substrate for glutathione reductase was previously considered, but at the low concentrations used for inhibition no experimental support could be obtained.[4] However, using 1 mM TNBS instead of GSSG in the assay system, an oxidation of NADPH was recorded at 340 nm. The rate of NADPH oxidation was 16 % of the activity obtained with GSSG as substrate. By difference spectrophotometry, using enzyme and NADPH in both cuvettes and TNBS in the sample cuvette only, the NADPH consumption due to addition of TNBS was followed as a decrease in absorbance at 340 nm. By addition of an NADPH-regenerating system, consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase, the decrease in absorbance was eliminated, showing that it originated from NADPH and not from TNBS. However, when a limiting amount of TNBS was used, the NADPH consumption exceeded that anticipated for a reaction with TNBS; *e.g.*, 2 μM TNBS caused the oxidation of 10 μM NADPH. For reduction of TNBS to 2,4,6-trinitrobenzene and HSO_3^- a 1:1 stoichiometry between TNBS and NADPH is expected. Reduction of the nitro groups, which requires 6 electrons per nitro group, was considered less likely to occur. Similar experiments were carried out with lipoamide dehydrogenase, using 0.1 mM NAD^+, 0.1 mM NADH, and 1 mM TNBS instead of lipoamide in the assay system. Also in this case oxidation of the pyridine nucleotide (NADH) was caused by addition of TNBS; the activity was 6 % of that obtained with lipoamide in the absence of TNBS.

An alternative reaction considered was a TNBS-induced oxidase activity. An oxidase activity of lipoamide dehydrogenase has previously been studied in some detail (*cf.* Ref. 5 and papers cited therein). By measuring the oxygen tension with a Clark electrode, it was found that untreated glutathione reductase had a negligible and lipoamide dehydrogenase a low oxidase activity. However, after addition of TNBS a very significant oxygen consumption was registered (Fig. 1). The velocity of oxygen reduction catalyzed by glutathione reductase in the presence of 0.1 mM NADPH and 1 mM TNBS (Fig. 1) corresponded to about 20 μmol O_2 reduced/min per mg of enzyme. The ratio of the rates of (oxygen consumption): (NADPH

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** Abbreviations: GSSG, glutathione disulfide; $NAD(P)^+$ and NAD(P)H, oxidized and reduced forms of nicotinamide adenine dinucleotide (phosphate); NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; TNBS, 2,4,6-trinitrobenzenesulfonate.

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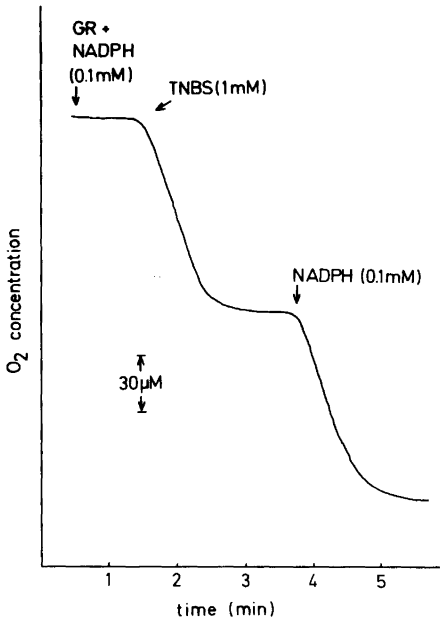

Fig. 1. Oxidase activity of glutathione reductase induced by TNBS. The assay system contained 0.1 M Hepes-OH buffer (pH 7.4), 1 mM ethylenediaminetetraacetate and 15 μg of glutathione reductase (GR) in a total volume of 3 ml. Additions of NADPH and TNBS were made at the time points indicated by arrows to give 0.1 mM and 1 mM concentrations, respectively. Oxygen consumption was monitored by use of a Clark electrode. The test system was kept at 30 °C and the initial O_2 concentration was 240 μM.

oxidation) was about 1:1, consistent with the formation of hydrogen peroxide from O_2:

$$NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O_2$$

However, the nature of the product of O_2 in the reaction has not yet been positively demonstrated. Nonetheless, the effect of TNBS at 1 mM concentration is largely due to induction of oxidase activity of the enzymes. However, dissipation of reducing equivalents by the oxidase activity is not the only explanation of the inhibitory effect on glutathione reductase of low concentrations of TNBS, because the transhydrogenase activity is not affected.[4]

NBD-Cl was found to inhibit glutathione reductase in the standard assay system (using GSSG as electron acceptor); 1 μM NBD-Cl gave 50 % inhibition. Lipoamide dehydrogenase was not inhibited at NBD-Cl concentrations up to 100 μM. With both enzymes an NBD-Cl-induced oxidation of reduced pyridine nucleotide was recorded at 340 nm. These results are qualitatively similar to those obtained with TNBS. Also the ratio between oxidation of NAD(P)H and added reagent was similarly high, indicating oxidase activity. However, spectral changes in the solution showed that NBD-Cl was reduced in an enzymatic reaction. Two strong absorption bands developed with peaks at 470 and 540 nm. The nature of the product(s) has not, so far, been clarified, but a change in the ratio of peak heights with time indicated that at least two products are formed. The same type of spectrum developed slowly in the absence of enzyme upon illumination of NBD-Cl in the presence of ethylenediaminetetraacetate, which is known to facilitate photochemical reductions. We therefore conclude that NBD-Cl not only induces oxidase activity in glutathione reductase and lipoamide dehydrogenase, but also serves as a substrate, which is reduced by the enzyme. A substrate function should also be considered for TNBS, although no evidence for this has been obtained.

The results of the present study show that the interaction of TNBS and NBD-Cl with the active-site dithiol in the reduced forms of glutathione reductase and lipoamide dehydrogenase induces oxidase activity. This finding suggests that the role of the dithiol in the native enzyme is not only to transmit electrons between the flavin and the substrate interacting with the dithiol/disulfide, but also to protect the flavin from reaction with oxygen. In glutathione reductase it is known from X-ray diffraction studies that one of the sulfur atoms is close to the 4a position in the isoalloxazine ring,[6] which is believed to react with oxygen in other flavoenzymes.[7]

Experimental. Crystalline glutathione reductase was prepared from human erythrocytes by a procedure slightly modified from Ref. 8. The assay system was described previously.[4] Lipoamide dehydrogenase from pig heart was purchased from Sigma Chemical Co.; its activity was assayed using 1 mM lipoamide and 0.1 mM NADH as substrates in the presence of 0.1 mM NAD^+.[9] TNBS and NBD-Cl were obtained from Sigma and Fluka, respectively, and were used without further purification.

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