Modification of Glutathione Reductase and Cross-linking of Its Subunits by the Bifunctional Reagent 1,5-Difluoro-2,4-dinitrobenzene *

VIJAYAKUMAR BOGGARAM and
BENGT MANNERVIK

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG**) by NADPH:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The enzyme molecule is composed of two identical subunits and has topologically discrete binding sites for GSSG (G-site) and NADPH (N-site).[1] The active center of a subunit contains flavin adenine dinucleotide and a reducible cystine disulfide.[2] These redox-active groups transmit reducing equivalents from NADPH in the N-site to GSSG in the G-site. The N-site appears to contain an arginine residue, which may have a role in the binding of NADPH.[3] The G-site contains a histidine residue near the disulfide bond of both the redox-active cystine and that of the bound GSSG.[4,5] This residue, which is essential for the reduction of GSSG,[4] may function as an acid-base catalyst in the G-site. An important structural feature of the G-site is the location of the flavin and the redox-active cystine disulfide to the polypeptide chain of one subunit, whereas the essential histidine is contributed by the neighbouring subunit.[1] This topological arrangement may provide one explanation of why glutathione reductase is dimeric. The goal of the present study was to cross-link the histidine residue with one of the thiol groups of the reduced protein disulfide in the G-site and, thereby, to further explore the role of these residues in the catalytic function of the enzyme.

1,5-Difluoro-2,4-dinitrobenzene is a bifunctional electrophilic reagent, which can cross-link residues at a distance of about 3 Å. This reagent inactivated homogeneous glutathione reductase from human erythrocytes irreversibly, and the rate of inactivation increased with reagent concentration. At 0 °C no significant inactivation of oxidized glutathione reductase was obtained with 50 μM 1,5-difluoro-2,4-dinitrobenzene. However, reduced enzyme (prepared by pretreatment with a 10-fold molar excess of $NaBH_4$) was inactivated >80 % in 60 min. Fig. 1 shows similar experiments at 30 °C At this temperature the oxidized enzyme was also inactivated, but with an initial rate which was about 25 times lower than that of the reduced enzyme. The progress curve of inactivation of reduced enzyme was biphasic as expected for modification of two classes of target residues. The related monofunctional reagent 1-fluoro-2,4-dinitrobenzene was a less effective inactivator even of reduced enzyme (about 15 % inactivation after 60 min using 50 μM reagent). The inactivation was established by measuring the capacity of GSSG reduction, but parallel measurements of the trans-

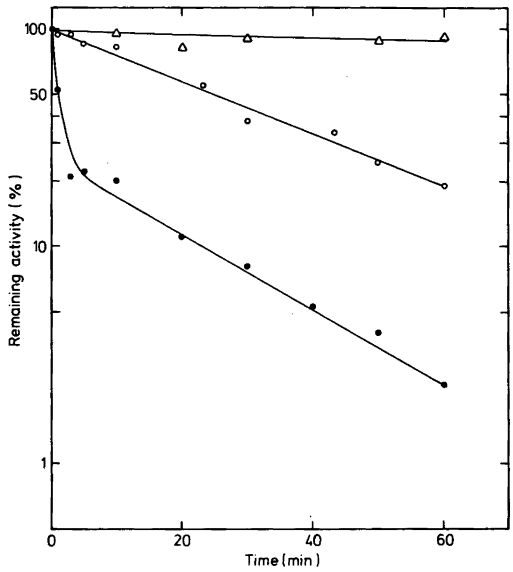

Fig. 1. Progress curves of the inactivation of glutathione reductase from human erythrocytes by 1,5-difluoro-2,4-dinitrobenzene. The enzyme (1 μM, equivalent to 2 μM flavin) was incubated at 30 °C with 50 μM 1,5-difluoro-2,4-dinitrobenzene in 0.2 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetate. Aliquots (10 μl) were taken at the times indicated for measuring the residual GSSG-reducing activity by use of a standard assay system (*cf.* Ref. 6). Oxidized enzyme (○) or reduced enzyme (●) (1 μM enzyme pretreated with 20 μM of $NaBH_4$) in the presence or absence (control) (△) of 1,5-difluorodinitrobenzene was used.

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** Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; $NADP^+$ and NADPH, oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate.

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hydrogenase activity, using NADPH and thionicotinamide adenine dinucleotide phosphate, showed only a minor inactivation. These results indicate that the G-site, but not the N-site, is affected by the inactivation (*cf.* Refs. 4 and 6). Further support for this assignment was obtained by the demonstration that 50 μM 2,4,6-trinitrobenzene sulfonate protected the enzyme against modification with 1,5-difluoro-2,4-dinitrobenzene. This reagent is believed to react with the dithiol in the G-site of the reduced enzyme.[6] Analysis by dodecyl sulfate-polyacrylamide gel electrophoresis[7] demonstrated that modified enzyme (< 5 % remaining activity) contained a major component of $M_r = 100\,000$ in contrast to the active enzyme which was resolved into its subunits ($M_r = 50\,000$) under identical conditions. At high 2-mercaptoethanol concentrations in the gel also the modified enzyme could be cleaved into components of $M_r = 50\,000$. The latter finding is consistent with the assumption that sulfhydryl and imidazole groups are cross-linked, because thiols are known to displace such groups from nitrophenyl residues. No larger derivatives ($M_r > 100\,000$) corresponding to dimers or polymers of the native enzyme were detected.

The results show that the two subunits of glutathione reductase are cross-linked by 1,5-difluoro-2,4-dinitrobenzene and that the modification affects the G-site of the enzyme. The data are also consistent with the assumption that a histidine residue (His-450) in one subunit is linked to one of the cysteine residues (Cys-41 or Cys-46) in the reduced form of the other subunit. The verification of this localization of the linkage requires isolation and identification of the modified peptides. Such studies are in progress.

Experimental. Crystalline glutathione reductase was prepared from human erythrocytes by a modification of a published procedure.[8] Enzymatic reactions, including GSSG reduction and transhydrogenase activity, were assayed as previously described.[6] 1,5-Difluoro-2,4-dinitrobenzene was purchased from Sigma Chemical Co. All chemicals used were of the highest purity available from commercial sources.

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