FLAVIN AND THIOL GROUPS OF YEAST GLUTATHIONE REDUCTASE

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Yeast glutathione reductase was first described by Rall and Lehninger (1952) and purified extensively by Racker (1955). It has now been found to contain a flavin prosthetic group and also a thiol group which is sensitive to -SH reagents only when exposed in the presence of TPNH* or DPNH.

In the presence of the active substrates TPNH or GSH (Fig. 1), or DPNH, there is a decrease in the enzyme's absorption of light between 450 and 505 mμ and a rise in the region above 505 mμ which is typical of the behavior of flavoproteins upon reduction under certain conditions (Beinert, 1957; Massey and Veeger, 1961). Non-substrate thiols, for example cysteine (Fig. 1) or mercaptoethanol, do not produce this change.

This enzyme has been found also to catalyze a "diaphorase" type of reaction, causing a reduction of 2,6-dichlorphenol indophenol by TPNH at about 2% of the rate of GSSG reduction. In contrast to lipoyl dehydrogenase (Searls and Sanadi, 1960; Massey and Veeger, 1960) which is similar to glutathione reductase in several respects, inhibitors of the disulfide reduction in this case also strongly inhibit the dye reduction.

The flavin has not been successfully dissociated from the enzyme or identified. It is of interest in this regard that the glutathione reductase of *E. coli* contains dissociable FAD as a prosthetic group (Asnis, 1955).

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* Abbreviations used are: TPNH and DPNH, reduced tri-and diphosphopyridine nucleotides; GSH and GSSG, reduced and oxidized forms of glutathione; FAD, flavin adenine dinucleotide.

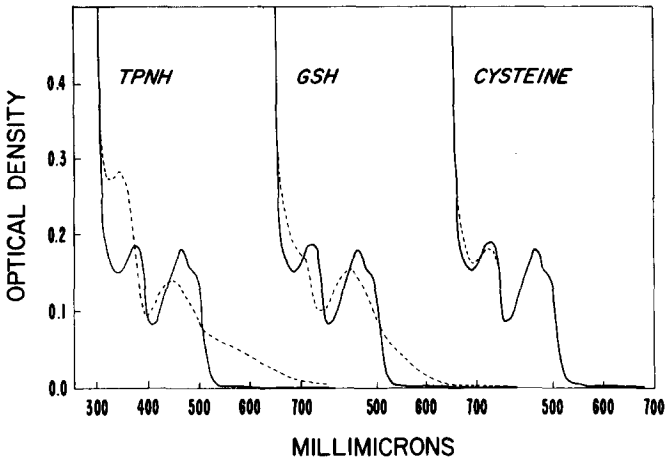

Fig. 1. Absorption spectrum of yeast glutathione reductase in the presence of TPNH, GSH and cysteine. The enzyme was prepared by a newly developed procedure to be described elsewhere and contained about 400,000 units of activity per mg, estimated according to Racker (1955). 1,500,000 units of enzyme in 1.0 ml of 0.2 M potassium phosphate buffer, pH 7.6, were placed in a small quartz cuvette with a 1.0 cm light path and scanned at room temperature with a Model 14 Cary spectrophotometer. In the separate tests indicated in the figure 0.01 ml of 0.012 M TPNH, 1.2 mg of cysteine (free base), and 3.2 mg of GSH were added, and the spectrum again scanned. The enzyme spectrum prior to other addition is indicated by a solid line and after addition of the indicated substance by a broken line.

When a concentrated solution of the enzyme is incubated with an -SH reagent in the absence of TPNH or DPNH and the reagent then removed by dialysis or reduced to an ineffective concentration by dilution, the enzyme is found to be fully active in a subsequent test (Table I). If, however, TPNH is present during the pre-test incubation the enzyme is inactivated by such inhibitory agents as N-ethyl maleimide (Table I), iodoacetamide or iodoacetate, divinyl sulfone, p-chloromercuribenzoate, and very dilute mercuric ions. Concentrations of the latter as low as 10^{-7} M are effective. Arsenite is a comparatively poor inhibitor. Presumably one or more thiol groups are exposed to inhibitor action by TPNH. The latter possibility is further supported by the increase in the enzyme's capacity, upon reduction by TPNH, to bind C^{14}-labeled N-ethyl maleimide (Table I). That the thiol exposed by TPNH is not a lipoic acid residue is suggested by the fact that

Table I

THE EFFECT OF TPNH ON THE INHIBITION AND LABELING OF YEAT GLUTATHIONE REDUCTASE BY N-ETHYL MALEIMIDE-1-C^{14}

Pre-test Incubation mixture	Units of enzyme recovered	Radioactivity of protein in counts/min	Counts/min in ether extract of hydrolyzed enzyme
TPNH absent	468,000	5650	55
TPNH present	0	7850	210

Approximately 500,000 units of enzyme, described in the legend of Figure 1, in 1.25 ml of 0.2 M potassium phosphate buffer, pH 7.6, were incubated for 30 minutes at room temperature with 2.5 µmoles of N-ethylmaleimide-1-C^{14}. The latter was obtained from Schwarz Bioresearch Inc. with a specific radioactivity of 3.6 µcuries per mg. In the indicated experiment 0.1 ml of 0.002 M TPNH was added. Following the incubation the solution was diluted to 10 ml with water and 0.01 ml removed for testing the enzyme activity by the procedure of Racker (1955). One tenth ml of 1 M acetic acid and 0.5 ml of calcium phosphate gel, 25 mg/ml, were then added and the stirred suspension allowed to stand for 5 minutes. The adsorbed enzyme was then centrifuged and washed an additional 8 times with 10 ml portions of water. The gel with adsorbed enzyme was then made up to 2.5 ml with water and 0.5 ml dried on a planchet for determination of radioactivity with a thin window counter. To the remaining 2 ml were added 2 ml of concentrated HCl and 12 mg of cysteine to minimize oxidative degradation. The acid solution was kept in a sealed tube at 105° for 15 hours, cooled, and extracted continuously with ether for two hours. The total ether extract was concentrated and transferred to a planchet for counting.

following acid hydrolysis of the labeled enzyme only a negligible quantity of the C^{14}-labeled material is extractable from acid solution by ether (Table I). In a control experiment the corresponding labeled derivative of lipoic acid was rapidly and completely extracted under the same conditions.

The present study was undertaken with the hope that it might help to elucidate the nature of a recently described more complex disulfide reducing system (Black et al., 1960). The latter also contains groups which are exposed to -SH reagents by TPNH. This similarity permits the surmise that the new system may consist of thiol-containing flavoproteins and that enzyme II of that system plays a role analogous to the electron transferring flavoprotein "ETF" of Crane and Beinert (1956).

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