On the Kinetics of Glutathione Reductase

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The kinetics of glutathione reductase (NAD(P)H:glutathione disulfide oxidoreductase EC 1.6.4.2) from different sources demonstrate a constant ratio of the apparent values of K_m and V, obtained at different constant concentrations of one substrate at variable concentrations of the other.[1-4] This finding was taken as evidence for a mechanism involving consecutive binary complexes of the enzyme and the substrates; a mechanism first described by Alberty [5] and later termed Ping Pong by Cleland.[6] However, the kinetics of glutathione reductase from porcine erythrocytes were not in agreement with a simple Ping Pong mechanism.[7] The data were not sufficiently extensive to elucidate the actual mechanism, but they have now been found to be consistent with experiments of Staal and Veeger,[8] which were interpreted according to an Ordered Bi Bi mechanism. The experiments described in the present communication were carried out in 1966 and have previously been presented in Swedish.[7]

Materials and methods. Solutions of GSSG were standardized enzymatically with glutathione reductase; also the GSSG present in GSH solutions was accounted for in this way. $NADP^+$ and NADPH were determined by their UV absorbancies. The barium salt of the thiosulfonate analogue of GSH [9] was converted into the sodium form by an equimolar amount of Na_2SO_4.

The assay of glutathione reductase was carried out in 1 ml cuvettes containing 90 mM sodium phosphate – 0.9 mM EDTA (pH 7.6), and substrates and inhibitors as indicated in legends to the figures. Enzyme activity is expressed as μmoles·min^{-1}.

Glutathione reductase was prepared from porcine erythrocytes twice washed with 0.17 M NaCl – 1 mM EDTA. The erythrocytes were usually kept frozen and were lysed by fourfold dilution with water, followed by the slow addition of a mixture of ethanol and chloroform (0.12 and 0.06 vol, respectively). The hemolysate was kept near 0° and was centrifuged 30 min after the start of the denaturation. After centrifugation, the supernatant was adjusted to pH 6.0 with 0.2 M acetic acid and then mixed with CM-Sephadex C-50 (1 g per 7.5 ml of erythrocytes), which had been allowed to swell in water. The slurry was poured into a glass column a few min after mixing, and the gel bed was then washed with 5 mM sodium phosphate – 0.1 mM EDTA (pH 6.0). The enzyme was eluted with 50 mM sodium phosphate – 1 mM EDTA (pH 6.5). After threefold dilution with water and adjustment to pH 6.0, the enzyme was retained by smaller columns of CM-Sephadex. By two such steps a 230 fold concentration was achieved. The enzyme was then chromatographed on a Sephadex G-200 column, followed by a DEAE-Sephadex A-50 column equilibrated with 5 mM Tris – 0.1 mM EDTA (pH 8.8). A linear KCl gradient (0 – 0.5 M) eluted the enzyme, which, after equilibration with 5 mM sodium phosphate – 0.1 mM EDTA (pH 6.0), was applied to a CM-Sephadex C-50 column. The elution was carried out with a combined concentration (0 – 0.5 M KCl) and pH (6.0 – 6.8) gradient. The enzyme was dialyzed against 5 mM sodium phosphate – 0.1 mM EDTA (pH 7.0) before use. The overall purification was 1600 fold (specific activity: 3.9 μmoles·min^{-1}·mg^{-1}).

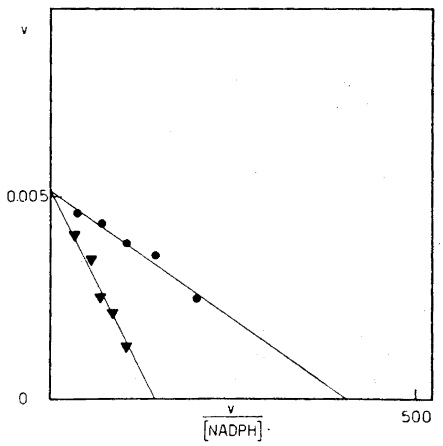

Fig. 1. Inhibition of glutathione reductase by $NADP^+$. Activity (v, expressed in μmoles·min^{-1}) plotted against v/[NADPH] (expressed in in μl·min^{-1}) in the presence (▼) or absence (●) of $NADP^+$ (0.123 mM) at a constant GSSG concentration (0.120 mM).

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Results and discussion. In accordance with earlier investigations on glutathione reductase,[1-4] it was found that the kinetics in the absence of inhibitors were consistent with a Ping Pong mechanism. It was therefore attempted to verify such a mechanism by inhibition experiments. Thus, it was shown that *p*-mercuribenzoate and the thiosulfonate analogue of GSH [9] were competitive inhibitors against GSSG and probably uncompetitive inhibitors against NADPH,[7] as required by a Ping Pong mechanism. However, product inhibition experiments carried out with $NADP^+$, in agreement with the results of Staal and Veeger,[8] demonstrated competitive inhibition against NADPH (Fig. 1) and apparently uncompetitive inhibition against GSSG (Fig. 2). Furthermore, GSH, used as a product inhibitor, was found to inhibit competitively against GSSG (Fig. 3) and apparently uncompetitively against NADPH (Fig. 4). From these experiments it was concluded that glutathione reductase from porcine erythrocytes did not react according to a Ping Pong Bi Bi mechanism.[7]

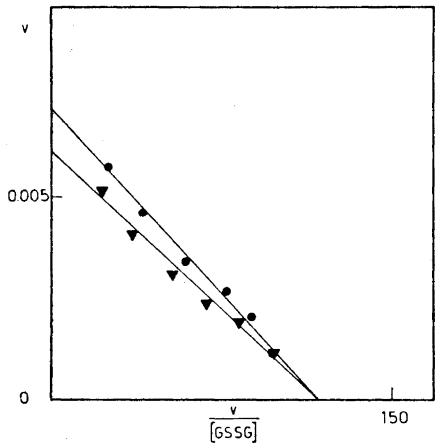

Fig. 2. Inhibition of glutathione reductase by $NADP^+$. Activity plotted against $v/[GSSG]$ in the presence (▼) or absence (●) of $NADP^+$ (0.123 mM) at a constant NADPH concentration (0.096 mM).

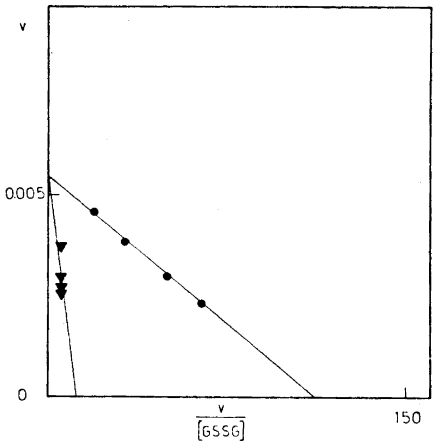

Fig. 3. Inhibition of glutathione reductase by GSH. Activity plotted against $v/[GSSG]$ in the presence (▼) or absence (●) of GSH (10.0 mM) at a constant NADPH concentration (0.032 mM).

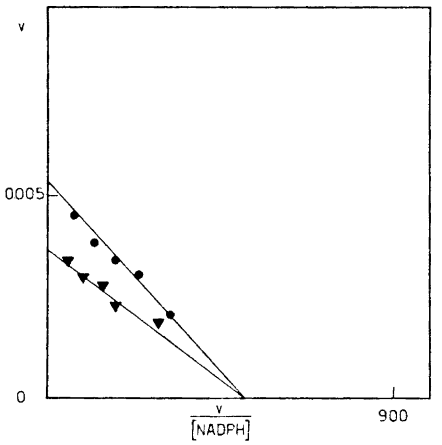

Fig. 4. Inhibition of glutathione reductase by GSH. Activity plotted against $v/[NADPH]$ in the presence (▼) or absence (●) of GSH (10.0 mM) at a constant GSSG concentration (0.584 mM).

The data presented in Figs. 2 and 4 are not accurate enough to distinguish between noncompetitive and uncompetitive inhibition, and the kinetics of glutathione reductase from erythrocytes may thus be that of an ordered two-substrate mechanism, as first suggested by Staal and Veeger.[8] This interpretation has new support from the

inhibition pattern of GSH reported in the present paper. The data are consistent with a Theorell-Chance mechanism,[6] but it is evident that a simple Bi Bi mechanism will not suffice for a full description of the kinetics, as two molecules of GSH and one molecule of $NADP^+$ are liberated in the enzymatic reaction. Further experiments are required to demonstrate whether the complete rate equation is second order with respect to GSH.

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Synthesis of 2-*O*-β-D-Glucopyranosyl-L-arabinose

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Graded hydrolysis of an alkaloid glycoside, isolated from a *Malaxis* sp. (Orchidaceae), yielded, *inter alia* an amorphous disaccharide, $[\alpha]_{578}^{22}+23°$ (water).[1] Structural studies indicated that it was 2-*O*-β-D-glucopyranosyl-L-arabinose, a previously unknown disaccharide. In the present communication the synthesis of this disaccharide is reported.

Benzyl 3,4-*O*-isopropylidene-β-L-arabinoside[2] in benzene was condensed with tetra-*O*-acetyl-α-D-glucopyranosyl bromide, in the presence of silver oxide. The condensation product was isolated by column chromatography on silicic acid, deacetylated, and the isopropylidene groups removed by mild acid hydrolysis. The resulting benzyl 2-*O*-β-D-glucopyranosyl-β-L-arabinopyranoside crystallised from ethanol, m.p. 194–195°, $[\alpha]_{578}^{22}+132°$ (water).

The structure of the glycoside was confirmed by methylation analysis. The glycoside was methylated by the Hakomori procedure,[3] hydrolysed and the resulting methylated sugars reduced into alditols with sodium borodeuteride. These were then analysed, as their acetates, by GLC[4]-mass spectrometry.[5] On an ECNSS-column, two peaks were obtained with T-values (retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) of 1.00 and 1.38. From its mass-spectrum and T-value the component in the first peak was characterised as the alditol acetate of 2,3,4,6-tetra-*O*-methyl-D-glucose. From its mass-spectrum, the component in the second peak was identified as the alditol acetate of a 3,4-di-*O*-methyl-pentose. The possibility that this component was the alditol acetate of a 2,3-di-*O*-methyl-pentose was excluded because of the deuterium labeling at C-1. The identification of 3,4-di-*O*-methyl-L-arabinose is in agreement with the expected structure.

Debenzylation of the glycoside by catalytic hydrogenation yielded a disaccharide, $[\alpha]_{578}^{22}+29°$, which did not crystallise.

The disaccharide on hydrolysis afforded equimolar amounts of D-glucose and L-arabinose. Its electrophoretic mobility in germanate buffer[6] at pH 10.7, $M_G=0.6$, was of the order of magnitude expected for arabinose substituted in the 2-position. The same value, $M_G=0.6$, was obtained for 2-*O*-methyl-D-galactose.

The low optical rotation of the disaccharide indicates a β-glucosidic linkage. The Koenigs-Knorr reaction, under the conditions used, is also known to give almost exclusively β-glucosides.

The synthetic disaccharide and that obtained on graded hydrolysis of the alkaloid glycoside, were indistinguishable