

Aerobic Oxidation of *p*-Hydroquinone by Horse Radish Peroxidase in the Presence of a Thiol and MnCl₂

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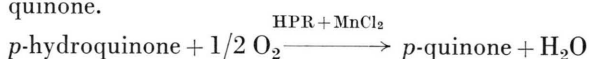
Peroxidase, Hydroquinone, Thiols

In the presence of MnCl₂ and a thiol (glutathione, cysteine, 2-nitro-5-thiobenzoic acid) horse radish peroxidase oxidizes *p*-hydroquinone to *p*-benzoquinone which in turn immediately adds the thiol present yielding 2-S-substituted *p*-hydroquinone.

It is well established that peroxidase (donor: H₂O₂ oxidoreductase EC 1.11.1.7) oxidizes a great number of compounds in the presence of H₂O₂. Peroxidase may also utilize molecular oxygen and act as oxidase or hydroxylase. Thus it was found that the endiol group of dihydroxyfumarate is aerobically oxidized to the diketo group and that the reaction may be activated by MnCl₂^{1,2}. In the presence of dihydroxyfumarate and oxygen peroxidase also catalyzes hydroxylation of various aromatic compounds³. Stonier *et al.*⁴ observed a rapid oxydation of glutathione by horseradish peroxidase when supplemented with MnCl₂ and dichlorophenol. The present investigation shows that horseradish peroxidase may also catalyze the oxidation of *p*-hydroquinone in the presence of a thiol, MnCl₂, and oxygen.

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Addition of horse radish peroxidase (HRP, Sigma) to a buffered solution (pH 6.0) containing *p*-hydroquinone, MnCl₂ and a thiol, such as glutathione (GSH), cysteine (CySH) or 2-nitro-5-thiobenzoic acid⁷, resulted in a rapid consumption of oxygen and a concomitant decrease of the thiol content. The estimation of the stoichiometry of the reaction (Table I) revealed that a half mole of oxygen was taken up per one mole of thiol consumed and that no significant amounts of disulfide were formed. This clearly indicates that the reaction is different from that described by Stonier *et al.*⁴ where GSH was oxidized to GSSG by peroxidase in a system supplemented with MnCl₂ and dichlorophenol. In the medium described here horse radish peroxidase appears to oxidize *p*-hydroquinone to *p*-benzoquinone which in turn immediately adds the thiol present yielding a 2-S-substituted *p*-hydroquinone.



The proposed mechanism is in agreement with the findings that the consumption of a half mole oxygen leads to the disappearance of one mole of the thiol, the rate of oxygen uptake is rather independent of the thiol supplied, no disulfide is formed in the reaction and that quinones easily react with thiols in a Michael type 1,4-addition-reaction to give mono-adducts⁷. Additional evidence for the postulated mechanism and the structure of the reaction product formed was obtained by TLC. When a freeze dried reaction mixture prepared by horse radish peroxidase + MnCl₂ catalyzed oxydation of *p*-hydroquinone + GSH was separated by TLC (silicagel, BuOH:HOAc:H₂O 4:1:1) the resulting chromatogram

Table I. Effect of horse radish peroxidase on a system containing hydroquinone, MnCl₂ and a thiol. The reaction mixture contained hydroquinone (0.25 mM), MnCl₂ (0.1 mM), the indicated thiol (0.25 mM) and horse radish peroxidase (0.016 mg) in a final volume of 3 ml 0.025 M phosphate-citrate buffer pH 6.0. Oxygen uptake was measured with a Clark-type electrode, the thiols were estimated according to⁵ and the disulfides with a slightly modified procedure given by Modig⁶.

Thiol	Initial rate of oxygen uptake [μmole/min]	After 10 min, reaction time			
		Oxygen uptake [μmol]	Thiol consumed [μmol]	Disulfide present [μmol]	mol O ₂ per mol thiol
Glutathione	0.157	0.379	0.75	<0.01	0.505
Cysteine	0.128	0.389	0.75	<0.01	0.518
2-nitro-5-thiobenzoic acid	0.126	0.320	0.75	0.02	0.426



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showed after spraying with either ninhydrin or Folin reagent only one spot and its $R_F = 0.23$ corresponded to the R_F -value of the reference sample prepared from *p*-benzoquinone and GSH. (To a solution of 0.4 mmol GSH in 20 ml 0.05 M phosphate buffer pH 6.0 was added dropwise within 15 min 0.4 mmol *p*-benzoquinone in 20 ml H_2O . The colorless solution was stored in a freezer.) No GSH or GSSG could be detected on the TLC. Omitting of one of the components from the reaction mixture resulted in a complete prevention or drastic reduction of the oxygen consumption. In brief the following results were found: 1. No measurable O_2 uptake in the absence of horse radish peroxidase or thiol, 2. no measurable O_2 uptake in the systems containing horse radish peroxidase + Mn + GSH or horse radish peroxidase + Mn + CySH, 3. a slow O_2 uptake (less the one tenth of the rate in the complete system) in the systems horse radish peroxidase + hydroquinone + GSH, horse radish peroxidase + hydroquinone + CySH, horse radish peroxidase + hydroquinone + 2-nitro-5-thiobenzoic acid and horse radish peroxidase + Mn + 2-nitro-5-thiobenzoic acid.

It appears that the essential function of the thiol is to act as scavenger for the inhibiting quinone thereby preventing its accumulation. When 2-nitro-

5-thiobenzoic acid is used as quinone scavenger the progress of the reaction may also be followed spectrophotometrically, as the absorbance of the yellow colored 2-nitro-5-thiobenzoic acid (λ_{max} 412 nm) decreases due to the coupled reaction with the quinone generated by the enzymatic oxidation. With this technique the dependence of the reaction rate from the substrate concentration was measured covering the range from 0.0083 to 16.6 mM *p*-hydroquinone. The Lineweaver-Burk plot of the data strongly suggests that horse radish peroxidase has two binding sites for *p*-hydroquinone; a low affinity binding site with $K_M = 0.83$ mM and high affinity binding site with $K_M = 0.14$ mM. The pH dependence of the rate of oxygen uptake shows a broad maximum at pH 6 and marked rate increase above pH 7.5. The position of the second maximum could not be ascertained as non-enzymatic oxydation of *p*-hydroquinone becomes high at $pH \geq 9.0$.

The present investigation gives further support for the idea⁸ that environment and/or substrates may modify the active site of peroxidase thus giving rise to unusual enzymatic activities.

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