Aerobic Oxidation of *p*-Hydroquinone by Horse Radish Peroxidase in the Presence of a Thiol and $MnCl_2$

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In the presence of $MnCl_2$ 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 peroxydase (donor: H_2O_2 oxidoreductase EC 1.11.1.7) oxidizes a great number of compounds in the presence of H_2O_2. 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 $MnCl_2$ [1, 2]. In the presence of dihydroxyfumarate and oxygen peroxydase also catalyzes hydroxylation of various aromatic compounds [3]. Stonier *et al.* [4] observed a rapid oxydation of glutathione by horseradish peroxydase when supplemented with $MnCl_2$ and dichlorophenol. The present investigation shows that horseradish peroxydase may also catalyze the oxidation of *p*-hydroquinone in the presence of a thiol, $MnCl_2$, and oxygen.

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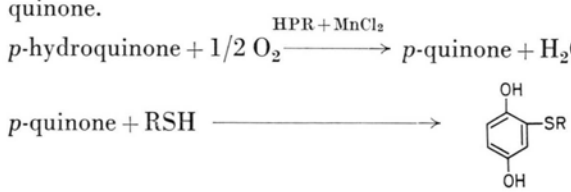
Addition of horse radish peroxydase (HRP, Sigma) to a buffered solution (pH 6.0) containing *p*-hydroquinone, $MnCl_2$ and a thiol, such as glutathione (GSH), cysteine (CySH) or 2-nitro-5-thiobenzoic acid [7], resulted in a rapid consumption of oxygen and a concomitant decrease of the thiol content. 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 peroxydase in a system supplemented with $MnCl_2$ and dichlorophenol. In the medium described here horse radish peroxydase 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 monoadducts [7]. 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 peroxydase + $MnCl_2$ catalyzed oxydation of *p*-hydroquinone + GSH was separated by TLC (silicagel, BuOH: HOAc : H_2O 4 : 1 : 1) the resulting chromatogram

Table I. Effect of horse radish peroxydase on a system containing hydroquinone, $MnCl_2$ and a thiol. The reaction mixture contained hydroquinone (0.25 mM), $MnCl_2$ (0.1 mM), the indicated thiol (0.25 mM) and horse radish peroxydase (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 to [5] and the disulfides with a slightly modified procedure given by Modig [6].

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

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 peroxydase or thiol, 2. no measurable O_2 uptake in the systems containing horse radish peroxydase + Mn + GSH or horse radish peroxydase + Mn + CySH, 3. a slow O_2 uptake (less the one tenth of the rate in the complete system) in the systems horse radish peroxydase + hydroquinone + GSH, horse radish peroxydase + hydroquinone + CySH, horse radish peroxydase + hydroquinone + 2-nitro-5-thiobenzoic acid and horse radish peroxydase + 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 follwed 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 peroxydase 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 $\geqq$ 9.0.

The present investigation gives further support for the idea [8] that environment and/or substrates may modify the active site of peroxydase thus giving rise to unusual enzymatic activities.

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