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OXIDATION OF HOMOGENTISIC ACID CATALYZED BY HORSE-RADISH PEROXIDASE

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SUMMARY

Horse-radish peroxidase catalyzes the oxidation of homogentisic acid in the presence of sulfhydryl compounds to form products similar to those obtained by the spontaneous reaction of benzoquinoneacetic acid with sulfhydryl agents. Other heme proteins, such as catalase, cytochrome *c*, hemoglobin and methemoglobin, do not catalyze this oxidation. Studies on substrate specificity have indicated that a number of aromatic compounds containing disubstituted hydroxy or amino groups in the para position are oxidized in this system. A scheme is presented illustrating a mechanism to explain the formation of thioether derivatives of homogentisic acid and sulfhydryl agents in the presence of horse-radish peroxidase. Similar reactions may be involved in the formation of ochronotic pigment in the connective tissues of alcaptonuric subjects or after topical application of phenol or resorcinol.

INTRODUCTION

It has been observed that horseradish peroxidase has the unusual property of catalyzing the oxidation of homogentisic acid. In the presence of sulfhydryl compounds such as glutathione, this oxidation results in the formation of products similar to those obtained by spontaneous reaction of benzoquinoneacetic acid, the corresponding quinone of homogentisic acid, with sulfhydryl compounds.

A study of the requirements and characteristics of this peroxidase catalyzed oxidation of homogentisic acid will be presented in this paper. The possibility that a similar peroxidative reaction is involved in the formation of ochronotic pigment in the connective tissues of alcaptonuric individuals will be discussed.

MATERIALS AND METHODS

Materials

Homogentisic acid was obtained from the Cyclo Chemical Corporation, Los Angeles, Calif. (U.S.A.). Reduced glutathione was obtained from the Nutritional Biochemical Corporation. Horse-radish peroxidase, grade A, RZ (Reinheitzahl), approx. 3, and crystalline beef-liver catalase were obtained from Worthington Biochemical Corporation.

Preparation of benzoquinoneacetic acid

Benzoquinoneacetic acid was prepared by oxidizing homogentisic acid with iodine by a modification of the method of NEUBERGER[1]. 0.9 ml of 0.05 M sodium phosphate buffer (pH 6.5) containing 760 μg of homogentisic acid was treated with 0.1 ml of 0.1 N I_2 in 5% KI solution. The mixture was allowed to stand at room temperature for at least 5 min for the stoichiometric oxidation of homogentisic acid to benzoquinoneacetic acid.

RESULTS

Requirements for the enzymic oxidation of homogentisic acid by horse-radish peroxidase

The oxidation of homogentisic acid with horse-radish peroxidase was followed manometrically as shown in Fig. 1. In the presence of horse-radish peroxidase and

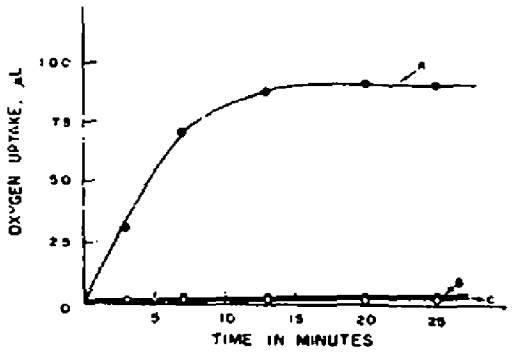

Fig. 1. Oxidation of homogentisic acid in the presence of horse-radish peroxidase. The main compartment of the Warburg vessels contained 0.1 ml of horse-radish peroxidase (100 μg) in 0.2 M sodium phosphate buffer (pH 6.5) 0.1 ml of previously neutralized reduced glutathione (20 μmoles) and additional 0.2 M sodium phosphate buffer (pH 6.5) to make a total fluid volume of 2.0 ml. The side arm contained 4.0 μmoles of homogentisic acid in the phosphate buffer, or buffer alone, in the control flasks. 0.2 ml of 20% KOH was in the center well. The flasks were equilibrated for 5 min at 37° and the contents of the side arm were tipped in to start the reaction. The shaking rate was 180 oscillations/min; the gas phase was air. Curve A, the oxidation of 4 μmoles of homogentisic acid with horse-radish peroxidase and 20 μmoles of reduced glutathione; curve B, horse-radish peroxidase omitted from the flasks; curve C, glutathione omitted from the flasks.

reduced glutathione, 4 μmoles of homogentisic acid were rapidly oxidized with the uptake of 90 μl of oxygen (Curve A). This represents the uptake of 1 μmole of oxygen/μmole of homogentisic acid oxidized. The oxidation did not occur if either horse-radish peroxidase (Curve B) or GSH (Curve C) were omitted. The initial rate of oxidation was linear with time and was proportional to the amount of horse-radish peroxidase present if the amount of enzyme were less than 20 μg. With larger amounts, the rate of oxidation was maximal and independent of horse-radish peroxidase concentration. The oxidation of homogentisic acid had a broad optimal pH range, from pH 6 to 8. Non-enzymic oxidation of homogentisic acid increased above pH 8.

Dialysis of horse-radish peroxidase for 16 h against dilute sodium phosphate buffer had no effect upon its catalytic activity. The rate of oxidation of homogentisic

acid was not decreased if all the constituents of the reaction mixture were in water doubly distilled from quartz. Metal chelating agents, such as EDTA and diethyldithiocarbamate had virtually no inhibitory effect on the oxidation of homogentisic acid. However, boiling horse-radish peroxidase for 30 min completely destroyed its ability to catalyze the oxidation. Other heme proteins, such as catalase, cytochrome *c*, hemoglobin, methemoglobin, as well as hematin and ferritin, could not substitute for horse-radish peroxidase in this system. In fact, catalase completely inhibits the oxidation of homogentisic acid by horse-radish peroxidase. This latter finding suggests that peroxide is a component in the oxidation.

Evidence has been presented (Fig. 1, Curve C) that a sulfhydryl compound is essential for the complete oxidation system. Experiments using various amounts of reduced glutathione (5, 10, 20 and 40 μmoles per vessel) indicated that for optimal activity the sulfhydryl compound needed to be present at twice the concentration of homogentisic acid. With lower relative amounts of glutathione, the oxygen uptake per μmole of homogentisic acid was reduced. Glutathione in excess of the 2 : 1 ratio had no effect on the oxygen uptake and the expected residual glutathione could be recovered at the end of the incubation period. If the ratio of glutathione to homogentisic acid was exactly 2 : 1, no residual glutathione (oxidized or reduced) was found in the flasks. This indicated that the sulfhydryl compound had reacted chemically with the substrate during the oxidation. It was found that other sulfhydryl compounds could replace reduced glutathione in this oxidation. These included cysteine, thioglycollic acid, homocysteine and CoA. The oxidized forms, oxidized glutathione and cystine, were completely ineffective and no oxidation of homogentisic acid occurred in the presence of these compounds. Furthermore, other reducing agents, such as ascorbic acid and 2,6-dichlorophenolindophenol could not substitute for the sulfhydryl compounds.

Substrate specificity

Other compounds were tested to determine whether they would be oxidized by horse-radish peroxidase and glutathione in place of homogentisic acid (Table I). 2,5-Dihydroxyphenylpyruvic acid, resorcinol and *p*-phenylenediamine were all oxidized at about the same rate as homogentisic acid. Phenol, 3,5-dihydroxybenzoic acid, 2,5-dihydroxyphenylalanine and hydroquinone were approximately half as active as homogentisic acid. None of the active compounds were oxidized unless glutathione was present in the incubation vessels. It was of interest that 2,5-dihydroxybenzoic acid (gentisic acid) was essentially inactive in this system. No *o*-dihydroxy compounds were oxidized, nor were the *o*- and *m*-phenylenediamine analogues. The incubation period was continued for at least 30 min to be certain that none of the inactive compounds would be oxidized after an initial lag period.

The nature of the product derived from homogentisic acid, horse-radish peroxidase and glutathione

At the end of the incubation period (after oxygen uptake had stopped) the contents of several experimental Warburg vessels were pooled and acidified with 5 N H_2SO_4 in order to study some of the chemical characteristics of the product formed

TABLE I

THE OXIDATION OF VARIOUS COMPOUNDS IN THE PRESENCE OF HORSE-RADISH PEROXIDASE AND GLUTATHIONE

The oxidation of the various compounds were followed manometrically as described under Fig. 1. The flasks contained 0.2 M sodium phosphate buffer (pH 6.5) 20 μmoles of reduced glutathione, 0.1 ml of horse-radish peroxidase (100 μg) and 4 μmoles of the compounds listed below.

Active substrates	*μl of oxygen uptake in 10 min**	*Inactive compounds***
Homogentisic acid (2,5-dihydroxyphenylacetic acid)	100	Gentisic acid (2,5-dihydroxybenzoic acid)
2,5-Dihydroxyphenylpyruvic acid	100	2,3-Dihydroxybenzoic acid
Resorcinol	100	3,4-Dihydroxybenzoic acid
p-Phenylenediamine	90	2,4-Dihydroxybenzoic acid
Phenol	60	2,6-Dihydroxybenzoic acid
3,5-Dihydroxybenzoic acid	60	*o*-Phenylenediamine
2,5-Dihydroxyphenylalanine	50	*m*-Phenylenediamine
Hydroquinone	30	*p*-Hydroxyphenylpyruvic acid
		3,4-Dihydroxyphenylalanine
		Phenylalanine
		Tyrosine
		Histidine
		Proline

* The initial rate of oxygen uptake was extrapolated to calculate the rate in a 10-min period. With some compounds, *i.e.*, homogentisic acid, the reaction was complete in less than 10 min.

** Inactive compounds are those oxidized at rates less than 1 μl/min.

from homogentisic acid and glutathione. It appeared probable that homogentisic acid was oxidized to its corresponding quinone (benzoquinoneacetic acid) which then reacted with glutathione to yield a 1,4-addition product (a thioether derivative). It is well known that many quinones easily undergo 1,4-addition reactions with sulfhydryl compounds[2-6]. The oxygen consumption (1 μmole) and the disappearance of glutathione (2 μmoles) for each μmole of homogentisic acid oxidized suggested that 2 molecules of glutathione had added in a thioether linkage to each molecule of homogentisic acid (see Fig. 2).

Products prepared chemically by mixing benzoquinoneacetic acid and gluta-

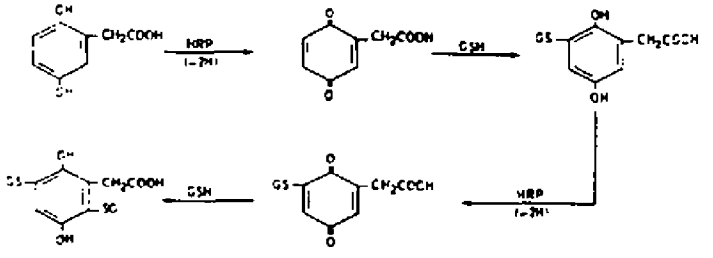

Fig. 2. Postulated scheme for the formation of a thioether derivative of homogentisic acid and glutathione catalyzed by horse-radish peroxidase.

thione were compared with the products obtained in the enzymic oxidation of homogentisic acid and glutathione. The products both reduced molybdate[1,6] and ammoniacal silver[7], as would be expected if they still retained a free *p*-dihydroxyphenyl structure. Both products were considerably more water soluble than homogentisic acid and could not be extracted into butanol from an acid solution. The R_F of the products were identical (0.13) and were much lower than for homogentisic acid[6] (0.70) in a butanol–acetic acid–water solvent system. The enzymically prepared and chemically prepared products were light yellow in neutral and acid solutions and turned dark brown in alkali. They were also inactive as substrates for homogentisic acid oxidase, the enzyme which converts homogentisic acid to maleylacetoacetic acid.

The enzymically and chemically prepared glutathione addition products also had similar absorption spectra in the ultraviolet (Fig. 3, Curve C). Both products

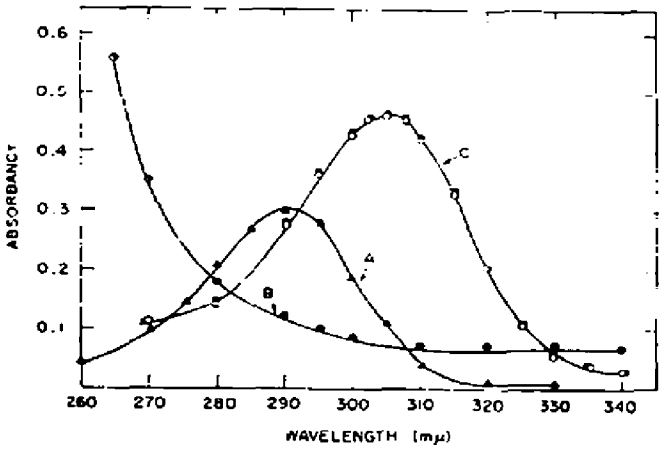

Fig. 3. Absorption spectra of homogentisic acid, benzoquinoneacetic acid and their sulfhydryl addition products. Curve A, spectrum of homogentisic acid in 0.2 M sodium phosphate buffer (pH 6.5). The concentration of homogentisic acid was 13 μg/ml, light path 1 cm. Curve B, spectrum of benzoquinoneacetic acid in 0.2 M sodium phosphate buffer (pH 6.5). The concentration of the quinone was 13 μg/ml. Curve C, the spectra of addition products prepared chemically with benzoquinoneacetic acid (■—■) and enzymically with homogentisic acid (○ – ○) were identical. Benzoquinoneacetic acid and glutathione were mixed in 0.2 M phosphate buffer (pH 6.5); concentration of the quinone, 13 μg/ml in aliquot analyzed spectrophotometrically. The enzymically prepared addition was analyzed after incubating homogentisic acid, glutathione and horseradish peroxidase manometrically as described in Fig. 1. The aliquot measured was equivalent to 13 μg/ml of homogentisic acid. The identical spectrum also appeared in the enzymic reaction and was followed spectrophotometrically in a cuvette containing horse-radish peroxidase (100 μg), reduced glutathione (510 μg) and homogentisic acid (13 μg/ml) and the same buffer; total fluid volume, 3.0 ml.

had identical peaks at 305 mμ and the absorbancy at the peak of the enzymic product had the value expected calculated from the absorbancy of the addition product prepared chemically by mixing benzoquinoneacetic acid and glutathione. The reaction between homogentisic acid, horse-radish peroxidase and glutathione could also be followed spectrophotometrically. During the reaction there was a disappearance of homogentisic acid (Curve A) and a concomitant appearance of a new spectrum identical to that of Curve C. There was no disappearance of the homogentisic acid

in the absence of either glutathione or horse-radish peroxidase. The spontaneous reaction of benzoquinoneacetic acid and glutathione could not be followed spectrophotometrically because of its rapidity. Less than 1 min after mixing, the absorption spectrum of benzoquinoneacetic acid (Curve B) was replaced by the spectrum of the addition product (Curve C).

DISCUSSION

A mechanism to explain the formation of thioether derivatives of homogentisic acid and glutathione catalyzed by horse-radish peroxidase has been illustrated in Fig. 2. The requirement for horse-radish peroxidase appears to be relatively specific, as the oxidation of homogentisic acid is not catalyzed by other heme proteins, such as catalase, cytochrome *c*, hemoglobin and methemoglobin. The observation that catalase inhibits the peroxidase-catalyzed reaction suggests that hydrogen peroxide (or an organic peroxide which can be decomposed by catalase) is utilized in the oxidation. Although it is reasonable to propose that a trace metal participates in the reaction by generating peroxide, perhaps by the decomposition of glutathione, there is no direct evidence for such a requirement. Prolonged dialysis of the peroxidase and metal binding agents did not reduce the enzymic activity. It is also evident from the manometric data that no initial lag occurred in the oxidation of homogentisic acid as might be expected if a metal catalyzed source of peroxide were required to initiate the reaction.

Most of the substrates oxidized in the horse-radish peroxidase system are para dihydroxyphenyl or diaminophenyl compounds. In addition, phenol, resorcinol and 3,5-dihydroxybenzoic acid are also oxidized. None of the ortho dihydroxyphenyl compounds tested, such as Dopa (3,4-dihydroxyphenylalanine), were active. However, ROSTON[9] has recently reported that tyrosinase catalyzes the formation of addition products of sulfhydryl compounds with an oxidized derivative of Dopa. While it appears that the substrate specificity of horse-radish peroxidase and tyrosinase are distinct, the mechanism of formation of these addition products may be quite similar.

Among the compounds oxidized by horse-radish peroxidase, at least three (homogentisic acid, phenol and resorcinol) are known to produce ochronotic pigmentation in connective tissues. Ochronosis, secondary to the chronic application of phenol for the treatment of skin ulcers, is well known[10], and similar prolonged treatment with resorcinol leading to ochronosis has recently been described[11]. Ochronosis in individuals with alcaptonuria is due to the accumulation of homogentisic acid and its further oxidation to ochronotic pigment in connective tissues. The complicated biochemical steps leading to ochronosis in alcaptonuria are not known, but recent investigations in this laboratory have indicated that oxidation of homogentisic acid to benzoquinoneacetic acid is probably the inital step in this process[12,13]. It is perhaps significant that gentisic acid (2,5-dihydroxybenzoic acid), the only *p*-dihydroxyphenyl compound tested and found not to be oxidized by horse-radish peroxidase, also does not produce ochronotic pigmentation after oral administration[14,15]. In view of these observations, it will be of interest to search for oxidases or peroxidases of mammalian tissues with properties and specificity similar to those described for horse-radish peroxidase.

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