SUBSTRATE SPECIFICITY OF ASCORBATE OXIDASE

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SUMMARY: Ascorbate oxidase oxidizes leuco 2, 6-dichloroindophenol to the blue quinoid dye and produces spectral changes in the UV spectra of certain substituted polyhydric and amino phenols at pH 5.7. The new peaks produced by the addition of enzyme to the dichlorohydroquinones (2,5 and 2,6) and hydroxyhydroquinone correspond to the respective p-quinones of these substrates. At pH 5.7, the enzyme does not oxidize hydroquinone, barely oxidizes chlorohydroquinone, but oxidizes 2,6- and 2,5-dichlorohydroquinone and hydroxyhydroquinone at a rate about 1/12 that of ascorbic acid, with the uptake of one gram atom of oxygen per mole of substrate. A correlation has been found between the concentration of anion present in solution at pH 5.7 and the rate of oxidation of compounds of the hydroquinone series by the enzyme. The results indicate that an anionic form of the substrate is an important requirement of the enzyme specificity.

It is generally accepted that ascorbate oxidase (AO)[2], the Cu-containing enzyme which catalyzes the aerobic oxidation of ascorbic acid to dehydroascorbic acid, is highly specific for compounds which contain a lactone ring carrying a vicinal enediol adjacent to a carbonyl group (1). Here we report the interesting result that both crude and highly purified preparations of AO catalyze the aerobic oxidation of leuco 2,6-dichloroindophenol as well as certain structural analogs of the leuco dye. Our studies show that AO is less specific than formerly believed, and that the enzyme oxidizes substituted hydroquinones at rates dependent on the concentration of the substrate monoanion at pH 5.7.

Materials and Methods

For most of the experiments, AO prepared from rinds of yellow summer squash (2), having a copper content of 0.21% (743 units/ug of Cu (3) and a specific activity (4) of 1692 units[3]/mg was used. The data of Fig. 1 were also confirmed using a highly purified preparation (5) of the enzyme having a specific activity

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[2] Abbreviations: AO, L-ascorbate: O_2 oxidoreductase EC 1. 10. 3. 3.

[3] One unit of AO is the amount of enzyme that causes an initial rate of oxygen uptake of 10 µl/min during the first ten minutes, when catalyzing the aerobic oxidation of 2.5mg (1.42×10^{-5} moles) of ascorbic acid in a 5.0 ml reaction volume at pH 5.7 and 25°C.

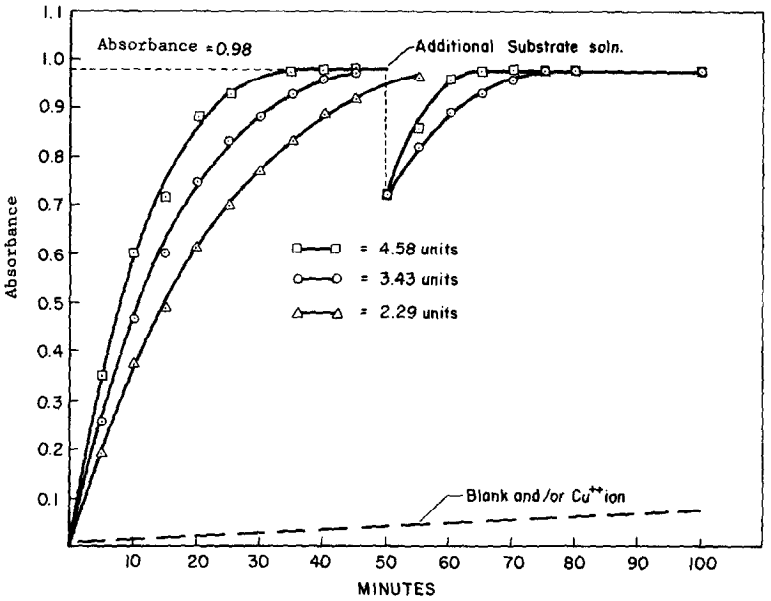

Fig. 1. The aerobic oxidation of a saturated solution of leuco 2,6-dichloroindophenol by AO in 0.2M McIlvaine's buffer, pH 5.7, at 25°C. A 0.1 ml aliquot of the enzyme was added at zero time to 3.0 ml of the buffered leuco dye (containing 0.5mg/ml gelatin) and the formation of the blue dye was followed by absorbance measurements at 605 nm.

of 3030 units/mg, and a copper content of 0.39% (825 units/ug of Cu). Oxygen uptake measurements were determined in Warburg respirometers using standard manometric techniques.

Leuco 2,6-dichloroindophenol (6), hydroxy hydroquinone (7), the dichlorohydroquinones (8) and nitrohydroquinone (9) were prepared by previously described procedures. The amino phenols were prepared by Sn-HCl reduction of the corresponding nitro compounds. A solution of 2,6-dichloroindophenol was prepared and standardized according to the method of Menaker and Guerrant (10).

The concentration of a saturated solution of the leuco dye in 0.2M McIlvaine's (citrate-phosphate) buffer (pH 5.7) at 25°C, was determined by measuring the absorbance in the presence of excess enzyme. The concentration of the saturated solution was found to be about 1.0×10^{-4}M. The molar absorptivity at 605 nm was det ermined to be 1.07×10^{4}.

Results and Discussion

Oxidation of Leuco Dye: As shown in Fig. 1, AO aerobically oxidizes leuco 2,6-dichloroindophenol to its blue (quinoid) form. The reaction is catalytic in that the oxidation is effected by an enzyme to substrate molar ratio in the order of 1:20,000, and the initial rate is essentially first order in enzyme. The theoretical absorbance value expected for complete oxidation of 1.0×10^{-4}M

TABLE I

UV ABSORPTION MAXIMA OF SOME POLYHYDRIC AND AMINO PHENOLS AND THEIR RELATIVE RATES OF OXIDATION BY AO

Compound	Initial Absorption Max. λ, nm	Relative Rate of Oxidation	Observed Spectral Changes
		Hydroquinones	
Hydroquinone	289	none	———
Chloro-	287	slow	287 peak decreases
2,6-Dichloro-	295	fast	new peak at 275
2,5-Dichloro-	298	fast	new peak at 273
Hydroxy-	287	fast	new peak at 263
Nitro	283	none	———
		Aminophenols	
4-Aminophenol	298	none	———
2-Chloro-	303	slow	303 peak decreases
2,6-Dichloro-	305	fast	new peak at 280
2,6-Dibromo-	283	intermediate	new peak at 290
2-Aminophenol	283	none	———
Leuco 2,6-Dichloro-	275-280	fast	peak shifts to 270
indophenol			plateau appears at 310
		Polyhydric Phenols	
Pyrogallol	267	intermediate	267 peak increases
4,5-Dichlorocatechol	295	intermediate	295 peak increases
Phloroglucionol	267	none	———

A qualitative description of the relative rates of reaction of various substrates with ascorbate oxidase on the basis of observed spectral changes can be given in the following terms:

(1) Fast - a strikingly significant decrease in initial absorption peak and appearance of a new peak within five minutes or less upon addition of 12-15 units of AO. (2) Slow - a barely perceptible decrease in initial absorption peak in 15 minutes upon addition of 12-15 units of AO. (3) Intermediate - a significant change in absorption spectrum in 15 minutes upon addition of 12-15 units of AO. (4) None - no change in the absorption spectrum with the maximum amount of enzyme employed in these experiments (500 units) in 15 minutes.

solution of the leuco dye is 0.99, which is in agreement with the experimental absorbance value of 0.98 shown in Fig. 1 for the completed enzyme reaction. Additional substrate added at 50 minutes confirms the presence of active enzyme in the system. No oxidation of the leuco dye beyond that of the blank system was observed in controls containing 0.02M Cu^{++} in place of enzyme (Fig. 1). This excludes the possibility that the observed catalytic oxidation was due to extraneous Cu^{++} ion, possibly associated with the AO preparation.

Inhibition studies were performed with the copper complexing agents, cyanide and diethyldithiocarbamate. The concentration of inhibitor required to produce 50% inhibition was the same for both the oxidation of ascorbic acid and leuco dye by two units of enzyme i.e., 10^{-6}M cyanide and 10^{-7}M diethyldithiocarbamate produced 50% inhibition in both cases.

Oxidation of Structural Analogues of Leuco Dye: Polyhydric and amino phenols structurally related to leuco 2,6-dichloroindophenol were tested as substrates for the enzyme. Approximately equal concentrations of the phenols were used, and the effect of AO on the ultraviolet absorption maximum of the compounds was observed spectrophotometrically. The results are presented in Table I.

A quantitative measure of the relative rates of reaction with ascorbate oxidase could not be readily made from the observed spectral changes. However, a qualitative description of the relative rates is given in the legend to Table I.

All of the reaction systems that showed significant spectral changes became yellow during the enzymatic oxidation. In the case of three of the hydroquinones listed in Table I, information was available concerning the absorbance characteristic of the corresponding quinones. Thus Mason (11) has reported that hydroxy-p-quinone at pH 5.4 has a principal absorption band at 260 nm. The spectra of authentic samples of the two dichloro-p-quinones (2,6- and 2,5-) showed maxima at 275 nm respectively. Consequently, in the case of these three phenols, it may be concluded (Table I) that the enzymatic oxidation by AO produced the corresponding p-quinones.

TABLE II

RELATIVE RATES OF OXIDATION, OXYGEN UPTAKE TOTALS, AND ACIDITY CONSTANTS OF COMPOUNDS OXIDIZED BY AO

Compound	pH	Gram Atoms O_2 per mole of Substrate	Relative Rates of Oxidation $ul O_2$/min/unit AO X 10	Acidity Constants
Ascorbic Acid	5.7	1.0	100.0	6.77×10^{-5} (17)
Hydroquinone	5.7	—	0.0	1.41×10^{-10} (14)
	7.2	—	0.5	
Hydroxyhydroquinone	5.7	1.0	8.3	1×10^{-7} (15)
2,6-Dichlorohydroquinone	5.7	1.0	8.3	0.5×10^{-7} (14)
2,5-Dichlorohydroquinone	5.7	1.0	8.3	0.55×10^{-7} (a)
Chlorohydroquinone	5.7	1.0	0.5	3.9×10^{-9} (a)
Catechol	5.7	—	0.0	———
	7.2	—	0.088	
Leuco 2,6-Dichloro-indophenol	—	—	(b)	1×10^{-7} (16)

A 1.00 ml aliquot of a 1.42×10^{-2}M solution of substrate was placed in the side-arm of a Warburg flask at 25°C while in the main compartment were placed 1.00 ml of AO (12 -4800 units), 2.00 ml of 0.2M McIlvaine's buffer pH 5.7, 0.50 ml of gelatin (5 mg/ml) and 0.50 ml of Cu-free water. The final volume was 5.00 ml. A control containing all the substances except enzyme was run concurrently.

(a) Calculated as follows: The K_a of chlorohydroquinone and 2,5-dichlorohydroquinone were approximated by assuming that the introduction of a Cl atom would have the same effect on the K_a of hydroquinone as on the K_a of phenol. On this basis, the K_a of 2,6-dichlorohydroquinone was calculated to be 1.9×10^{-7}, which is in reasonable agreement with the value reported by Boxendale (14)(0.5×10^{-7}).

(b) Due to the poor solubility of leuco 2,6-dichloroindophenol in H_2O, the relative rate of oxidation by AO could not be determined manometrically. However, as shown in Table I, addition of AO caused a change in the uv spectrum of the leuco dye which was quite comparable to the change produced in the uv spectra of 2,6- and 2,5-dichlorohydroquinone and hydroxyhydroquinone during the first five minutes.

Manometric Determination of Relative Rates of Oxidation and Oxygen Uptake Totals: Several of the phenols of the hydroquinone group listed in Table I were sufficiently soluble in water so that it was possible to quantitatively

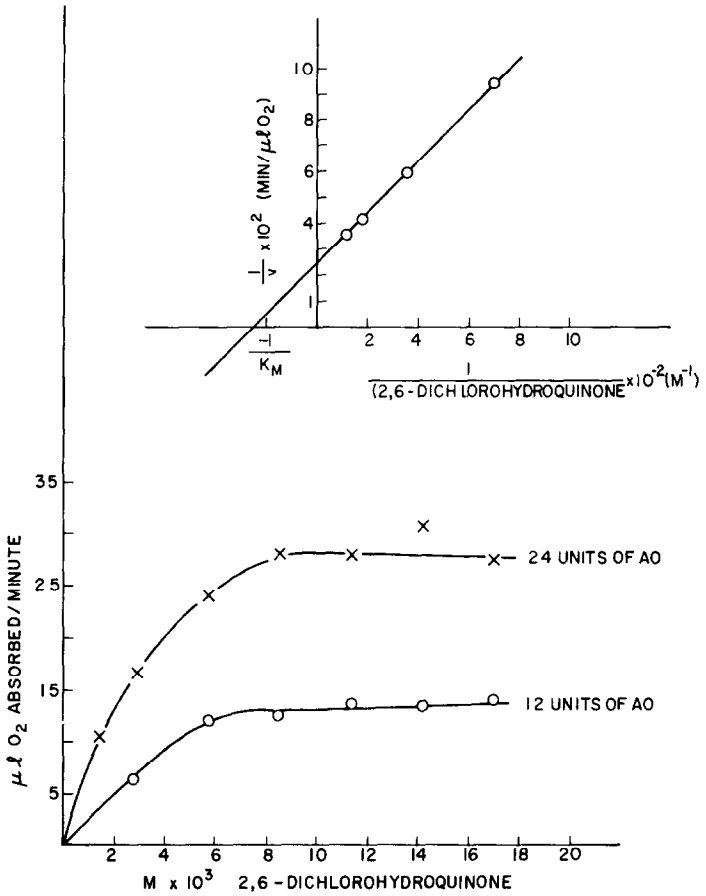

Fig. 2 (Lower) The effect of varying substrate concentration on the microliters of oxygen absorbed during the oxidation of 2,6-dichlorohydroquinone by ascorbate oxidase. A 1.00 ml aliquot of ascorbate oxidase (12 or 24 units) was placed in the side-arm of a Warburg Flask, while in the main compartment were placed appropriate aliquots of 2.84 X 10^{-2}M solution of 2,6-dichlorohydroquinone, 2.00 ml of 0.2M McIlvaine's buffer pH 5.7, 1.0 ml of gelatin (5 mg/ml) and sufficient Cu-free water to bring the final volume of solution to bring the final volume of solution to 10.0 ml.

(Upper) Double reciprocal plot of initial velocity (ul 0_2/min) as a function of 2,6-dichlorohydroquinone concentration in presence of 24 units of AO.

measure the relative rates of AO oxidation, and oxygen uptake totals by direct manometric means. The rate data in Table II reveal that the oxidation of ascorbic acid by AO was approximately 12 times faster per unit of enzyme than the oxidation of hydroxyhydroquinone, 2,5- and 2,6-dichlorohydroquinone, and 200 times faster than chlorohydroquinone, under similar conditions. The total oxygen uptake

during the enzymatic oxidation of these compounds corresponds to one gram atom of O_2 per mole of substrate, which is the same as that observed with ascorbic acid.

K_M and V_{max} Values: Based on the Experimental results shown in Fig. 2, it is evident that the AO catalyzed oxidation of 2,6-dichlorohydroquinone follows Michaelis-Mention Kinetics. The K_m value for 2,6-dichlorohydroquinone, under the conditions indicated, was determined as 4.0×10^{-3}M with a $V_{max}=3.6 \times 10^{4}$ moles/min/mole. These values may be compared with the corresponding values for the substrate, ascorbic acid, $K_m = 2.4 \times 10^{-4}$M and $V_{max}=6.5 \times 10^{5}$ moles/min/mole enzyme (12).

Effect of pH on Oxidation of Substrates by AO: It has been found that the fraction of leuco dye oxidized by enzyme per minute increases with increasing pH (13). Since the K_a of the leuco dye is 1×10^{-7} (Table II), it follows that the concentration of monoanion of the leuco dye also increases from pH 5.3 to 7.4.

Hydroquinone and catechol are not oxidized at pH 5.7 by AO (Table II) even when very large amounts of the enzyme (4800 units) are present. At pH 7.2, however, where the concentration of hydroquinone and catechol anion is appreciably larger than that at pH 5.7, a perceptible oxidation by AO was observed.

Acidity Constants of Compounds Oxidized by Ascorbate Oxidase: In Table II are listed the acidity constants (K_a values) and the relative rates of oxidation (by AO at pH 5.7) of the several hydroquinones we have studied. Similar information is given in the table for the customary substrate (ascorbic acid) and for leuco 2,6-dichloroindophenol. It is apparent from these data that at pH 5.7 only the hydroquinones having a K_a value of about 10^{-7} were enzymatically oxidized at a significant rate (about 1/12 that of ascorbic acid). The much lower rate of oxidation of chlorohydroquinone, and the complete lack of oxidation of hydroquinone, are consistent with the view that, because of their much lower K_a values, there was an insufficient concentration of their respective anionic forms at pH 5.7 for interaction with the enzyme. Supporting this view is the observation

(Table II) that at pH 7.2 (corresponding to a higher concentration of its anionic form) hydroquinone does undergo a perceptible oxidation.

Conclusions: As a result of the present study, it is apparent that anions other than ascorbate are susceptible to AO catalyzed aerobic oxidation. The enzyme is therefore less specific with respect to the structural features of the substrate anion, than formerly believed. It is suggested that the structural requirements for an AO substrate may include only (a) an enolic system, (b) existence primarily in an anionic form at physiological pH, and (c) the capacity for oxidation to a quinoid type of product via a free radical (semiquinoid) intermediate (18).

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