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OXIDATION OF AROMATIC AMINES BY PEROXIDASE AT pH 14

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Key Word Index—Peroxidases; alkaline oxidation; aromatic amine.

Abstract—Horseradish peroxidase catalyzes the peroxidation of *p*-anisidine and other aromatic amines at pH 14. Sensitivity to KCN and thermal inactivation are characteristic of classical heme-enzyme catalysis.

INTRODUCTION

Peroxidases (EC 1.11.1.7) are ubiquitous heme-containing glycoproteins which catalyze oxidations of an unusually broad structural spectrum of H-donors by H_2O_2. They have been implicated in a variety of biochemically important processes including lignification [1], auxin inactivation [2] and thyroxin synthesis [3, 4]. Historically, the highly reactive polyphenol pyrogallol, served as the H-donor [3, 4], and its increasing instability toward molecular oxygen with rising alkalinity set an upper operational limit on the pH at which the reaction could be followed. From pH 4.5 to 8.8, the rate of autoxidation of pyrogallol increases by more than 1000-fold [5]. Furthermore, the products of the reaction in alkaline media consist of a mixture of brown to black polymeric quinones, not the orange benztropolone formed under acidic conditions [6].

Sensitivity to air oxidation under alkaline conditions is, of course, characteristic of phenols generally, but it has been shown with some peroxidases that clear-cut optima do in fact exist below pH 7. The oxidation of phenylpropanoids to lignin-like polymers does not proceed at pH 8.5 and is lower at pH 7 than at 4.5 [7], the chloroperoxidase of *Caldariomyces* halogenates optimally at pH 3 and peroxidizes optimally at pH 5–6 [8, 9]. The highest pH levels reported are 8.8 for iodide oxidation [10] and 8.5 for homovanillic acid oxidation [11].

Both chloroperoxidase [8] and horseradish peroxidase [12] have been studied in modified aqueous media, and in some wholly non-aqueous polar solvents [13]. These findings led to the demonstration that peroxidase can catalyze the oxidation of aromatic amines in 15 M aqueous ammonia (pH 12.2) [14], the most alkaline operational condition yet reported for the enzyme. Aqueous NH_3 is, of course, a unique solvent distinct from solutions of Group Ia bases in many respects. Accordingly, our study has been extended to include more extremely alkaline environments up to and beyond the limits of the Sørenson scale.

Table 1. Formation of coloured oxidation products by peroxidase-H_2O_2 in alkaline media and inhibition by KCN

Substrates	NaOH (N)	Colour in alkaline H_2O_2*		
		Control	+Enzyme	+Enzyme KCN
Aniline	0.1	c	YB	c
	1.0	c	YB	c
	5.0	pY	pY	pY
p-Anisidine	0.1	c	RO	pY
	1.0	c	RO	pY
	5.0	Y	Y	Y
Benzidine	0.1	c	BlG	c
	1.0	c	GB	pY
	5.0	c	c	c.
Diphenylamine	0.1	c	c	c
	1.0	c	c	c
	5.0	c	c	c

* 2 hr at 24°; 5 mM substrates, 10 mM H_2O_2, 0.1 mg/ml enzyme and 10 mM KCN. Y = yellow, B = brown, Bl = blue, G = grey, R = red, O = orange, p = pale, c = colourless.

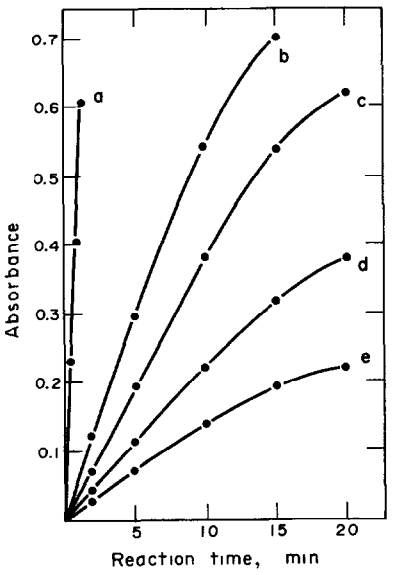

Fig. 1. Time course of 5 mM *p*-anisidine oxidation in reaction mixtures containing 10 mM H_2O_2 and 0.1 mg/ml peroxidase. Curves a–e represent pH 6, 11, 12, 13, 14 respectively.

RESULTS AND DISCUSSION

Many enzymes are active well into the alkaline range and optima as high as pH 11 have been reported [15]. Nevertheless, persistence of enzyme activity far into the alkaline range is not common. The performance of peroxidase is even more impressive, because it has been shown to retain appreciable catalytic ability (in phenol oxidation) in formic and glacial acetic acids [14], hence to span an extremely wide range of H-ion activity. In addition, peroxidases can operate in alcohols and saturated salts, and at temperatures as low as −48°.

Unlike phenols, which characteristically undergo rapid oxidation in alkaline media, the aromatic amines used here were relatively stable (table 1) toward H_2O_2 (and air) at alkalinities at least equal to pH 14. Thus, the rapid formation of coloured oxidation products on addition of enzyme is striking evidence for catalysis (Fig. 1).

Unlike the ammonia system, the NaOH media did not support diphenylamine oxidation, even at pH 13–14. As was observed in the earlier study of peroxidations in 15 M aqueous NH_3, KCN inhibits the process completely, although a higher concentration was required than in ammonia (Fig. 2).

Enzyme activity was also inhibited by its pre-incubation at 97–98° for 4 hr; by elevation of the NaOH concentration to 5 N; and by pre-incubation of enzyme at 34° at pH 14 for at least 4 days (table 2). Here, reducing and oxidizing substrates were added to aliquots of enzyme in N NaOH drawn from the bath and assayed as usual.

These data show that the activities described here are those of a conventional heme-enzyme sensitive to cyanide and subject to thermal denaturation. The micromolar concentrations of iron introduced as the heme moiety of the enzyme account for the activity resistant to heat or alkalinity. The relative importance of denaturative processes *per se* vs hydrolytic degradation in determining the loss of activity at 34°, pH 14 is not known, but many enzymes are far less stable when maintained at 34° in media at ordinary pH values used in enzymology. It is interesting to note in passing that horseradish peroxidase has been shown to possess non-specific superoxide dismutase activity in the autoxidation of epinepherine at pH 10.2 [16]. There is no indication of this activity using aromatic amines under the alkaline conditions employed here.

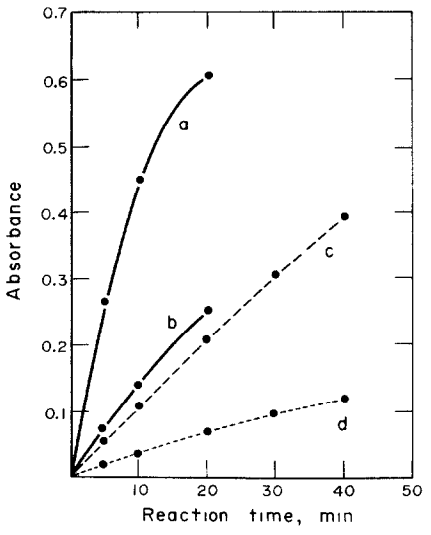

Fig. 2. Effect of 5 mM KCN on the course of *p*-anisidine and benzidine oxidations at pH 13. Conditions as given in Fig. 1. Curves a and c: *p*-anisidine and benzidine controls, respectively. Curves b and d; *p*-anisidine + KCN and benzidine + KCN respectively.

Table 2. Effect of pre-incubation of peroxide at pH 14 in subsequent catalytic activity toward *p*-anisidine and benzidine

Pre-incubation time (hr)	Rate of substrate oxidation (Δabs/min)*			
	p-anisidine		benzidine	
	4°	34°	4°	34°
0	0.015	0.015	0.0061	0.0061
24	0 015	0.006	0.0059	0.0025
48	0.013	0.003	0.0053	0 0014
96	0 011	0.001	0.0046	0 0005
144	0.009	0.001	0.0038	0.0004

* Rates at 475 mm (*p*-anisidine) and 600 nm (benzidine) based on aliquots of enzyme soln withdrawn at specified times and made up to complete reaction mixtures with amines and H_2O_2. Assays were conducted at 24°.

The physiological significance of this exotic capability is not general, but its exobiological and specialized ecological possibilities are nonetheless obvious in relation to the successful cultivation of bacteria, fungi and other organisms above pH 12 in ammoniacal systems [17]. The existence of a complex protein catalyst capable of remaining active under a wide array of severe environmental conditions cannot be overlooked in any consideration of macromolecular evolutionary processes.

EXPERIMENTAL

Calbiochem B grade horseradish peroxidase, R. Z. 0.69, containing 62 purpurogallin units/mg was used in this study. Reaction mixtures contained 0.1 mg/ml of enzyme. Aromatic amine substrates were purchased as crystalline hydrochlorides but were converted to their free bases for use in these experiments Substrates were stored in the absence of light and O_2 at 4° Although a number of amines were screened as substrates, the four used here—aniline, *p*-anisidine, benzidine, diphenylamine—were selected because they had proven to be peroxidase substrates in 15 M aq. NH_3 [14]. They were used in the present experiments at a concn of 5 mM. The kinetics of *p*-anisidine oxidation were followed photometrically at 475 nm, and of benzidine at 600 nm. The oxidant, H_2O_2, was present at 10 mM. Reactions were carried out at 24° in appropriate solns of reagent grade NaOH prepared and stored under CO_2-free conditions. The data presented here are based on duplicate or triplicate determinations in 2 or 3 experiments.

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