Indoleacetic Acid Oxidase Activity of Apoperoxidase

Abstract. *The conventional activity of electrophoretically purified horseradish peroxidase toward guaiacol, pyrogallol, 2,6-dimethoxyphenol, and benzidine is abolished by removal of the heme prosthetic group with a mixture of cold acetone and hydrogen chloride. The apoenzyme, though devoid of peroxidase activity, retains its activity as an indoleacetic acid oxidase when it is supplied with 10^{-5} mole of manganous ion and 2,4-dachlorophenol per liter. This oxidase activity is cyanide-sensitive; azide also inhibits under specific conditions of both* p*H and cofactor concentration. Partial restoration of the peroxidase activity by recombination of apoprotein with heme produces no effect on the oxidase activity, except that cofactors are no longer absolutely required. Therefore, it appears that the activity of peroxidase as an indoleacetic acid oxidase need not directly involve the heme prosthetic group, or that manganous ions and dichlorophenol can substitute for the heme group in the reaction between indoleacetic acid and oxidase.*

There is considerable evidence that indoleacetic acid (IAA) oxidase is a peroxidase (*1*). However, there are no obvious quantitative relations between the peroxidative and IAA-oxidative function of this enzyme; also, correlations between the activity function (PZ) and purity function (RZ) have not yet been produced (*2*). The action of monophenols and manganous ions (Mn^{++}) as cofactors in the IAA oxidation has been repeatedly shown in vitro (*3*), although Hinman and Lang (*4*) demonstrated complete oxidation of IAA with horseradish peroxidase when cofactors were omitted.

Weinryb (*5*) suggested that peroxidase activity requires participation of ligands in addition to heme at the catalytic site (*5*). Bastin (*6*), on the basis of kinetic experiments, concluded that the peroxidative and IAA-oxidative sites are identical. This situation is complicated by the fact that even highly purified crystalline peroxidase can be shown to consist of several isozymic variants (*7*). Studies (*8*) with the isoperoxidases of peas showed that the relative rates of IAA oxidation and guaiacol peroxidation were not the same among the different isozymes. While trying to purify these isozymes from tobacco roots, Sequeira and Mineo (*9*) isolated a fraction which possessed IAA-oxidase activity but no peroxidase activity.

We performed experiments on the dissociation of peroxidase molecules into apoenzyme and heme to compare the electrophoretic mobilities of isoapoenzymes with their corresponding holoenzymes. Unexpectedly, these experiments yielded data indicating that, while the intact hemoprotein is required for guaiacol oxidation, the apoperoxidase alone can catalyze IAA oxidation in the presence of Mn^{++} and phenolic cofactors.

Electrophoretically purified horseradish peroxidase (*10*) was used in all experiments, although heterogeneous preparations of this enzyme gave similar results. Dissociation of the holoenzyme into apoprotein and the heme prosthetic group was performed essentially by Theorell's method (*11*). Two milliliters of the enzyme (1.7 mg of protein) were slowly dropped into 400 ml of a solution of acetone and HCl (0.33 ml of concentrated HCl per 100 ml of acetone) in a dry-ice acetone bath. The white apoprotein precipitate was collected by centrifugation at 10,000*g* at 3°C, washed several times with the cold solution of acetone and HCl, centrifuged again, and allowed to dry. This precipitate, suspended again in 1 ml of 0.2*M* phosphate buffer, *p*H 5.8, is termed "standard apoprotein" preparation.

The heme fraction was prepared from the original acetone-HCl supernatant after removal of the acetone by vacuum aspiration at room temperature. The few remaining yellow drops (essentially heme in concentrated HCl) were neutralized with NaOH, and the volume was brought up to 1 ml. This solution is termed "standard heme" preparation. The "standard reconstituted" enzyme is generated by mixing equal volumes of standard heme and standard protein solutions.

Peroxidative activity was measured by the oxidation of 5 m*M* guaiacol in the presence of H_2O_2 (5 m*M*) in 0.1*M* phosphate buffer, *p*H 5.8. Three milliliters of the mixture of guaiacol and H_2O_2 were used, and the reaction was initiated by the addition of 0.1 ml of the enzyme at appropriate dilutions. The optical density was observed at 15-second intervals at 470 nm in a Bausch and Lomb Spectronic-20 colorimeter.

The kinetics of IAA oxidation were determined by changes in ultraviolet absorbency (*12*), and end-point determinations of the remaining IAA were checked with Salkowski reagent (*13*). The ultraviolet determinations of IAA-oxidase activity were performed in a Perkin-Elmer Model 350 recording spectrophotometer. The final reaction mixture of 1 ml contained $3 \times 10^{-4}M$ IAA and 0.1*M* phosphate buffer, *p*H 5.8. Reaction mixtures lacking or containing cofactors at varying concentrations were also studied. After initiation of the reaction by the addition of the enzyme, complete sweeps of the

Table 1. Activity of holoenzyme, apoenzyme, and reconstituted enzyme as peroxidase and IAA oxidase. Peroxidase was determined in a reaction mixture (total volume, 3 ml) of 5 m*M* guaiacol, 5 m*M* H_2O_2, and 0.1*M* phosphate buffer, *p*H 5.8. The reaction was initiated by the addition of 0.1 ml of enzyme or heme solution at appropriate dilutions. Activity of peroxidase is given as ΔOD (change in optical density) per minute at 470 nm. IAA oxidase was determined in a reaction mixture (final volume, 1.0 ml) containing $3.0 \times 10^{-4}M$ IAA and 0.01*M* phosphate buffer, *p*H 5.8. The plus cofactor samples also contained $10^{-5}M$ dichlorophenol and $10^{-5}M$ $MnCl_2$. Activity of IAA oxidase is given as ΔOD at 254 nm during the first 30 minutes of reaction. For IAA oxidase activity of reconstituted enzyme minus cofactors, there was no change in the 280-nm IAA peak, no shift of the 245-nm trough, and no appearance of 261-nm isosbestic point. However, there was a decrease in the depth of the trough with time (indicated by ± in the table).

Test material	Dilution of standard solution	Acitvity (ΔOD/min)
As a peroxidase		
HP peroxidase	1:100	1.30
Protein*	1:10	0.01
Heme*	None	0.07
Reconstituted enzyme*	1:10	0.38
Protein†	1:50	0.35
Heme†	1:50	0.31
Reconstituted enzyme†	1:50	1.28
As an IAA oxidase		
HP peroxidase minus cofactors	1:10	0.90
HP peroxidase plus cofactors	1:10	0.77
Highly purified apoprotein minus cofactors	1:5	0
Highly purified apoprotein plus cofactors	1:5	0.93
Reconstituted enzyme minus cofactors	1:2	±
Reconstituted enzyme plus cofactors	1:2	1.05

*Highly purified. † Purified.

Table 2. Azide inhibition of IAA-oxidase activity. Cofactors were $10^{-5}M$ $MnCl_2$ and $10^{-5}M$ dichlorophenol. The IAA concentration was $3.0 \times 10^{-4}M$, and the reaction was carried out at *p*H 4.8. For reconstituted enzyme minus cofactors, O.D. was measured as an increase at 254 nm ($t_0 - t_{20}$ minutes). Enzyme was added at *t* (time) minus 15 seconds. Similar units were obtainable by following decrease of the 280-nm IAA peak.

Test material	Presence of cofactors	ΔO.D. per minute		Inhibition (%)
		No azide	$2 \times 10^{-4}M$ azide	
HP peroxidase	—	0.42	0.20	52
HP peroxidase	+	.20	.18	10
Peroxidase protein	—	0		
Peroxidase protein	+	.44	.25	48
Reconstituted enzyme	—		0	
Reconstituted enzyme	+	.36	.21	40

ultraviolet spectrum were made at appropriate intervals for up to 2 hours. Increased absorbency at 254 nm was used as the principal criterion of the destruction of IAA and the formation of 3-methyleneoxindole (*12*).

The original peroxidase had an iron content of 0.06 percent, which compares with an iron content of 0.13 percent for horseradish peroxidase and 0.06 percent for lactoperoxidase originally reported by Theorell and Åkesson (*14*). The highly purified apoprotein contained no detectable iron as determined by x-ray fluorescence analysis. This apoperoxidase also had no detectable activity toward guaiacol. Under our conditions of repeated washings with a solution of acetone and HCl, only about 1/40th of the original peroxidative activity could be regenerated by the readdition of the heme (Table 1). When preparations were washed less extensively, there was contaminating peroxidase activity in both heme and apoprotein fractions, and almost half of the initial activity could be regenerated in the recombined holoenzyme.

When these experiments were repeated to measure IAA-oxidase activity, the results were quite different in that both the apoprotein and the reconstituted enzyme displayed IAA-oxidase activity in the presence of dichlorophenol and $MnCl_2$. The cofactors enhance the initial rate of oxidation of IAA when intact peroxidase is used (Fig. 1); however, a much higher final peak at 254 nm, as well as larger changes at other wavelengths, were observed when the cofactors were omitted. Reaction mixtures, both lacking and containing cofactors, gave a negative Salkowski color test at the end of the reaction (30 minutes for the minus-cofactor group, with an absorbency of 1.85; 60 minutes for the reaction-containing cofactors, with an absorbency of 1.75).

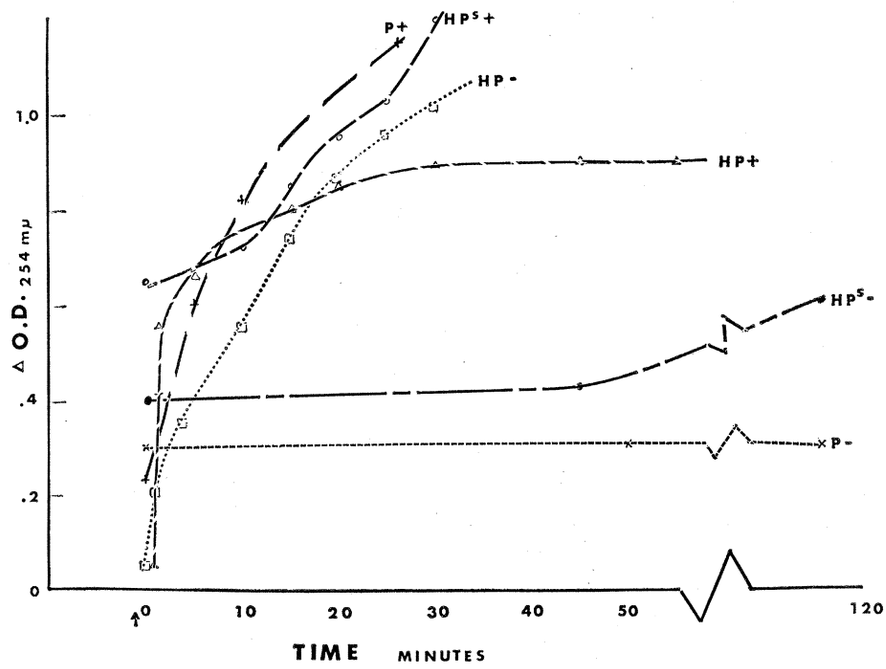

Fig. 1. Appearance of 3-methyleneoxindole in a reaction mixture containing $3 \times 10^{-4}M$ IAA, *p*H 5.8. The presence or absence of cofactors ($10^{-5}M$ $MnCl_2$, $10^{-5}M$ DCP) is indicated by plus or minus. *HP*, initial holoenzyme of horseradish peroxidase; HP^s, resynthesized holoenzyme; *P*, apoprotein. The enzyme or protein was added 15 seconds before the initial O.D. reading (arrow).

Figure 1 also shows that Mn++ and dichlorophenol are required for IAA-oxidase activity of the apoprotein. After about 15 hours, the reaction mixture showed a typical methyleneoxindole peak at 254 nm and was Salkowski-negative. When cofactors were omitted from this reaction mixture, there was no indication of IAA destruction even after 48 hours. The IAA-oxidase activity of the reconstituted hemoprotein is enhanced by the cofactors, but a slow reaction proceeds without them.

Neither hydrogen peroxide (10^{-3} to $10^{-5}M$) nor α-naphthaleneacetic acid (a synthetic functional analog of the plant hormone, IAA) had any observable effects on any of the IAA-oxidizing systems studied here. Cyanide ($10^{-3}M$) inhibited both the IAA-oxidase function and the peroxidative reaction.

Azide was inhibitory, but only at certain *p*H's. The peroxidative and IAA-oxidative functions have quite dissimilar *p*H optima, but this *p*H dependence may reflect nothing more than the acid-base properties of the particular hydrogen donors used. In fact, there is little or no IAA-oxidase activity under alkaline conditions, whereas peroxidase activity, although spanning a wide range (*p*H 3.0 to 12.0) had an optimum at about *p*H 8.8. Azide was inhibitory only in the acid range (*15*), thus interfering with IAA-oxidase activity. Peroxidation was effectively inhibited at acid *p*H's, but at *p*H 9, even $0.1M$ sodium azide had no effect on the reaction. In regard to the relation between inhibitors and cofactors (Table 2) in the IAA-oxidase reaction, the reconstituted enzyme apparently behaves more like the purified apoprotein than the original holoenzyme.

Other enzymes which appear to possess more than one active site have been described (*16*), and a number of enzymes which require activation by the addition of small molecules are

known (*17*). Peroxidase is unique in this respect only in that it seems to possess one function, that of an IAA oxidase, in the nonheme protein portion of the molecule, and a second function, involving guaiacol peroxidation, which depends on a heme-protein attachment. These sites appear to be distinct, since readdition of heme to apoprotein has no effect on the IAA-oxidase activity of the latter.

Peroxidase contains a sizable carbohydrate component (about 15 percent of its dry weight) which remains associated with the protein fraction during these experiments (*18*). Other investigators (*5*) suggested a second active site (the heme-protein complex generally being considered the primary site) of peroxidase which may involve the carbohydrate-protein complex.

The cyanide inhibition of a nonheme-dependent reaction can be explained on the basis that CN^- is an effective free-radical chain terminator (*19*)—some oxidase and peroxidase reactions are believed to function by means of such a mechanism (*20*)—or by the fact that CN forms a complex with Mn^{++} at the active site. The azide effect may be more difficult to understand, although Weinryb (*15*) demonstrated a reaction between azide and amino acid residues which alters the environs of the catalytic center in horseradish peroxidase. Chance (*21*), from his studies on azide inhibition of other hemoprotein systems, concluded that the azide may be capable of attachment only during a transition between two different configurations of the protein, possibly at a histidyl locus. It is generally assumed that azide attaches as a ligand to the heavy metal of the prosthetic group. In our purified apoprotein preparation, there is no detectable iron; nevertheless, Mn^{++} is required for the apoprotein to function as an IAA oxidase, although Mn^{++} cannot replace the heme for peroxidase activity. It is possible that the azide might block the active site formed between the apoprotein and Mn^{++} and hence could act in the classic manner. The *p*H effects may well be due to conformational changes of the protein produced by the charge alteration, as well as reflecting the acid-base properties of the substrates.

The nonperoxidase IAA oxidase reported by Sequeira and Mineo (*9*) may be an artifact resulting from the removal of the heme during the processes involved in isolation. Alternatively, the apoprotein may exist in the free form in vivo. We have attempted to detect free apoperoxidase protein in pea tissue homogenate, but without success.

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Phosphorylative Inactivation of Aminoglycosidic Antibiotics by Escherichia coli Carrying R Factor

Abstract. *Kanamycin and paromamine are inactivated by an enzyme solution prepared from* Escherichia coli *carrying R factor; and the 3-hydroxyl group of 6-amino-6-deoxy-*D*-glucose moiety of kanamycin or the 3-hydroxyl group of glucosamine moiety of paromamine is phosphorylated. These inactivated products and dihydrostreptomycin inactivated by the enzyme solution are converted to the original antibiotics by treatment with alkaline phosphatase.*

Okamoto and Suzuki (*1*) reported that a multiple drug-resistant *Escherichia coli* K12 (R-5) produced an enzyme that inactivates kanamycin. The strain used by these authors was obtained by transmission of R factor (R-5) from a naturally isolated drug-resistant strain of *Shigella sonnei* which was resistant to chloramphenicol, tetracycline, dihydrostreptomycin, sulphanilamides, and kanamycin. These authors suggested that this resistant strain would produce an enzyme that acetylates kanamycin, because of necessity of coenzyme A, acetate, and adenosine triphosphate for the inactivation. As reported earlier, we isolated the inactivated kanamycin and confirmed the fact that amino group of 6-amino-6-deoxy-D-glucose moiety of kanamycin was acetylated. The relation of inactivation to resistance can be shown by the fact that this resistant organism is sensitive to kanamycin C which contains D-glucosamine instead of 6-amino-6-deoxy-D-glucose, and that the enzyme extracted from this organism does not inactivate kanamycin C. We later found that the mode of the inactivation by *E. coli* K12 carrying another R factor was different. We now report on the phosphorylation and inactivation of kanamycin, paromamine, and dihydrostreptomycin by an enzyme solution prepared from a resistant strain of *E. coli* carrying R factor.

Escherichia coli K12 ML 1629 was obtained by transmission of R factor from a naturally isolated drug-resistant strain of *E. coli* to *E. coli* K12 ML 1410 which was resistant to nalidixic acid. The inhibitory concentrations of kanamycin, paromomycin, and neomycin toward *E. coli* ML 1629 were higher than 320 μg/ml, that of paromamine was 320 μg/ml, and that of streptomycin was 20 μg/ml. The inhibitory concentrations for *E. coli* K12 were as follows: kanamycin, 1.25 μg/ml; paromamine, 40 μg/ml; par-