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Purification and Properties of Amine Oxidase from Epicotyls of *Pisum sativum*

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ABSTRACT

A procedure has been developed for the purification of amine oxidase (E.C. 1.4.3.4) from etiolated pea epicotyls (*Pisum sativum* cv. Little Marvel). The enzyme is sensitive to copper chelating reagents and carbonyl reagents, but is not inhibited by sulfhydryl reagents. The purified enzyme has a molecular weight of 1.85×10^5, as determined by sedimentation equilibrium centrifugation, and has been shown to be specifically stimulated by phosphate.

Recent studies in this laboratory have indicated the possibility that IAA synthesis in *Pisum* could proceed through tryptamine and that the enzyme responsible for catalyzing this reaction could be purified (10, 14). As part of an investigation of IAA biosynthesis in *Pisum*, an enzyme which forms IAA from tryptamine was purified and found to be an amine oxidase. In view of the wide interest in amine oxidases from mammals and the one amine oxidase purified from a higher plant, it was decided to characterize this enzyme for a further understanding of the plant enzyme and for a comparison with the mammalian enzymes.

Amine oxidase from plants was first purified and studied by Mann, who found that the enzyme was a pink, copper-containing protein which was sensitive to carbonyl reagents (12). The visible absorption spectrum of this enzyme had an absorbance maximum at 500 nm (13). This absorbance maximum could be shifted to lower wavelengths by incubation of the enzyme with substrate under anaerobic conditions or by incubation of the enzyme with copper-chelating reagents. These spectral shifts could be reversed by incubation under aerobic conditions in the first case and cupric ions in the second (9). The enzyme was found to be active in catalyzing the oxidative deamination of a wide variety of substrates (9). The purified enzyme had an $s_{20,w}$ of 7.7S, measured from sedimentation velocity centrifugation, and a molecular weight of 9.6×10^4, calculated from electron micrographs (9). Recently, Yamasaki *et al.* (24) have shown that the binding of oxygen to amine oxidase from peas is dependent upon amine concentration.

Amine oxidase from bovine plasma has been studied extensively by Yasunobu and co-workers (21–23) who have found that the visible absorbance spectrum, copper content, and sensitivity to carbonyl reagents are similar to the plant enzyme. In addition, the enzymes from bovine and porcine plasma have been found to contain pyridoxal phosphate (4, 23). The amine oxidase from porcine plasma has a molecular weight of 1.95×10^5 (5), and the amine oxidase from bovine plasma has a molecular weight of 1.7×10^5 (1). The enzyme from bovine plasma has a subunit molecular weight of 8.05×10^4 (1).

The study reported here describes a purification procedure for the amine oxidase from etiolated pea epicotyls, confirmation of copper and R-COR as functional groups in catalysis, the molecular weight as determined by ultracentrifugation, and the requirement of phosphate for maximal catalysis.

MATERIALS AND METHODS

Seeds of *Pisum sativum* L. cv. Little Marvel were soaked for 48 hr in aerated tap water and grown either hydroponically or in vermiculite for 4 to 5 days in the dark. At the end of this growth period the fourth internode was elongating. The epicotyls were cut at the first node and immediately frozen with Dry Ice.

Enzyme Assays. The formation of IAA from tryptamine by the purified enzyme was determined by the *Avena* curvature bioassay as described by Lantican and Muir (10).

Amine oxidase was assayed spectrophotometrically as described by Tabor *et al.* (19). The increase in absorbance at 250 nm due to benzaldehyde formation was measured at 25 C with a Gilford 2000 spectrophotometer or with a Cary 14 spectrophotometer.

The spectrophotofluorometric assay was based on a method suggested by Andreae (2). An Aminco-Bowman spectrophotofluorimeter, with a controlled temperature cuvette compartment maintained at 25 C, connected to a Sargent multirange recorder, was used. Reaction mixtures of 1 ml volume contained substrate, 0.1 mg of horseradish peroxidase, 10 nmoles of scopoletin, and 0.125 μmole of tris buffer adjusted to the desired pH. The reaction was initiated by the addition of enzyme. Activity was determined as a decrease in scopoletin fluorescence at 465 nm using an excitation wavelength of 370 nm.

Specific activity is defined as micromoles of product formed per mg protein per min, for purposes of standardization. Protein was measured using either the biuret reaction (8) with bovine serum albumin as standard, or by the method of Warburg and Christian (20).

Enzyme Purification.

Preparation of Crude Extract. Two kilograms of frozen pea epicotyls were powdered in a prechilled Waring Blendor. This powder was added with 50 mM potassium phosphate buffer, pH 6.9, in the ratio of 0.33 ml buffer per g of tissue to an Eppenbach model MV-6-3 colloid mill (Gifford-Wood Company) with constant blending at a gap setting of 76. The

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temperature was maintained below 10 C by circulation of coolant through the colloid mill. When all of the powder had been added and free circulation of the slurry through the mill was achieved, the gap setting was gradually reduced to 16. Additional homogenization at top speed was allowed for 10 min, at which point the slurry was removed from the Micromill and strained through a nylon bag. All procedures were performed at 4 C unless otherwise indicated. The crude filtrate was centrifuged at 27,000*g* for 30 min, and the precipitate was discarded.

Protamine Sulfate Treatment. Protamine sulfate was added to the crude supernatant solution in the ratio of 1 g of protamine per 10 g of supernatant protein. A 5% (w/v) suspension of protamine sulfate adjusted to pH 6.5 was added slowly with constant stirring. Thirty minutes after the final addition of protamine, the solution was centrifuged at 27,000*g* for 30 min, and the precipitate was discarded.

Ammonium Sulfate Fractionation. Solid ammonium sulfate (209 g per liter) was added slowly with stirring to the supernatant solution from the protamine sulfate treatment and allowed to stand for 1 hr. The precipitate was removed by centrifugation and discarded. Additional solid ammonium sulfate (200 g per liter initial supernatant volume) was added to the 35% ammonium sulfate supernatant solution. One hour after the final addition of ammonium sulfate the precipitate was collected by centrifugation at 27,000*g* for 1 hr, dissolved in 200 ml of 50 mM potassium phosphate, pH 6.9, dialyzed against the same buffer for 12 to 16 hr, and clarified by centrifugation.

Ethanol Fractionation. The clarified preparation was placed in a beaker immersed in a mixture of ice and salt at −7 C. Absolute ethanol, prechilled to −7 C, was added slowly with vigorous stirring until the solution was 10% (v/v) in ethanol. This solution was centrifuged, and the supernatant solution was made 36% (v/v) in ethanol. One hour after the final ethanol addition, the solution was centrifuged at 27,000*g* for 30 min. The precipitate was collected and dissolved in 100 ml of 50 mM potassium phosphate, pH 6.4. The dialyzed solution was clarified by centrifugation.

Phosphocellulose Chromatography. The dialyzed fraction which had precipitated between 10 and 36% ethanol was applied to a 2- × 12.5-cm column of phosphocellulose equilibrated with 20 mM potassium phosphate, pH 6.4. The column was washed with equilibrating buffer until the eluate had no further absorbance at 280 nm. The column was then washed with 50 mM potassium phosphate, pH 6.9, and eluate fractions of 2 ml were collected. The amine oxidase activity was associated with a pink bank of material eluted by this buffer. The active fractions were pooled, concentrated to 2 ml with a membrane concentrator (Amicon Corp.), and dialyzed against the elution buffer.

Phosphocellulose Rechromatography. The concentrated material was applied to another column of phosphocellulose (1 × 20 cm), equilibrated in the same manner. The column was then eluted with equilibrating buffer and fractions of 1 ml were collected. The fractions with highest specific activity were pooled. All physical measurements were made on this material.

Sedimentation Measurements. Sedimentation velocity measurements were made in a Spinco model E analytical ultracentrifuge at 59,780 rpm using schlieren optics and Kodak metallographic photographic plates. Sedimentation coefficients were calculated as described by Schachman (17). High speed sedimentation equilibrium experiments were performed according to the procedure of Yphantis (25) as described by Lindell and Stellwagen (11).

Electrophoresis. Polyacrylamide gel electrophoresis experiments were performed using the running gel as described by Davis, with the sample applied to the top of the gel as a 10% (w/v) sucrose solution (6). Gel electrophoresis at low pH was performed according to the method described by Panyim and Chalkley (15). Neutral pH gels were made using phosphate buffer instead of the tris-glycine buffer described by Davis (6).

Table I. *Purification Scheme for Amine Oxidase from Pea Epicotyls*

Procedure	Total Protein[1]	Total Activity[2]	Specific Activity[3]	Purification	Recovery
	mg	*units*			%
Crude extract	38,880	93,400	0.0024		100
Protamine sulfate treatment	9,460	139,500	0.015	6	149
35—65% Ammonium sulfate precipitation	4,662	129,700	0.028	12	139
10–36% Ethanol precipitation	697	177,000	0.254	106	189
Phosphocellulose chromatography	171	130,500	0.761	317	140
Phosphocellulose rechromatography	39	29,500	1.005	419	31

[1] Protein was determined by the biuret reaction using bovine serum albumin as standard.

[2] A unit of activity is defined by the formation of 1 μmole of benzaldehyde per minute.

[3] Specific activity is defined as μmoles product per minute per milligram protein.

Materials. The chemicals used in this study were obtained from the following sources: tryptamine hydrochloride and indoleacetic acid from Regis Chemical Company; dimedone, benzylamine, acrylamide, N,N,N′,N′-tetramethylethylenediamine, and N,N′-methylenebisacrylamide from Eastman Organic Chemicals; protamine sulfate from Krishell Laboratories; cuprizone from K & K Laboratories, Inc.; neocuproine from G. F. Smith Chemical Company; phosphocellulose (Cellex-P) from Bio-Rad Laboratories; putrescine dihydrochloride from Calbiochem; and scopoletin from Mann Research Laboratories. Visking dialysis tubing was boiled in 1 mM EDTA before use.

RESULTS

Purification Procedure. The purification procedure is presented in Table I. This purification procedure results in an enzyme preparation which is homogeneous as determined by several hydrodynamic and electrostatic properties. In the final step two protein peaks are observed. One of these peaks represents enzyme of high specific activity and the other is enzyme of low specific activity. The latter is considered to be amine oxidase which has been partially modified by the purification procedure.

Using the spectrophotofluorometric assay, the enzyme purified by this procedure has the highest specific activity reported for pea amine oxidase. In addition, this enzyme will form auxin from tryptamine.

Sedimentation Measurements. Purified amine oxidase from pea epicotyls exhibits a single symmetrical boundary during sedimentation velocity centrifugation (Fig. 1). From measurements of the boundary displacement with time of centrifugation, a sedimentation constant ($s_{20,w}$) was calculated to be 7.7S.

The molecular weight of amine oxidase was calculated from high speed sedimentation measurements by the method of

Yphantis (25). A typical protein gradient at equilibrium is illustrated in Figure 2. Assuming that the partial specific volume ($\bar{v}$) of pea amine oxidase is the same as that calculated for bovine plasma amine oxidase, 0.76 ml/g (1), a molecular weight of 1.84 ± 0.06 × 10^5 was calculated for pea amine oxidase.

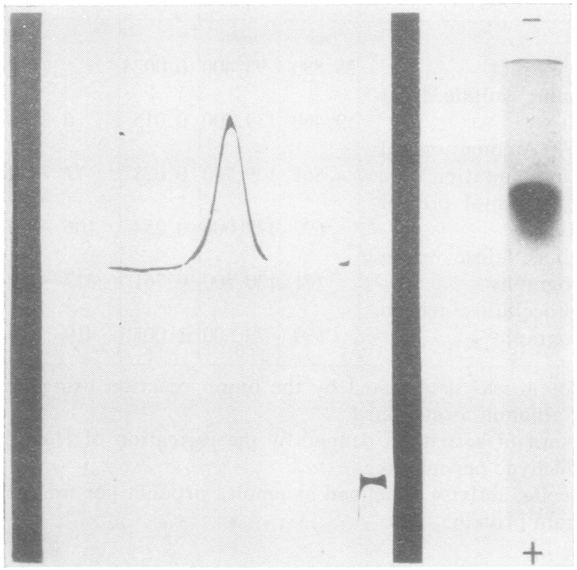

FIG. 1. Sedimentation velocity (left) and electrophoresis (right) patterns shown by amine oxidase from peas. The purified enzyme was sedimented in the Spinco Model E ultracentrifuge at 59,780 rpm. The solvent was 50 mM potassium phosphate, pH 6.4, and the protein concentration was 4.5 mg per ml. The photograph was taken 32 min after attaining speed. The electrophoretic pattern is for 50 μg of protein run at pH 9 for 16 hr at a constant current of 2 milliamps per gel.

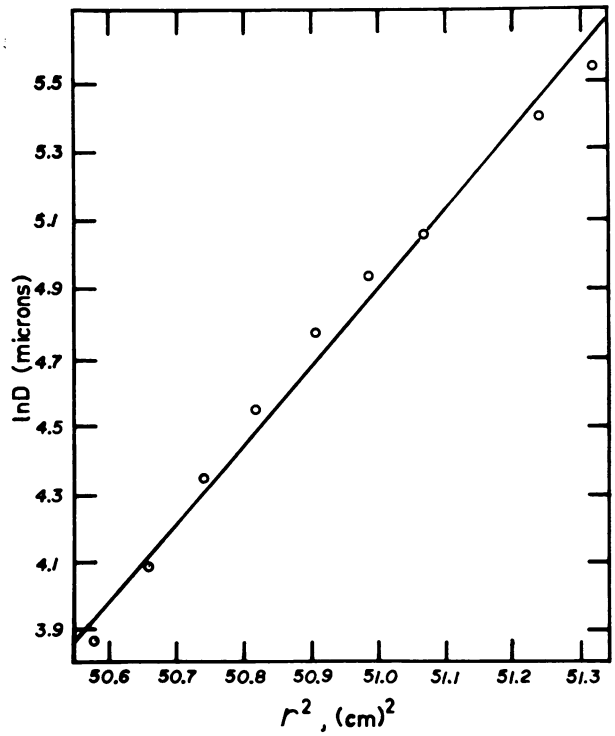

FIG. 2. Equilibrium sedimentation of the purified enzyme. The ordinate is the natural logarithm of fringe displacement (ln D) in microns and the abscissa is the distance from the center of rotation in cm^2 (r^2). The solvent was 20 mM potassium phosphate, pH 6.4, and the protein concentration was 0.1 mg per ml. Centrifugation was for 20 hr at 15,220 rpm at 20 C. Values of ln D greater than 50 microns were used for calculation.

Table II. *The Rate of Oxidative Deamination of Amines by Amine Oxidase*

Amine	pH	K_m	V_{max}
		M	*μmoles/min·mg protein*
Spermidine	7.0	1.9×10^{-3}	480
Putrescine	7.0	8.2×10^{-5}	332
Spermine	7.0	1.3×10^{-3}	46
Tryptamine	7.0	3.2×10^{-3}	26
Benzylamine	8.6	2.0×10^{-4}	19

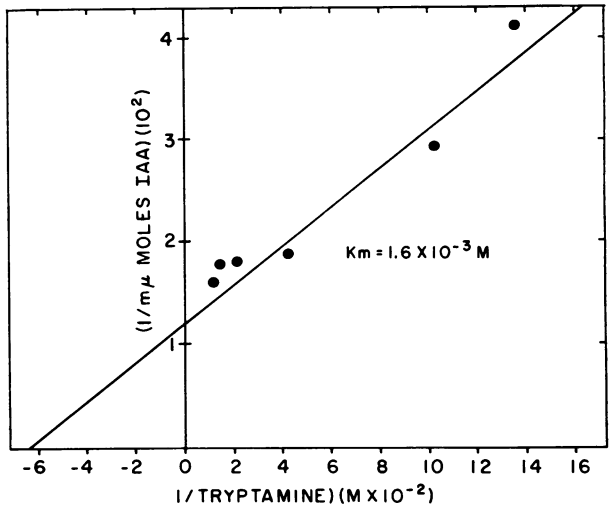

FIG. 3. A double reciprocal plot of the dependence of auxin synthesis on the concentration of tryptamine in the assay mixture. Reaction mixtures of 5 ml volume, pH 6.9, varying in tryptamine concentration were incubated with 200 spectrophotometric units of enzyme preparation (0.55 mg protein) for 30 min at room temperature, stopped by a boiling water bath, adjusted to pH 6.0, and auxin was determined by the *Avena* curvature bioassay. Auxin is expressed as IAA equivalents based on a standard curve. Each point represents the average response of 10 coleoptiles to each assay solution.

Electrophoresis. Purified pea amine oxidase migrates as a single band when subjected to zone electrophoresis in a polyacrylamide gel at pH 9 (Fig. 1), 7.4, and 3.4.

Absorption Spectrum. Purified pea amine oxidase exhibits absorbance maxima at 279 nm and 490 nm and shows an inflection at 292 nm. The enzyme has an A_{280}/A_{260} ratio of 1.84 and an extinction at 500 nm for a 0.1% solution of 0.145.

Kinetic Measurements. Amine oxidases show a broad substrate specificity (26). This enzyme was investigated for its specificity towards several amines considered to be of possible biological importance. Diamines as well as monoamines are suitable substrates; the diamines putrescine and spermidine are most active as substrates (Table II). Tryptamine oxidative deamination catalyzed by the enzyme was also measured by the *Avena* curvature bioassay. The *Km* for tryptamine as determined with the bioassay (Fig. 3) was 1.6 mM, which agrees reasonably well with the *Km* determined using the spectrophotofluorometric assay (3.2 mM). A Hill plot (3) of the data from which the *Km* for benzylamine was calculated gives a Hill coefficient (n) of 1.17, indicating no interaction between binding sites.

The presence of copper as a component of the active site

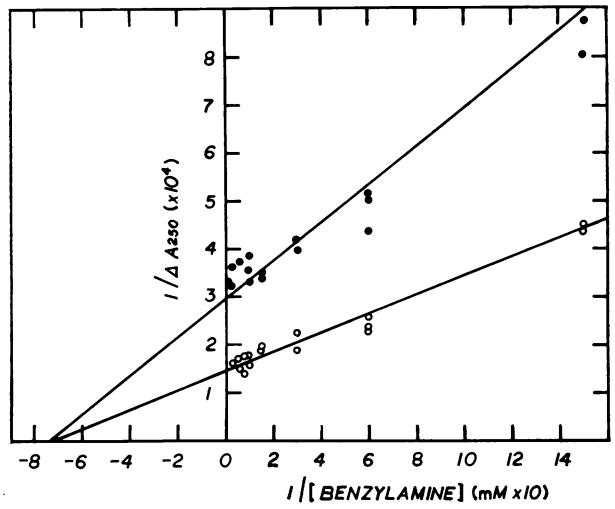

FIG. 4. The effect of a copper chelator, cuprizone, on amine oxidase from peas. Assays were performed using the spectrophotometric assay described in "Materials and Methods." Cuprizone and enzyme were allowed to react for 10 min before substrate was added to initiate the reaction; the chelator concentration was 0.1 mM and substrate concentration (benzylamine) was varied. Incubation with chelator (●); incubation without chelator (○).

of the enzyme is illustrated by the data shown in Figure 4. The cupric chelator, cuprizone, decreases the maximal velocity of catalysis but does not change the Michaelis constant. The cuprous chelator, neocuproine, has no effect.

The presence of a carbonyl group at the active site is indicated by the complete inhibition of catalysis by hydroxylamine at 1×10^{-5} M and semicarbazide at 1×10^{-6} M. Dimedone did not inhibit catalysis below 1×10^{-2} M. Hydroxylamine and semicarbazide are general carbonyl reagents, whereas dimedone is specific for aldehydes (18).

Some amine oxidases are considered to be sulfhydryl enzymes (7). The possible requirement of a sulfhydryl group for catalytic activity by the enzyme examined in this investigation was tested using reagents which are known to react with sulfhydryl groups. The data shown in Table III indicate that a sulfhydryl group is not required for the catalytic activity of this enzyme. Although inhibition by sulfhydryl reagents is observed, it is only observed at very high concentrations of inhibitor. Enzymes with an active sulfhydryl group (such as alcohol dehydrogenase) are inhibited 100% by concentrations of inhibitor which are lower than those giving little or no inhibition here.

It was observed that phosphate was required for maximal catalytic activity. This requirement was dependent on the buffer used (Fig. 5). The fact that this stimulation was specifically induced by phosphate was indicated by the lack of stimulation by arsenate, sulfate, and a number of other ions. The Michaelis constant for phosphate was determined to be 0.9 mM. Phosphate did not change the Michaelis constant for substrate binding. In tris buffer, no requirement for phosphate was apparent (Fig. 5), and the Michaelis constant for substrate was the same in tris as in borate-phosphate buffer.

DISCUSSION

The procedure reported here results in a purified pea amine oxidase of a specific activity apparently 10-fold higher than previously reported. The high specific activity was measured by the spectrophotofluorometric assay which requires very small amounts of enzyme and which provides for decomposition of the H_2O_2 produced by oxidative deamination. It is possible that the diluted enzyme and the removal of H_2O_2 combine to give higher specific activities than can be obtained with the spectrophotometric or manometric assays.

The characteristics of this enzyme with respect to substrate specificity, sensitivity to cupric chelators and carbonyl reagents, and lack of sensitivity to sulfhydryl reagents are consistent with the previously reported characteristics for the enzymes from pea cotyledons and mammalian plasma. The visible spectra of all these amine oxidases are very similar. The pea amine oxidase was previously reported to have a molecular weight of 9.6×10^4; however, this was estimated for an enzyme which had apparently been dissociated during preparation for electron microscopy (9). The molecular weight of pea amine oxidase reported here is 1.84×10^5, which

Table III. *The Effect of Sulfhydryl Reagents on the Catalytic Activity of Amine Oxidase*

The enzyme (0.135 μg) was incubated for 1.5 hr in 1 ml solution of the desired concentration of inhibitor and buffered by 0.25 M tris. The solutions containing N-ethylmaleimide were at pH 7.0 and the solutions were dialyzed overnight at 4 C with several changes of buffer (0.25 M tris, pH 7.0). The assays were performed by adding to the above reaction mixtures 10 μl of 0.1 M diaminobutane, 20 μl of 5×10^{-4} M scopoletin, and 0.1 ml of horseradish peroxidase (stock solution 1.0 mg per ml). Initial velocity (V_0) was measured as the decrease in percent transmittance per minute.

Condition	V_0	Inhibition
	ΔT/min	%
N-Ethylmaleimide		
10^{-2} M	1.37	66
10^{-3} M	2.20	47
5×10^{-4} M	3.77	10
Iodoacetic acid		
10^{-2} M	3.3	21
10^{-3} M	2.13	49
5×10^{-4} M	2.57	38
Iodoacetamide		
10^{-2} M	4.9	0
10^{-3} M	4.27	0
Control	4.17	

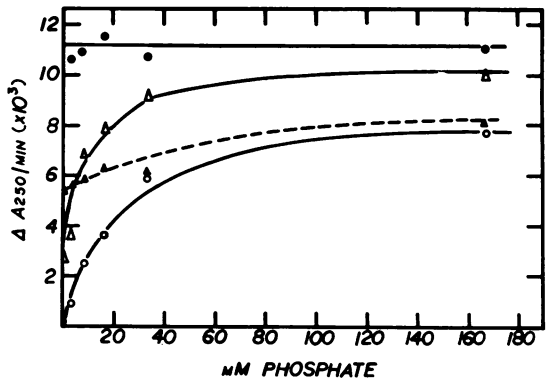

FIG. 5. The effect of phosphate on the initial velocity of enzymatic catalysis of benzylamine oxidation by amine oxidase from peas. Various amounts of phosphate were added as 1 M dibasic potassium phosphate, pH 8.6, with the assay volume remaining constant at 3 ml. The buffer concentrations were all 80 mM and pH 8.6. Two microliters of enzyme preparation were added to start each reaction, and the initial velocity was followed with a Cary 14 spectrophotometer using the expanded scale slide wire with a full scale deflection equivalent to 0.100 absorbancy unit. ●: Tris; ○: glycine; ▲: bicarbonate; and △: borate.

is consistent with the molecular weight reported for the mammalian plasma enzymes, and also consistent with the molecular weight estimation by Muir and Lantican for the enzyme which forms auxin from tryptamine (14). The subunit composition and the partial specific volume have not yet been determined for the pea enzyme; however, they are expected to be similar to the mammalian enzymes.

The requirement of inorganic phosphate for maximal catalytic activity except in the presence of tris has not been previously reported. Dog kidney glutaminase is activated by phosphate. This activation led Sayer and Roberts to postulate that phosphate attaches to a cationic site on this enzyme, and that the glutamine amino group attaches to an anionic site on the phosphate (16). They have suggested that this enzyme forms an E-P complex and then an E-P-S complex (where P represents phosphate, E enzyme, and S substrate). Failure to cover the cationic site also leads to rapid inactivation. The report by Yamada and Yasunobu that bovine plasma amine oxidase is stimulated by phosphate (21), which they attributed to an ionic strength phenomenon, raises the question of whether or not the requirement for phosphate might be a characteristic of all the amine oxidases or at least the class which are not flavoproteins. It is interesting to speculate that the phosphate stimulation is related to amine oxidase regulation of the levels of amines which interact with nucleic acids, and that either the level of inorganic phosphate or the phosphate groups on the nucleic acids might activate the enzyme. Certainly, further studies on phosphate binding to the enzyme and the site of phosphate binding would be helpful in extrapolation to the *in vivo* control of this enzyme.

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