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Oxygen Consumption during the Indole-3-Acetic Acid Oxidation Catalyzed by Peroxidase. Effect of the Enzyme/Substrate Ratio

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Oxygen consumption during the oxidation of indole-3-acetic acid (IAA) by peroxidase has been studied, in conditions of no limitation by oxygen. For different enzyme/substrate ratios the quotient O_2 consumed/IAA oxidized shows rather small differences and it is always less than unity. During the reaction, however, the quotient undergoes important variations because oxygen uptake ceases long before the decarboxylation of IAA. The results are discussed in the light of previous proposed mechanisms.

Key words: Decarboxylation, E/S ratio, Indole-3-acetic acid, Peroxidase, Indole-3-methanol, Oxygen.

The biochemistry of the oxidation of IAA catalyzed by peroxidase (E.C. 1.11.1.7) has been frequently matter of research owing to the role that the process can play in the regulation of the hormone level in plants (14, 15).

IAld and the oxindoles, HMO and MO, have been the most frequently reported products of oxidation and hence a stoichiometry of the reaction involving one mole of oxygen per mole of IAA could be expected. However the confirmed formation of a less oxidized product, i.e., IM (2, 5, 12) points to a stoichiometry different from 1:1, as has been found (9, 12).

The reaction rate as well as the relative composition of the mixture of products (included IM) are under the influence of several factors, among which the ratio of concentrations enzyme/substrate (E/S) is one of the most relevant (6, 7, 8, 11, 13). Consequently the stoichiometry of oxygen consumption will depend on the E/S ratio as has been recently reported (16). A reconsideration

* Abbreviations: IAA, indole-3-acetic acid; IAld, indole-3-aldehyde; HMO, 3-hydroxymethyloxindole; MO, 3-methyleneoxindole; IM, indole-3-methanol; HRP, horseradish peroxidase; Fe_p^{3+}, ferriperoxidase; Co-I and Co-II, intermediate active compounds of peroxidase; R'OOH, hydroperoxide form of skatole.

of the so far proposed mechanisms of reaction becomes, then, necessary.

In this paper, the oxygen consumption during the oxidation of IAA at different E/S ratio, is studied. The aim is to justify the stoichiometry obtained, according to the type of products.

Materials and Methods

Enzyme. HRP from SIGMA, type VI (two basic isoenzymes), RZ = 3.1, was dissolved in a phosphate buffer 0.1 M pH 6.3. The concentrations were estimated on the basis of a molecular weight of 40,000, and further corrected by absorbance of solutions at 403 nm using the molar extinction coefficient $\varepsilon_{mM}=102$.

Measure of O_2 uptake. An oxygraph with Clark electrode was used. The reaction mixtures contained a phosphate buffer 0.06 M, pH 6.3 and variable amounts of HRP and IAA in a final volume of 3 ml. Before the start of the reaction, the vessel was air saturated by bubbling at 30°C, so that the initial content of oxygen was 0.63 μmoles. A recorder coupled to the oxygraph directly supplies the kinetics of O_2 consumption, and from this curve the initial reaction rate (vi) and the total O_2 uptake were calculated as follows: vi is graphically obtained as the slope at zero time; the total O_2 uptake at the end of the reaction is confirmed in each case by addition of new IAA and HRP in the previously aired media (only addition of IAA, but not that of HRP, restarted the O_2 uptake).

Measure of IAA decarboxylation. The determination of the unreacted IAA was made by the addition of $^{14}C_1$-IAA to the reaction medium. The radioactivity in the medium at different times was measured by liquid scintillation counting of 50 μl aliquots, in a Rack Beta Model 1211. The IAA decarboxylated during the reaction is calculated by subtracting the unreacted IAA from the initial IAA. The scintillation liquid contains 4 g PPO, 0.2 g POPOP, 667 ml toluene and 333 ml Triton X100. The $^{14}C_1$-IAA (48 mCi/mmol) was obtained from Amersham and radiochemical purity was checked by thin layer chromatography.

Results

Kinetics of oxygen uptake. The evolution observed in the oxygen uptake shows a progressive decrease of the reaction rate, irrespective of the concentration of enzyme and substrate assayed by us (fig. 1).

This decrease is not due to limitation by oxygen since addition of new enzyme restores the initial rate. A possible limitation by substrate (IAA) can be judged from the results shown in table I: additions of the new substrate result in increases of the rate of oxygen uptake, as expected, and the results are similar, both in accumulative (in the same reaction medium) or in non-accumulative (additions to different media) conditions, but this effect decreases gradually along time

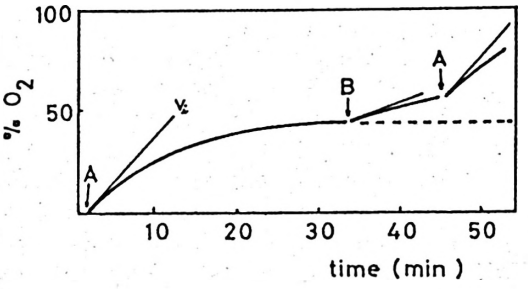

Fig. 1. *Oxygen uptake during enzymatic degradation of IAA.*
The reaction medium (3 ml) contained 0.45 μmol of IAA and 0.63 μmol of O_2. Arrows indicate additions of: (A) 0.93 nmol of HRP and (B) 0.5 μmol of IAA. The dotted line represents the course of the reaction in absence of any addition. The initial rates of O_2 consumption (vi) after each addition are represented. Experiment replicated three times.

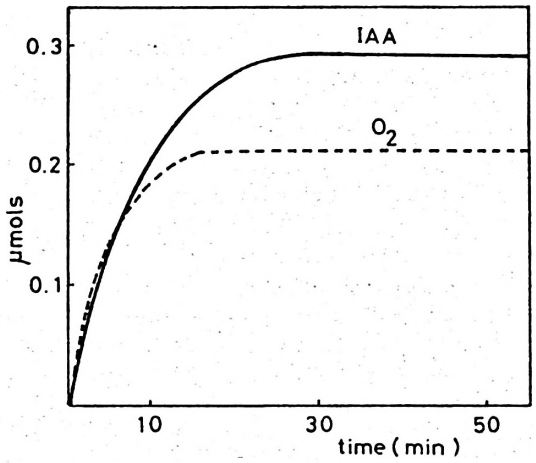

Fig. 2. *Oxygen uptake and IAA decarboxylation during the reaction.*
The concentrations of HRP and IAA were 0.5 µM and 0.1 mM respectively. For the IAA decarboxylation measure, 0.16 µCi of $^{14}C_1$-IAA were added to the medium.

Figure 2 shows that oxygen uptake ceases long before the total IAA decarboxylation is reached. Thus at the end of the reaction there is IAA decarboxylation without simultaneous oxygen uptake.

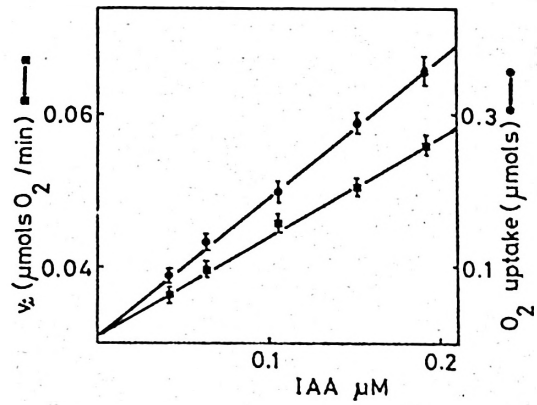

Fig. 3. *Initial rate of reaction (vi) and total O_2 uptake versus IAA concentration.*
HRP remains constant (0.624 µM). The calculations of vi and total O_2 uptake after 35 min of reaction are as expressed in Materials and Methods. The standard errors are presented.

Table I. *Effect of IAA added during the reaction on the oxygen uptake.*
The initial medium is analogous as in figure 1. After different times of reaction (10, 20 and 30 min), 0.45 µmoles of IAA were added either on the same medium (accumulative: A), or on different media (non accumulative: no A). In each case the O_2 consumed during a period of 10 min after the addition, is expressed. The control (C), shows the O_2 consumption every 10 min from the start of reaction. The experiment was repeated three times with additions at different reaction times. The data presented are representative.

Time Interval (min)	O_2 uptake (µmols)		
	A	no A	C
0-10	0.142	0.142	0.142
10-20	0.139	0.139	0.071
20-30	0.101	0.094	0.030
30-40	0.072	0.063	0.007

Influence of the E/S ratio. When the concentration of enzyme is kept constant (fig. 3) a range of initial concentrations of substrate was chosen so that the initial

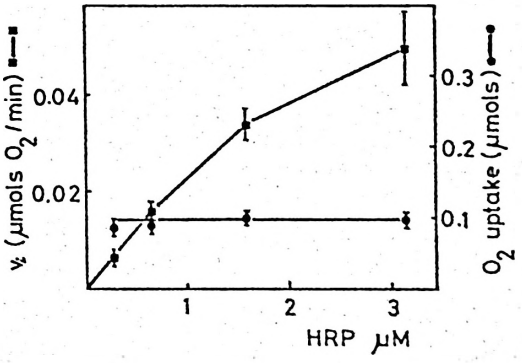

Fig. 4. *Initial rate of reaction (vi) and total O_2 uptake versus HRP concentration.*
IAA is 0.0416 mM in all the media. The calculations of vi and total O_2 uptake are as expressed in Materials and Methods. The end of the reaction (for calculations of total O_2 uptake) depends on the HRP concentration: for 3.12, 1.56 and 0.624 µM the respective times are 7, 14 and 35 min. For 0.25 µM the rate is unappreciable after 20 min. The standard errors are presented.

rate of the reaction varies linearly with it. After 35 minutes all the substrate has been consumed in every case and the total oxygen uptake is always proportional to the initial amount of substrate. In these conditions the mean oxygen consumption is 0.675 mols per mol of IAA.

For a fixed concentration of substrate (fig. 4) the initial rate of reaction is proportional to the concentration of enzyme (except when the latter is high, where the substrate is presumably limiting). At the end of the reaction the amount of oxygen consumed is (in all the cases) independent of the concentration of enzyme and the mean value of O_2/IAA is 0.73.

Discussion

Conditions with no limitation of oxygen (fig. 1), are necessary to evaluate the stoichiometry of the oxygen consumption. On the other hand results in table I show that the decline of the rate at the end of the reaction cannot be explained only in terms of limitation by IAA. If a possible inhibition by products is discarded (1) one must conclude that the enzyme loses part of its catalytic power along the reaction. Some authors (3, 4, 10) demonstrated molecular modification of peroxidase during the oxidation of IAA. At the end of the reaction, however, all the substrate has been oxidized (fig. 2) and therefore the degradation of the enzyme does not modify the stoichiometric calculations.

In a previous paper (2), it was established that the products with a higher level of oxidation (IAld and HMO) cease to be produced and IM accumulates simultaneously, after some minutes of the reaction. The present results show that in the same conditions, at this time of the reaction, the oxygen uptake becomes practically null while the decarboxylation of IAA continues (fig. 2). This implies that the further formation of IM takes place with no parallel consumption of oxygen. NAKAJIMA and YAMAZAKI (9) show when the oxygen uptake stops in their conditions of reaction, about 50 % of the initial IAA is in the form of peroxide and although they do not find IM as a product, they consider that the peroxide may be the precursor of IM.

The relative amounts of the products closely depend on the E/S ratio, as has been recently stated (13) so that when the ratio is high, IM is formed in preference while the production of the more oxidized products severely declines. One should expect, then, that with different E/S ratios, the observed quotient O_2 uptaken/IAA consumed would change according to the following:

$$\text{IAA} + O_2 \rightarrow \text{IAld or oxindoles}$$
$$\text{IAA} + \tfrac{1}{2}\, O_2 \rightarrow \text{IM}$$

If: A = mols of (IAld + oxindoles)
B = mols of IM

then

$$\frac{O_2\ (\text{mols})}{\text{IAA (mols)}} = \frac{A + 0.5\,B}{A + B} = M$$

and hence:

$$\frac{A}{B} = \frac{M - 0.5}{1 - M}$$

Note that very similar values of M (for example, 0.7 and 0.75) would correspond to not so similar values (0.66 and 1.0) of the ratio: more oxidized/less oxidized products.

Even so, the results in figure 3 and 4 (black circles) do not show considerable differences of M (O_2/IAA) for very different E/S ratios. A possible explanation must take account of the fact that, as has been found by us and others, several secondary products are formed in the reaction besides IAld, oxindoles and IM.

The present results can be interpreted according to the reaction mechanism by

NAKAJIMA and YAMAZAKI (9). They proposed for the formation of IM the reaction:

$$Fe_p^{3+} + R'OOH \rightarrow R'OH + Co\text{-}I \quad [1]$$

where R′ stands for the decarboxilated IAA, so R′OH is IM. In their scheme the production of the peroxide is as follows:

$$R\cdot + O_2 \rightarrow R'OO\cdot + CO_2 \quad [2]$$

$$R'OO\cdot + RH \rightarrow R'OOH + R\cdot \quad [3]$$

where RH means IAA.
If, as we found, the formation of IM at the end of the reaction is not accompanied by the uptake of oxygen, one must conclude that, the reactions [2] and [3] take place very slowly when E/S ratio is high.

Regeneration of Fe_p^{3+} in [1] involves the formation of free radicals of IAA:

$$CoI + RH \rightarrow CoII + R\cdot$$
$$CoII + RH \rightarrow Fe_p^{3+} + R\cdot$$

and this explains why IAA, but not oxygen, is consumed (fig. 2), the active forms of the enzyme Co-I and Co-II (and finally the peroxide) being the oxidant, and the products of oxidation, the free radicals of IAA (R ·). These free radicals are now stabilized in ways different from [2] and [3], and that includes decarboxilation (fig. 2). SUZUKI and KAWARADA (17) found reaction products that could be the result of different forms of stabilization of the free radicals. Those products contribute to the final balance of oxidized products and, therefore, to the O_2/IAA ratio.

Resumen

Se estudia la estequiometría del consumo de oxígeno durante la oxidación de ácido indol-3-acético (AIA) por peroxidasa, en condiciones en que no existe limitación por O_2. Al variar la relación Enzima/Sustrato, el cociente O_2 consumido/AIA oxidado no cambia de forma apreciable, manteniéndose siempre inferior a la unidad. No obstante, dicho cociente experimenta variaciones importantes durante la reacción ya que el consumo de oxígeno cesa mucho antes que la descarboxilación de AIA. Los resultados se discuten teniendo en cuenta los mecanismos de reacción previamente propuestos.

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