GENERATION OF ELECTRONIC ENERGY IN THE PEROXIDASE CATALYZED OXIDATION OF INDOLE-3-ACETIC ACID

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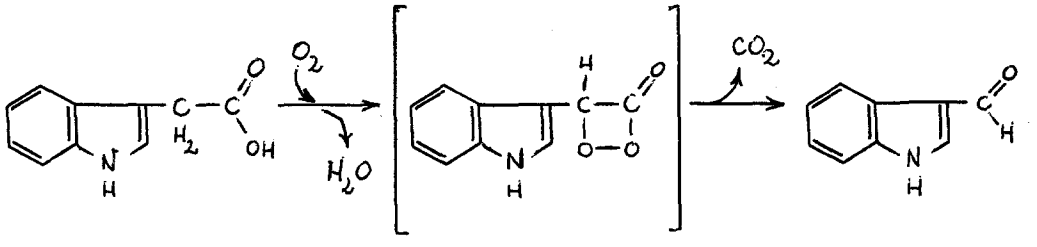
SUMMARY: The horseradish peroxidase-catalyzed aerobic oxidation of indol-3-acetate results in the conversion of the enzyme to a green form. The spectral changes observed after the oxidation of small amounts of substrate may be reproduced approximately by irradiation of the enzyme at 290 nm. The possibility that electronic energy is generated in the indoleacetate/peroxidase/O_2 system, and is responsible for the change is supported by the chemiluminescence, albeit very weak, observed in the presence of sodium 9,10-dibromoanthracene-2-sulfonate, and of eosin. It appears that the chemienergized species is indol-3-aldehyde in its triplet state.

The possible formation of non-emissive excited eletronic states in biochemical systems is under investigation in this laboratory (1-6). One such potential system pointed out (6) is the peroxidase catalyzed oxidation of the auxin IAA by molecular oxygen (7). In proper conditions, the products formed are as expected from the cleavage of a dioxetane intermediate, namely IA and CO_2, allowing the aldehyde to be generated in an electronically excited state (8).



Abbreviations: HRP, horseradish peroxidase; IAA, indole-3-acetic acid; IA, indole-3-carboxaldehyde; ANSA, 8-anilino-1-naphtalene-sulfonic acid, ammonium salt.

We report here the results of an investigation of this system by the "self-damage" and by the "leakage" approach. Both strongly suggest that an electronically excited product is generated in the IAA/HRP/O_2 system.

Materials and Methods

HRP and deuterium oxide (99,8%) were obtained from Sigma Chemical Company. IAA from BDH Ltd. was recrystallized from water. Superoxide dismutase was obtained as described by McCord and Fridovich (9). The sodium salts of anthracene-2-sulfonic acid and of its 9,10-dibromo derivative were prepared by standard methods. Eosin was from Merck.

The reactions were run in a spectrophotometric cell at 20°C; the conditions employed being essentially those used by Yamazaki, Ohishi and Yamazaki (10). The buffer was 0.05M acetate, pH 4.0, and the peroxidase concentration, 1.6×10^{-5}M. The reaction was started by adding IAA. The concentration of the latter was 1.0×10^{-4}M in the spectrophotometric studies, and 4.0×10^{-4}M for chemiluminescence measurements.

Absorption spectra were taken on a Zeiss DMR-21 Recording Spectrophotometer using 1 cm. cells. Chemiluminescence was measured on an Aminco Bowman Spectrophotofluorometer with the exciting source off.

HRP was irradiated with 290 nm light with a 150 watt xenon lamp at 90 cm distance.

RESULTS

Self-damage approach. AS a result of the reaction it catalyzes, the enzyme HRP becomes spectrally altered (10), the changes being greater at pH 4.0 than at pH 5.0. The alteration might have been induced by an electronically excited product or by its electronic

energy. Therefore we tested whether the spectral changes might be induced simply by irradiation of the peroxidase with UV light. By such an approach it had been inferred in an earlier work (4) that excited methylglyoxal is generated in the myoglobin-catalyzed aerobic oxidation of acetoacetate. Also in the IAA/HRP/O_2 system now investigated the spectral changes produced as a result of reaction of small amounts of substrate are similar to those produced by ultraviolet irradiation (Fig. 1). The similarity is remarkable considering that the spectrum of the transformed enzyme is affected by the presence of products. Light of 480 nm was ineffective in altering the peroxidase.

That the alteration of peroxidase during the reaction was not due to IAA or to IA or to possible intermediate radicals (OH$^{\cdot}$ and $HO_2^{\cdot}/O_2^{-}$) is indicated by the following facts: (i) anaerobic incubation of HRP with IAA did not affect the enzyme (ii) IA did not alter HRP (iii) the OH$^{\cdot}$ radical scavengers, benzoate (11) and formate (12), at 5×10^{-3} and 0.01M respectively, did not protect the enzyme (iv) 3.3×10^{-7}M superoxide dismutase (9) had hardly any effect upon the system.

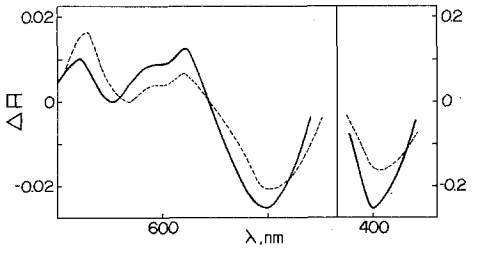


Figure 1. Differential spectra of horseradish peroxidase at pH 4.0 relatively to a freshly prepared solution: ——— after 2.0 hours irradiation at 290 nm; - - - - after catalyzing the aerobic oxidation of IAA.

To investigate the possibility that the electronic energy would be transferred to oxygen with formation of $^1\Delta_g$ singlet oxygen, which would then attack the enzyme, the reaction has also been studied in D_2O. In D_2O the lifetime of $^1\Delta_g$ $^1O_2$ is increased by one order of magnitude (13) so that greater damage might be expected to occur. It has been found that the spectral changes are approximately the same in H_2O and D_2O.

The leakage approach. Even if an excited singlet state is non-emissive, it is conceivable and widely believed that a very small part of the chemically generated electronic energy may leak out in the form of radiation. Therefore we looked for low level emissions. We also used sensitizers because of the possibility of energy transfer, even from the triplet state, if the acceptor has heavy atoms (14-16). No emission could be detected in the 335 nm region where IA was found to fluoresce maximally; nor was any emission detected at 490 or at 525 nm in the presence of the hydrophobic probe ANSA. A very weak but definite emission occurred in the presence of sodium 9,10-dibromo-anthracene-2-sulfonate in the 440-450 nm, region, where this sensitizer fluoresces maximally (λ_{max} = 445 nm). A representative example appears in Fig. 2. Emission could also be detected at 575 nm in the presence of eosin (Fig. 2). No emission occurred in the presence of 1×10^{-4}M anthracene-2-sulfonate (λ_{max} = 410 nm) or of 4×10^{-4}M riboflavin (λ_{max} = 525 nm).

DISCUSSION

Our results strongly suggest that a product of the HRP catalyzed oxidation of IAA is formed in an electronically excited state and transfers the energy to the enzyme. The chemienergized product

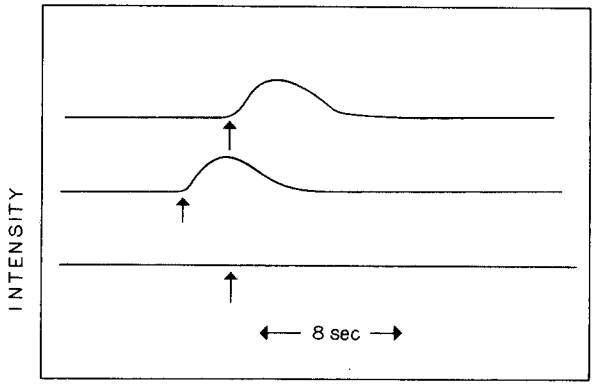


Figure 2. Light emission by the IAA/HRP/0_2 system. Upper curve; in the presence of sodium 9,10-dibromoanthracene-2-sulfonate, approximately 3.0×10^{-5}M. Middle curve; in the presence of eosin, 4.0×10^{-4}M. Lower curve: no sensitizer added. The arrows denote the addition of IAA.

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is presumably IA; it is unlikely that it is the other expected product, methylenoxindole. It is true that in the system under investigation products expected from a dioxygenase type cleavage of the indole nucleus may also be formed (17). However although this cleavage may also involve a dioxetane intermediate, and therefore might generate an excited product (8), it only occurs to a limited extent (17).

Our results are consistent with, but do not prove, that the chemienergized species is IA. Evidence in this regard has been obtained from studies with model systems to be reported later. These studies clearly show that properly substituted indoleacetic acid and non indolic auxins, produce the electronically excited aldehyde in aprotic solvents.

In view of the fact that the 9,10-dibromoanthracenesulfonate sensitizes the chemiluminescence whereas the anthracenesulfonate

does not, it is very possible that the dibromoderivative receives energy from IA triplets and not from singlets. IA phosphoresces maximally at 435 nm (18) and therefore the transfer is energetically feasible. The inference of triplet-singlet energy transfer is strongly reinforced by two facts. First, that eosin -a molecule containing heavy atoms- also allowed emission to occur. Second, and not less important, is that IA shows a ϕ_p / ϕ_f ratio which is unusually high for an indole compound (18).

The observed energy transfer from enzymically generated triplet species to the excited singlet of an acceptor with heavy atoms is of considerable interest in connection with the hypothesis that thyroid hormones may promote energy transfer from biochemically generated triplet species to the excited singlet of the hormone (1) or more likely, to the excited singlet of the hormone receptor (6).

The generation of eletronic energy during the enzyme-catalyzed aerobic oxidation of IAA is of obvious interest in connection with a possible role of such energy in the growth--promoting activity of auxins.

It is interesting that in some other systems also, peroxidases lead to products expected from the cleavage of a dioxetane intermediate; and therefore, at least potentially, to generation of electronic energy. Thus from aromatic pyruvates, oxalate and the corresponding aromatic aldehyde are generated (19, 20); from aromatic derivatives of acetaldehyde (21-23), formic acid and the aromatic aldehyde are generated. These considerations coupled with the fact that luciferase enzymes are often peroxidases, strengthen the inference that, apart from emission, the IAA/HRP/O_2 system is similar to bioluminescent systems. Here as in the case of the acetoacetate/myoglobin/O_2 reaction (4) conditions are appropriate for "photochemistry without light" (24) as revealed by self-damage.

The question arises -and is under investigation- if such self-damage might also be responsible for certain other cases of hemeprotein alterations as a result of the reaction in which they participate.

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