PEROXIDASE CATALYZED ELECTRONIC EXCITATIONS IN BRASIL AND CANADA: ISOBUTYRALDEYDE AND INDOLE-ACETIC ACID. THE IMPORTANCE OF THE INITIATION STEP

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The importance is emphasized of understanding the initiation step in free radical chain reactions, including those involving electronic excitation. Three examples are discussed in detail. The first is the reaction of isobutyraldehyde with molecular oxygen, catalyzed by horseradish peroxidase, in which either compound I formation or the rate of conversion of the keto to enol form of isobutyraldehyde is rate limiting, depending on the available supply of hydroperoxyl oxidant. The second example is the reaction of the plant growth hormone indole-3-acetic acid with molecular oxygen, again catalyzed by horseradish peroxidase. Available evidence appears to favor a rate-controlling step in which a trace of the iron in the enzyme is reduced from iron(III) to iron(II) by the indole-3-acetic acid. These horseradish peroxidase-catalyzed reactions have been studied in collaboration with the group of Prof. Cilento in São Paulo. The importance of understanding, and indeed addressing the question as to what is, the initiation step is also discussed in terms of the evolution of the present understanding of iron-catalyzed oxidative damage caused by species derived from hydrogen peroxide.

Keywords: horseradish peroxidase; isobutyraldehyde; indole-3-acetic acid; chemiexcitation; peroxidase kinetics.

INTRODUCTION

I shall summarize in a somewhat personal account, appropriate in a dedication to an esteemed colleague and dear friend, some of the highlights of a research collaboration which has extended over a period of 16 years on the mechanisms of light emitting reactions catalyzed by HRP,[1] and discuss some implications of the work relevant to other areas.

Collaborative research is at its best, in my view, when two groups have overlapping interests and complementary experimental approaches. Then it is possible to reach certain goals which would be difficult, if not impossible, for either group by itself.

The seeds of the collaboration were sown in 1976 when Giuseppe Cilento visited the laboratory of Karl Kopecky in the Department of Chemistry at the University of Alberta. Kopecky and Mumford were the first to synthesize a dioxetane by use of reactants which are not electronically excited[1]. Dioxetane synthesis using singlet oxygen was accomplished by Bartlett and Schaap[2]. Joe Cilento presented a seminar at the University of Alberta on his research and described reactions of HRP in which light is emitted. I had been working on the kinetics and mechanism of HRP-catalyzed reactions since 1965, and I had never heard of such a thing! My error, and I set about to correct and compensate. In 1977 I was in Joe Cilento's laboratory in São Paulo, again in 1984 and again in 1991. (The *Paulistas* say that I come back every seven years to eat another *feijoada*.) Let me describe what happened.

At first, not very much. A clerk in the Canadian capital city, Ottawa, sent applications for the Natural Sciences and Engineering Research Council of Canada exchange program with Brasil to Moscow, and with Russia to Brasilia. When I finally arrived in São Paulo, we conducted some preliminary experiments. Work on the isobutyraldehyde reaction with horseradish peroxidase was still in its infancy[3,4], and in the six-week period I was present in 1977, we did not obtain publishable results. However, contact was established, and we did not give up. Details follow on what was accomplished and the relevance of the work.

EXPERIMENTAL

The principal techniques used in São Paulo were photon counting and measurements of oxygen consumption. In Edmonton measurements were routinely made on the millisecond time scale, either rapid spectral scans or kinetic measurements at a single wavelength, using stopped flow equipment. The kinetic measurements were either in the transient state, looking at an elementary reaction, part of the overall reaction cycle, or in the steady state. There is an advantage in using a stopped flow apparatus for steady state measurements since the dead time for the experiments is reduced 1000-fold over that obtained using a conventional spectrophotometer.

My return to São Paulo in 1984 was the start of one of the most exciting, memorable collaborations in my career. Joe Cilento had a graduate student, Cornelia Bohne, and a postdoctoral fellow, Willi Baader, waiting to work with me. Languages in daily use were English, Portuguese and German, with my contributions in only one, to my embarrassment.

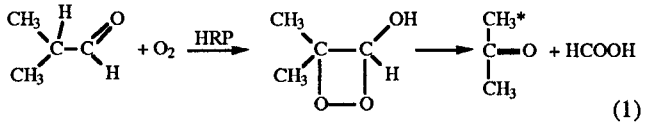
In my 1991 visit to São Paulo, Mariza Pires de Melo and Jorge Escobar were awaiting my arrival. The languages of choice were English, Portuguese and Spanish. This time a much tougher problem was tackled and as a result our results were not so definitive. There was one big improvement over 1984: Fax machines were now available. Almost immediately I involved Diana Metodiewa in my laboratory in Edmonton in the same project; and thanks in part to Fax transmission, two laboratories on opposite sides of the world were merged into one.

RESULTS

Isobutyraldehyde

Over the period 1977-1984 a great deal was learned by the Cilento group about the reaction of IBAL with HRP[5-7]. In this amazing reaction, all that is required is to mix the peroxidase with the aldehyde in an aerated solution, and light emission can be observed for periods up to ten minutes! The emission spectrum was shown to be that of triplet state acetone, a re-

sult that some said was impossible. The triplet state was thought too be short-lived, too readily quenched, to emit light. The conclusion that emission occurs from triplet state acetone is correct. Acetone and formic acid are the principal products.



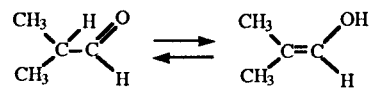
(1)

In 1984 we decided to tackle the related problems of the mechanism of the initiation reaction and the mechanism of participation of the HRP intermediate compounds, which exist in a higher oxidation state than that of the native enzyme.

A crucial experiment was performed by Cornelia Bohne. Aldehydes are very readily autoxidized[8], and if at any point in the purification procedure, even a trace of air comes in contact with the sample, some oxidation product is formed. She demonstrated that with ultra-pure IBAL, upon mixing with an aerated peroxidase solution at 35°C, no immediate reaction occurred. Using samples of this degree of purity, we demonstrated that immediate light emission occurred upon addition of hydrogen peroxide. Furthermore, trace autoxidation of IBAL, prior to mixing with peroxidase, could sustain light emission for some time. The IBAL is present in very large excess compared to the enzyme, so even a trace of autoxidation product is still in excess of the enzyme. Thus HRP-I formation, formed by the reaction of HRP either with the peracid autoxidation product of IBAL, or with added hydrogen peroxide, is an obligatory step in the reaction.

(2)

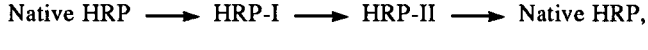
Keto compounds are not natural substrates for peroxidases, but phenols are. It appeared likely that the enol form of IBAL, in analogy with a phenol, is reactive, and this turned out to be the case. Under conditions where sufficient oxidant is present to maintain a steady-state concentration of compound I, the formation of the enol form of IBAL is rate-limiting[9,10].



(3)

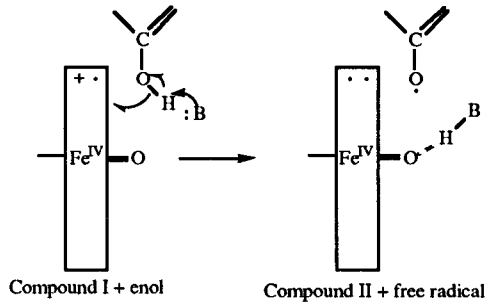
The hydrogen atom (electron plus proton) from the hydroxyl group of the enol is donated to compound I reducing it to compound II. The electron is transferred to the porphyrin π cation radical and the proton to a distal basic group in the active site.

The stage was now set to measure some rate constants, and to examine whether a normal peroxidatic cycle occurred. Cornelia Bohne, still a graduate student, came to Edmonton in 1986 for a five-month period, where she worked with one of my graduate students, David MacDonald. We were able to use the transient state reaction of HRP-I with various aldehydes as a means of obtaining the phosphate-catalyzed keto-enol equilibrium constant and rate constants for both the forward and reverse reactions[11]. Furthermore we demonstrated that both compounds I and II react with the enol form of isobutyraldehyde to generate triplet acetone. Peroxidase reacts via a normal cycle native



and not by a shuttle mechanism involving oxidation of compound II to I[12].

With [HRP-I] in excess of [enol] an initial burst in HRP-I



(4)

disappearance was observed, in which the equilibrium concentration of enol is consumed. This is followed by a steady state phase, in which keto-enol conversion is the rate determining step. Thus we made a prediction that there should be an initial burst in light emission from the enol form of IBAL, which had not been detected with the photon counter in São Paulo because the dead time was too long. We had sophisticated instruments available in Edmonton, however: human eyes! Three in my group waited in total darkness until their eyes were adapted. Stable solutions of pre-formed HRP-I and IBAL were mixed, and not only the initial burst of light but the subsequent steady state light emission were observed. The eyeball-IBAL experiment was a total success!

Indole-3-Acetic Acid

IAA is a plant growth hormone. It is an example of a class of peroxidase substrates which, like IBAL, consumes molecular oxygen, thus making it appear that peroxidase is an oxygenase. In the case of IBAL, both Cilento's group and our combined groups have shown clearly that the so-called oxygenase activity is a result of scavenging of free radicals by molecular oxygen. Furthermore the free radicals are produced in the course of a normal peroxidatic cycle involving compounds I and II. Thus the "oxygenase" activity does not directly involve the enzyme but consists of reactions of molecular oxygen with products of the enzyme reaction. It appeared likely that the "oxygenase" activity displayed by HRP in its reaction with IAA also consisted, in part at least, of free radical scavenging by oxygen[13]. However does the mechanism of enzyme action involve a normal peroxidase cycle[14], or an abnormal cycle[15], or some combination of both[16]? All three answers have been claimed by three different groups, none of whom studied the light emission. If one summarizes the research of Joe Cilento's group in a simple statement it is light emission and electronic excitation. His group had studied the IAA light-emitting reaction intensively[17], and we agreed to attempt to unravel mechanistic details of peroxidase involvement.

Scrupulous purification of IAA to eliminate any trace of autoxidation product did not change the pattern of light emission and oxygen uptake, which although it was negative evidence, pointed strongly to an initiation step other than compound I formation. There is consensus that peroxidase, in which the iron is reduced from the ferric to ferrous state, Fe^{II}-HRP, is formed during the course of the reaction[14-16]. There is not consensus about whether the reduction can occur by IAA itself or only from a free radical derived from IAA[14].

I do not regard the very careful but complicated anaerobic experiments using glucose and glucose oxidase or ascorbate and ascorbate oxidase, plus catalase, plus carbon monoxide, plus IAA, plus HRP, as definitive evidence against native HRP reduction by IAA[14]. A trace of oxygen would be converted to hydrogen peroxide which could react with both HRP and catalase. Reduction of HRP and/or catalase by IAA to ferrous form(s) should lead to trapping by carbon monoxide, but is this going to occur in the presence of oxidized forms of one

or two enzymes? Carbon monoxide binding is a reversible process and combined with the reversible equilibrium between Fe^{II}-HRP and native HRP in the presence of IAA, detection of the CO adduct may be impossible. Upon removal of molecular oxygen the mechanism of sustaining the HRP-IAA reaction is removed. Therefore the absence of a CO adduct in a strict (but complicated) anaerobic system, compared to the ready trapping of the CO complex in a steady state aerobic reaction[14,16] may not have significance with regards to the initiation step. It is fair to say that these anaerobic experiments provide negative evidence for direct reduction of HRP by IAA.

The equilibrium between Fe^{II}-HRP and native HRP is undoubtedly real, according to the laws of thermodynamics, and it is undoubtedly shifted far in favour of the native enzyme. The question remains: does it provide the initiation step for a very complicated reaction? With due respect for an esteemed colleague[14], I believe it does. The initiation step remains unproven, and may some bright, skilled experimentalist provide a definitive answer.

I have concentrated on the initiation step in great length because I regard it of paramount importance. Our combined Brasil-Canada results on the very important IAA-peroxidase reaction have been published[18,19], and while we have not provided definitive answers, we have added new evidence to a large body of literature. A capsule summary: there is no doubt that compounds I, II and III, Fe^{II}-HRP, superoxide, singlet oxygen, hydrogen peroxide and IAA-derived free radicals are intermediates in the overall peroxidase-oxidase reaction with IAA. The light emission reactions are not part of the main reaction pathway as is light emission in the IBAL-peroxidase reaction. The skatole radical is not a primary product[20]; it is not a source of light emission[21].

DISCUSSION

Initiation (and termination) reactions are of crucial importance in free radical chain reactions. The length of the chain, the number of times the cycle is repeated, is enhanced by an efficient initiation step and diminished by efficient termination steps. The focus on initiation reactions will now be shifted to the topic of iron-catalyzed oxidative damage, and its prevention, in biological systems. This research area is so vast that a comprehensive, concise summary is not possible. I shall focus on certain aspects of oxidative damage by superoxide, hydroperoxyl and hydroxyl radicals and their precursors, and certain aspects of prevention of oxidative damage.

The discovery of an enzyme function for the metalloprotein formerly known as erythrocuprein[22] caused great excitement and stimulated a new era of research on oxidative biological damage. Work stimulated by the discovery of superoxide dismutase activity also led to areas of controversy which unfortunately sometimes led to bad feelings. As a scientist raised in the culture of chemistry, but immersed in aspects of the culture of biochemistry for nearly 30 years, I believe that I may be able to offer a few germaine comments.

At first it was thought that superoxide, or its protonated form the hydroperoxyl radical, was the direct cause of oxidative damage. First it was pointed out that superoxide dismutes spontaneously at a rapid rate, which is fastest (rate constant of $2 \times 10^7\ M^{-1}s^{-1}$) in the vicinity of pH 5[23].

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (5)$$

However, superoxide dismutase does provide an increase in the dismutation rate constant to about $10^9\ M^{-1}s^{-1}$ [24].

Second it was pointed out that superoxide is not an efficient oxidizing agent. Attention then shifted to a reaction first proposed when free radical chemistry was still in its infancy[25], the Haber-Weiss reaction

$$O_2^{\cdot-} + H_2O_2 + H^+ \longrightarrow {}^{\cdot}OH + H_2O + O_2 \qquad (6)$$

Note that a stable molecule, hydrogen peroxide, is now involved in addition to free radicals. It was felt that if superoxide itself were not so harmful, its removal was still required because otherwise the hydroxyl radical could be generated. There is universal agreement that hydroxyl radicals are among the most reactive chemical species which are not electronically excited. Hydroxyl radicals will abstract a hydrogen atom from any available source; they will diffuse only a few molecular diameters before reacting. Unfortunately the Haber-Weiss reaction does not occur[26]! The reaction which does occur is the following

$$^{\cdot}OH + H_2O_2 \longrightarrow HO_2^{\cdot} + H_2O \qquad (7)$$

Thus the very reactive hydroxyl radical generates the much less reactive protonated superoxide radical, the hydroperoxyl radical, in its reaction with hydrogen peroxide.

The next hypothesis was called the iron-catalyzed Haber-Weiss reaction. Two of the elementary reactions in the ferric ion-catalyzed decomposition of hydrogen peroxide are:

$$Fe^{3+} + HO_2^{\cdot} \longrightarrow Fe^{2+} + O_2 + H^+ \qquad (8)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3}+ + {}^{\cdot}OH + OH^- \qquad (9)$$

$$HO_2^{\cdot} + H_2O_2 \longrightarrow {}^{\cdot}OH + O_2 + H_2O \qquad (10)$$

I pointed out that although the sum of the two reactions in eqs 8 and 9 do add up to the Haber-Weiss reaction, it is incorrect to consider only two elementary reactions out of an overall reaction mechanism, and to ignore other elementary steps, including initiation and termination[27]. It is correct to discuss the entire mechanism of the ferric ion-catalyzed decomposition of hydrogen peroxide, but note the transformation: now one is discussing the decomposition of a stable molecule, hydrogen peroxide, which leads to free radical products. I am further gratified to see that instead of using only part of the iron(III)-catalyzed decomposition of hydrogen peroxide, there is increasing use of the term "Fenton chemistry"[28] which refers to the ferrous ion-catalyzed decomposition of hydrogen peroxide. Note that eq 9 shows the first step of the Fenton reaction in which a hydroxyl radical is formed from hydrogen peroxide, and the superoxide anion is nowhere to be seen.

Thus there was a cultural clash, in which biochemists were acutely aware of the overwhelming evidence for iron-catalyzed (and copper-catalyzed) oxidative damage and they were searching for explanations. Chemists were saying get the chemistry right, a remark not widely appreciated by a group doing their best in research on complicated systems. The integrity on both sides was unquestionable, and from an objective viewpoint one can see that some controversy was inevitable. One hopes that the two cultures are now so intertwined in this area of research that there will be fewer future misunderstandings in the future.

There are many substances present in living tissue which can reduce iron(III) to iron(II); and the emphasis now appears to center on Fenton chemistry. Hydrogen peroxide by itself may not be highly reactive, but in the presence of a metal ion catalyst it is. It should not be ignored as a potential primary source of oxidative damage.

Two very recent, important articles discuss feasible, detailed, but incomplete mechanisms for the harmful role of superoxide in oxidative chain reactions[29,30]. Initiation steps are ignored. Free radicals originate from stable molecules, which may be the product of abnormal reactions occurring *in vivo*.

Identifying these obnoxious stable precursors may pave the way for a new era of preventive medicine.

SUMMARY AND ACKNOWLEDGEMENT

Free radical oxidative damage should not be discussed solely in terms of elementary reactions which do not include the initiation and termination steps. I have offered three examples where an understanding of the initiation step is crucial to an understanding of the overall mechanism: the reactions of isobutyraldehyde and indole-3-acetic acid with peroxidase, in which light emission is observed, and the iron-catalyzed decomposition of hydrogen peroxide. Work in the area of biological oxidative damage, involving molecules in their electronic ground states, is now entering a mature phase. Thanks in large measure to the pioneering work of Joe Cilento, work on the mechanisms of oxidative damage involving electronically excited molecules is well underway. I am grateful to Joe Cilento for introducing me to the latter area of research, and for a wonderful collaboration spanning 16 years. *Muito obrigado!*

ABBREVIATIONS

HRP horseradish peroxidase; HRP-I, HRP-II and HRP-III compounds I, II and III of HRP; Fe^{II}-HRP, HRP reduced to its ferrous form; IBAL isobutyraldehyde; IAA indole-3-acetic acid.

REFERENCES

1. Kopecky, K. R. and Mumford, C.; *Can. J. Chem.* (1969), **47,** 709.
2. Bartlett, P. D. and Schaap, A. P.; *J. Am. Chem. Soc.* (1970), **92,** 3223.
3. Duran, N.; Faria Oliveira, O. M. M.; Haun, M. and Cilento, G.; *J. Chem. Soc. Chem. Commun.* (1977), **13,** 442.
4. Faria Oliveira, O. M. M.; Haun, M.; Duran, N.; O'Brien, P. J.; O'Brien, C. R.; Bechara, E. J. H. and Cilento, G.; *J. Biol. Chem.* (1978), **253,** 4704.
5. Bechara, E. J. H.; Faria Oliveira, O. M. M.; Duran, N.; Casadei de Baptista, R. and Cilento, G.; *Photochem. Photobiol.* (1979), **30,** 101.
6. Cilento, G.; *Acc. Chem. Res.* (1980), **13,** 225.
7. Rivas-Suárez, E. and Cilento, G.; *Biochemistry* (1981), **20,** 7329.
8. Zhaikov, G. E.; Howard, J. A. and Ingold, K. U.; *Can. J. Chem.* (1969), **47,** 3017.
9. Dunford, H. B.; Baader, W. J.; Bohne, C. and Cilento, G.; *Biochem. Biophys. Res. Commun.* (1984), **122,** 28.
10. Baader, W. J.; Bohne, C.; Cilento, G. and Dunford, H. B.; *J. Biol. Chem.* (1985), **260,** 10217.
11. Bohne, C.; MacDonald, I. D. and Dunford, H. B.;*J. Am. Chem. Soc.* (1986), **108,** 7867.
12. Bohne, C.; MacDonald, I. D. and Dunford, H. B.; *J. Biol. Chem.* (1987), **262,** 3572.
13. Campa, A.; Salim-Hanna, M. and Cilento, G.; *Photochem. Photobiol.* (1989), **49,** 349.
14. Nakajima, R. and Yamazaki, I.; *J. Biol. Chem.* (1979), **254,** 872.
15. Ricard, J. and Job, D.; *Eur. J. Biochem.* (1974), **44,** 359.
16. Smith, A. M.; Morrison, W. L. and Milham, P. J.; *Biochemistry* (1982), **21,** 4414.
17. Campa, A.; in *Peroxidases in Chemistry in Biology,* (1991), Vol. II, Chapt. 2 (Everse, J.; Everse, K. E. and Grisham, M. B. eds.) CRC Press, Boca Raton, Florida, pp 25-50.
18. Metodiewa, D.; Pires de Melo, M.; Escobar, J. A.; Cilento, G. and Dunford, H. B.; *Arch. Biochem. Biophys.* (1992), **296,** 27.
19. Pires de Melo, M.; Escobar, J. A.; Metodiewa, D. and Dunford, H. B.; *Arch. Biochem. Biophys.* (1992), **296.**
20. Mottley, C. and Mason, R.; *J. Biol. Chem.* (1986), **261,** 860.
21. Escobar, J. A.; Vásquez-Vivar, J. and Cilento, G.; *Photochem. Photobiol.* (1992), **55,** 895.
22. McCord, J. and Fridovich, I.; *J. Biol. Chem.* (1969), **244,** 6049.
23. Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L. and Ross, A. B.; *J. Phys. Chem. Ref. Data* (1985), **14,** 1041.
24. Fridovich, I.; *Adv. Enzymol.* (1986), **58,** 61.
25. Haber, F. and Weiss, J.; *Proc. Royal Soc. (London)* (1934), **A147,** 332.
26. Barb, W. G.; Baxendale, J. H.; George, P. and Hargrave, K. R.; *Trans. Faraday Soc.* (1957), **47,** 462, 591.
27. Dunford, H. B.; *Free Radical Biol. Med.* (1987), **3,** 405.
28. Fenton, H. J. H.; *J. Chem. Soc.* (1894), 899.
29. Winterbourn, C. C.; *Free Radical Biol. Med.* (1993), **14,** 85.
30. Koppenol, W. H.; *Free Radical Biol. Med.* (1993), **14,** 91.

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