Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 878-885

AMBIGUITY ASSOCIATED WITH USE OF SINGLET OXYGEN TRAPPING AGENTS IN MYELOPEROXIDASE-CATALYZED OXIDATIONS

Andrew M. Held and James K. Hurst

Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, 19600 N. W. Walker Rd., Beaverton, Oregon 97005.

Received February 10,1978

SUMMARY: It is shown that hypochlorous acid preferentially oxidizes 2,5-dimethylfuran, histidine, β-carotene and 1,4-diazabicyclo[2.2.2]octane in the presence of hydrogen peroxide without intermediary formation of singlet-excited molecular oxygen. It is therefore highly unlikely that the protective action of these compounds towards myeloperoxidase-catalyzed chlorination reactions is due to singlet oxygen deactivation or removal, and putative evidence based upon these effects for singlet oxygen participation in bactericidal reactions of myeloperoxidase-containing leukocytes is equivocal.

INTRODUCTION:

The sheer complexity of oxidation occurring in many biological organelles renders direct identification of discrete reaction mechanisms exceedingly difficult. One useful indirect approach to the problem comprises addition of exogenous agents capable of reacting with transitory intermediates which might be generated in the overall oxidation process. From observed modifications in reactivity the existence of particular reaction species and, hence, the significant involvement of biological reactions which produce them, can often be inferred. Thus, e.g., in polymorphonuclear leukocytes (1), this sort of evidence has been obtained which is compatible with the participation of superoxide anion (2-4), hydrogen peroxide (3,5), hydroxyl radical (3,6), singlet molecular oxygen (7-9) and hypochlorous acid (10-12) in post-phagocytic reactions. The underlying premise of the method is that the trapping reactions are selective for the intermediate; when the chemical reactivity of the probes is not well understood (13-15), the analysis is prone to error.

ABBREVIATIONS USED: DMFu 2,5-dimethylfuran; $^1\Delta O_2$, dioxygen in its first-excited singlet state; DABCO, 1,4-diazabicyclo[2.2.2]octane.

0006-291X/78/0813-0878$01.00/0

Singlet oxygen formation has been proposed in the myeloperoxidase-catalyzed peroxidation of chloride ion from observations of chemiluminescence (16) and chemical trapping experiments (17). Specifically, in the latter studies, oxidation of 2,5-diphenylfuran gave cis-dibenzoylethylene, the same product as that obtained from reaction with $^1\Delta O_2$; the reaction was inhibited by singlet-oxygen quenchers and showed an inverse solvent deuterium isotope effect, as expected from the increased lifetime of $^1\Delta O_2$ in this medium. Because parallel reaction behavior was observed with hypochlorous acid in the absence of myeloperoxidase and hydrogen peroxide, the authors postulated that HOCl also decomposes spontaneously to form singlet oxygen which undergoes subsequent reaction in normal fashion.

In this communication, we demonstrate that oxidative reactions of HOCl with organic substrates do not proceed by intermediary formation of molecular oxygen. Based upon these observations, our recent kinetic studies of HOCl-H_2O_2 redox reactions (18) and a relatively sparsely documented literature, we propose an alternative explanation of the dynamic behavior of the myeloperoxidase-H_2O_2-Cl^- system which does not require obligatory participation of singlet oxygen. Implications towards the bactericidal action of myeloperoxidase-containing phagosomal particles are discussed.

EXPERIMENTAL:

Materials. Hypochlorite solutions were prepared by reaction of Cl_2 with HgO (19) or by vacuum distillation at 40° of commercial 5% hypochlorite solutions acidified to pH 6 with phosphoric acid. Commercial 2,5-dimethylfuran (DMFu) was purified by column chromatography over alumina. Other chemicals were reagent grade and used without further purification; β-carotene used was Sigma Type IV. Water was purified by reverse osmosis-ion exchange chromatography.

Methods. Oxygen concentrations were measured polarographically using a Clark-type electrode (Yellow Springs Instrument Co., Model YS-4004). The reactions of HOCl with H_2O_2 and DMFu were studied using either a Gibson-Durrum instrument or a variable speed drive assembly coupled to 0.1-1.0 optical cells mounted in a Cary 16 recording spectrophotometer. Details of the instrumentation and procedures have been published (18).

RESULTS:

Kinetic Summary of HOCl Oxidations of H_2O_2 and 2,5-Dimethylfuran. The

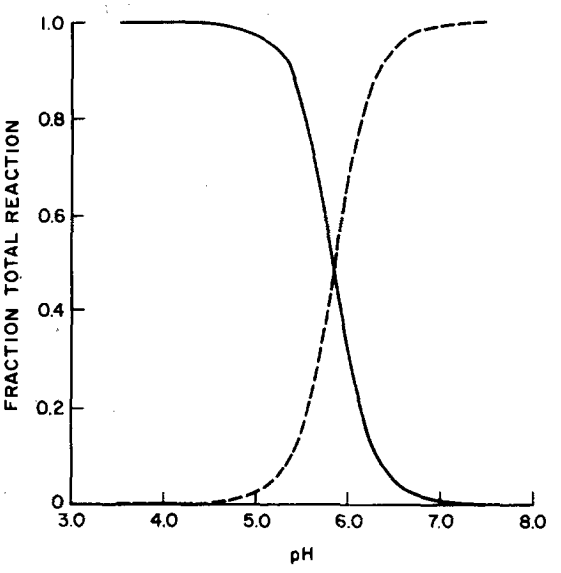

Figure 1: Relative rates of H_2O_2 oxidation by HOCl by the various pathways. Conditions: (Cl^-) = 80 mM, (H_2O_2) = 0.6 mM, 37°. Solid line, k_2-pathway; dashed line, k_3-pathway; the k_1-pathway (not shown) is 0.4% of the k_2-pathway over this pH-range; rates are extrapolated from data in ref. 18. At lower (H_2O_2), the breakpoint in the curves is shifted towards higher pH values.

following points from our study (18) of the reaction are germane to the biological reactivity of HOCl:

[1] Oxidation of H_2O_2 by HOCl proceeds by three concurrent pathways, whose rate laws are of the forms: $d(O_2)/dt = k_1(H_2O_2)(Cl_2) + k_2(HOCl)(H^+)(Cl^-) + k_3(HO_2^-)(HOCl)$. Relative rates by the various pathways under conditions approximating those found in phagosomes of neutrophilic leukocytes are plotted as a function of pH in Figure 1.

[2] The rate law for the k_2-pathway is identical to that for formation of molecular chlorine; nonetheless, kinetic analysis reveals that reaction does not proceed by rate-limiting conversion to Cl_2, and must therefore involve reaction of H_2O_2 with an intermediate species, $[H_2OCl_2]$, whose formation is rate-limiting.

[3] Reaction by the k_3-pathway gives nearly quantitative formation of $^1\Delta O_2$ as determined by reaction with the acceptor molecule, 2,5-dimethylfuran (DMFu). Singlet oxygen yields could not be determined for the other pathways because

direct oxidation of DMFu by HOCl is prohibitively rapid under these conditions.

[4] Oxidation of DMFu by HOCl in weakly acidic solutions is given by the rate law: $-d(DMFu)/dt = [k_4 + k_5(Cl^-)](HOCl)(H^+)$. The term for the chloride-dependent pathway is identical to that for the k_2 pathway in the H_2O_2-HOCl reaction, i.e., $k_2 = k_5$, implying that the intermediate $[H_2OCl_2]$ is common to the two reactions. In solutions containing $(Cl^-) \simeq 0.1M$, furan oxidation occurs almost entirely (≃98%) by the chloride-dependent pathway.

[5] Oxidation of H_2O_2 gives stoichiometric formation of O_2, which is not a product of DMFu oxidation. It is therefore possible to determine their relative reactivities with $[H_2OCl_2]$ from measurement of oxygen yields in competitive reaction of these compounds for HOCl, i.e., assuming the reaction scheme

$$HOCl + H^+ + Cl^- \overset{k_2}{\rightleftharpoons} [H_2OCl_2] \xrightarrow[H_2O_2]{k_6} O_2 + H_2O + 2HCl$$
$$\xrightarrow[DMFu]{k_7} \text{Oxidation products}$$

where $k_6(H_2O_2)/k_7(DMFu) = O_2$ formed /(HOCl reacted-O_2 formed). No evidence of O_2 formation attributable to reaction by the k_2-pathway was found under competitive reaction conditions with $(H_2O_2)/(DMFu) \simeq 10^2$, implying $k_6/k_7 \leqslant 10^{-3}$. Absence of measurable oxygen formation could not be ascribed to trapping of $^1\Delta O_2$ initially formed in H_2O_2 oxidation by dimethylfuran (20).

Competitive Reaction of Singlet Oxygen Quenching Agents with H_2O_2 for HOCl. In experiments similar to those described in paragraph [5], we have measured O_2 yields in the presence of β-carotene, histidine or the tertiary amine, 1,4-diazabicyclo[2.2.2]octane (DABCO). Results are given in Table I. For comparative purposes, quenching efficiencies reported for the reactions forming cis-dibenzoyl ethylene are tabulated. Medium conditions are comparable in the different studies, excepting our solutions contained H_2O_2 in place of the furan.

DISCUSSION:

The inhibitors used in our study deactivate singlet-excited oxygen principally by physical mechanisms, forming molecular oxygen in its electronic

ground state (21-23). If the inhibitors were functioning in this capacity in the HOCl-H_2O_2 reaction, no loss in oxygen yields would accompany their addition. The substantial reduction in O_2 yields observed (Table I) can therefore only be attributed to chemical reduction of HOCl by the inhibitors in competition with H_2O_2.

This conclusion is consistent with kinetic behavior reported for hypochlorous acid with similar organic compounds. Reaction of HOCl with the free base forms of amines (24,25) and amino acids (25) to give chloramines occurs at rates approaching diffusion-controlled limits. Although reaction with carotenoid compounds has not been studied, electrophilic substitution at other unsaturated carbon centers occurs readily (26). It is perhaps pertinent to the biological reactivity of HOCl to note that aromatic substitution (27) and oxidation of organosulfur compounds (28) in the presence of chloride ion proceed according to rate laws identical to the k_2-pathway, namely, rate = $k_2(HOCl)(H^+)(Cl^-)$. Also, in the only study reported examining the influence of solvent isotopic composition on organic reactions of HOCl, it was found that D_2O enhances chlorination of an aromatic ether by ca. 2-fold (29), an effect comparable in magnitude to those measured for diphenylfuran oxidations (17).

Recognizing the dynamic properties of HOCl discussed above, it is evident that all previously noted observations on the HOCl-diphenylfuran reaction which are concordant with singlet oxygen participation can also be explained by the direct competitive oxidation of the reaction components by HOCl. In addition, the large chloride ion-specific reaction enhancing effects previously described in diphenylfuran oxidation (17) find ready explanation in the chloride ion-dependent rate law for dimethylfuran oxidation. A basis for distinguishing between the alternative interpretations is provided by consideration of the kinetics of O_2 formation by hypochlorous acid decomposition; this reaction is immeasurably slow, either in the presence (20) or absence (30) of furans, and therefore cannot serve as a source of singlet oxygen.

The observation of selective oxidation of inhibitors by HOCl in the

TABLE I. Competitive Inhibition of HOCl Oxidation Reactions

Inhibitor	O_2 formed [a,c] ($HOCl + H_2O_2$) nmol	% Inhibition		
		$HOCl + H_2O_2$ [a]	HOCl + diphenylfuran [b]	Myeloperoxidase + H_2O_2 + Cl^- + diphenylfuran [b]
None	660			
β-Carotene (10μM)	211	68	60	89
Histidine (96μM)	33	95	98	95
DABCO (900μM)	125	81	98	90

[a] This study; [b] ref. 17; [c] medium conditions: 0.025M sodium acetate, pH 5.0, (Cl^-) = 0.1M, 25°; solvent for the runs using β-carotene was 20% ethanol. Reaction was initiated by adding HOCl (30μM) to solutions containing either 50μM H_2O_2 (β-carotene) or 100μM H_2O_2 (histidine, DABCO) and the inhibitor.

presence of H_2O_2 (Table I) also calls to question the reliability of tests presumed diagnostic for singlet oxygen in myeloperoxidase-catalyzed chloride oxidation reactions, particularly since reaction conditions are nearly identical in the two studies. While the issue of substrate activation by myeloperoxidase is unresolved (11), it has been shown that the enzyme does not require organic substrate for turnover in Cl^- peroxidation and that HOCl formed is freely diffusible into solution (31). Assuming the subsequent reactions are uncatalyzed, appreciable formation of singlet oxygen simply could not occur in the myeloperoxidase-H_2O_2-Cl^- system because HOCl formed would react preferentially with furan or inhibitors.

Finally, the protective action of DABCO, furans and carotenoid pigments on bactericidal (7) and cytotoxic (8) effects of the myeloperoxidase system and polymorphonuclear leukocytes (9) is subject to the same interpretation. Incorporation of chloride ion into insoluble fractions of phagocytizing neutrophilic granulocytes has been demonstrated (32), consistent with direct chlorination of biological substances by HOCl. Best estimates place reaction conditions during HOCl production within the phagosomal vacuoles at

(Cl^-) ≈ 0.08M, pH 3.5-5 (33), (H_2O_2) ≤ 0.6 mM (34) (this value estimated from rates of formation of H_2O_2 under conditions where its subsequent reactions are largely inhibited (35,36) - consequently, it is likely an upper limit). Uncatalyzed reactions of HOCl will therefore generally proceed according to the k_2-pathway (Figure 1). Given the high reactivity of biological amines and amino acids, sulfhydryl compounds (37), aromatic and other unsaturated carbon groups under these conditions, the question of which reactions occur is likely more a matter of proximity to redox partners (38) than of their relative reactivitites.

ACKNOWLEDGEMENTS:

The authors are indebted to Howard S. Mason and Walter Bruyninckx for stimulating discussions concerning leukocyte function. Financial support was obtained from grants from the National Institutes of Health #GM-20943, 18458 and Air Force Office of Scientific Research #75-2821.

REFERENCES and NOTES:

1. For a recent review, see e.g. Klebanoff, S. J. (1975) Semin. Hematol. 12, 117-142.
2. Babior, B. M., Kipnes, R. S. and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741-744.
3. Johnston, R. B., Keele, B. B., Misra, H. P., Lehmeyer, J. E., Webb, L. S., Baehner, R. L. and Rajagopalan, K. V. (1975) J. Clin. Invest. 55, 1357-1372.
4. Patriarca, P., Dri, P., Kakinuma, K., Tedesco, F. and Rossi, F. (1975) Biochem. Biophys. Acta 385, 380-386.
5. McRipley, R. J. and Sbarra, A. J. (1967) J. Bacteriol. 94, 1417-1424.
6. Tauber, A. I. and Babior, B. M. (1977) J. Clin. Invest. 60, 374-379.
7. Klebanoff, S. J. (1975) in "The Phagocytic Cell in Host Resistance," (Bellanti, J. A. and Dayton, D. H., eds), pp. 45-56, Raven Press, New York.
8. Klebanoff, S. J., Clark, R. A. and Rosen, H. (1976) in "Cancer Enzymology" (Schultz, J. and Ahmad, F., eds.), pp 267-288, Academic Press, New York.
9. Krinsky, N. I. (1974) Science 186, 363-365.
10. Strauss, R. R., Paul, B. B., Jacobs, A. A. and Sbarra, A. J. (1971) Infect. Immun. 3, 592-602.
11. Stelmaszynska, T. and Zgliczynski, J. M. (1974) Eur. J. Biochem. 45, 305-312.
12. Zgliczynski, J. M., Stelmaszynska, T., Domanski, J. and Ostrowski, W. (1971) Biochim. Biophys. Acta 235, 419-424.
13. Baldwin, J. E., Swallow, J. C. and Chan, H. W. S. (1971) Chem. Comm., 1407.
14. Takayama, K., Noguchi, T., Nakano, M. and Migita, T. (1977) Biochem. Biophys. Res. Comm. 75, 1052-1058.
15. Hodgson, E. K. and Fridovich, I. (1976) Arch. Biochem. Biophys. 172, 202-205.

16. Allen, R. C. (1975) Biochem. Biophys. Res. Comm. 63, 675-683; ibid, 684-691.
17. Rosen, H. and Klebanoff, S. J. (1977) J. Biol. Chem. 252, 4803-4810.
18. Held, A. M., Halko, D. J. and Hurst, J. K., submitted for publication.
19. Cady, G. H. (1957) Inorg. Syntheses 5, 156-166.
20. The ratio of rate constants for physical deactivation of $^1\Delta O_2$ vs. reaction with DMFu is $\beta=5 \times 10^{-4}$M. Under the experimental conditions, (DMFu) = $1.9\text{-}5.7 \times 10^{-4}$M, so that only 27-53% $^1\Delta O_2$ arising from H_2O_2 oxidation would be trapped.
21. Foote, C. S., Denney, R. W., Weaver, L., Chang, Y. and Peters, J. (1970) Ann. NY Acad. Sci. 171, 139-148.
22. Ouannes, C. and Wilson, T. (1968) J. Amer. Chem. Soc. 90, 6527-6528.
23. Matheson, I. B. C., Etheridge, R. D., Kratowich, N. R. and Lee, J. (1975) Photochem. Photobiol. 21, 165-171.
24. Ellis, A. J. and Soper, F. G. (1954) J. Chem. Soc., 1750-1755.
25. Morris, J. C. (1967) in "Principles and Applications of Water Chemistry" (Faust, S. D. and Hunter, J. V., eds.) pp. 23-53, Wiley, New York.
26. de la Mare, P. D. and Bolton, R. (1966) "Electrophilic Additions to Unsaturated Systems," Chapter 6, Elsevier, Amsterdam.
27. Soper, F. G. and Smith, G. F. (1926) J. Chem. Soc., 1582-1591.
28. Lordi, N. G. and Epstein, J. (1958) J. Amer. Chem. Soc. 80, 509-515.
29. Swain, C. G. and Ketley, A. D. (1955) J. Amer. Chem. Soc. 77, 3410.
30. Lister, M. W. and Petterson, R. C. (1962) Can. J. Chem. 40, 729-733.
31. Harrison, J. E. and Schultz, J. (1976) J. Biol. Chem. 251, 1371-1374.
32. Zgliczynski, J. M. and Stelmaszynska, T. (1975) Eur. J. Biochem. 56, 157-162.
33. Jensen, M. S. and Bainton, D. F. (1973) J. Cell Biol. 56, 379-388.
34. Roos, D. (1977) Trends Biochem. Sci. 2, 61-64.
35. Homan-Muller, J.W.T., Weening, R. S. and Roos, D. (1975) J. Lab. Clin. Med. 85, 198-207.
36. Zatti, M., Rossi, F. and Patriarca, P. (1968) Experientia 24, 669-670.
37. Knox, W. E., Stumpf, P. K., Green, D. E. and Auerbach, V. H. (1948) J. Bacteriol. 55, 451-458.
38. Klebanoff, S. J. (1970) in "Biochemistry of the Phagocytic Process," (Schultz, J. ed.) pp. 90-92, North-Holland, Amsterdam.