Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5195–5197, September 1983
Biochemistry

Chloroperoxidase generation of singlet Δ molecular oxygen observed directly by spectroscopy in the 1- to 1.6-μm region

[(0,0) transition/chloride peroxidase/hydrogen peroxide/chloride ion]

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Communicated by Michael Kasha, May 9, 1983

ABSTRACT **The (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ singlet molecular oxygen chemiluminescence emission from a biological reaction system, chloroperoxidase (EC 1.11.1.10) acting on hydrogen peroxide at low pH (phosphate buffer, pH 2.85) with Cl^- as a cosubstrate, was recorded with an ultrasensitive IR spectrometer. The strong chemiluminescence emission peak observed at 1.30 μm provides clear evidence of the enzymatic generation of (excited) singlet oxygen from peroxide in this system.**

The observation of $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ singlet molecular oxygen chemiluminescence emission generated by the chloroperoxidase–peroxide system is reported here. A strong emission peak at 1.3 μm (0,0 band) is detected by our ultrasensitive IR spectrometer (1–3) with 30 μM chloroperoxidase and 10% (wt/wt) aqueous hydrogen peroxide at low pH (2.85) with Cl^- as cosubstrate, at room temperature. This near IR emission spectrum constitutes clear evidence of the generation of the electronically excited species of molecular oxygen in a biological system. The current observation of singlet oxygen generation as a consequence of an enzymatic reaction indicates that a key step in biological oxidation of peroxide has been neglected in mechanistic discussions.

Numerous investigators, including one of the present authors, have proposed singlet oxygen generation in enzymatic reactions involving such enzymes as xanthine oxidase (4, 5), quercetinase (6), horseradish peroxidase (7), catalase (8), and myeloperoxidase (9). The evidence that was previously available was the analysis of the reaction products of singlet oxygen scavengers—e.g., diphenylisobenzofuran—in these systems. Aside from being inherently indirect, this technique suffers from two serious drawbacks: (*i*) the chemical scavengers are not the normal enzyme substrates and may not reach proximity to the enzyme active site, and (*ii*) other small oxygen-containing species (O_2^-, $HO^\cdot$, $HOO^\cdot$) also react with these scavengers. Another commonly used technique is to monitor any visible emission of light accompanying the enzymatic reaction and to measure the response of this emission after the addition of various quenchers and enhancers of singlet molecular oxygen to the reaction. Again this method is flawed by nonspecificity, the emission studied being primarily in the visible region and indeterminate in origin, because of excitation transfer, and no definitive spectroscopic basis was found to establish the identity of the emitting species as being oxygen. The results of these studies were therefore inconclusive and ambiguous (10).

Over the last 4 years we have constructed two ultrasensitive near-infrared spectrometers, one operating in the 1.00- to 2.50-μm region and based on a lead sulfide detector and the other, a more sensitive germanium photodiode detector, operating between 1.00 and 1.60 μm. The advent of this instrumentation (1–3) has opened up a window on the near-infrared spectral region so that luminescence studies of reactions suspected of generating intermediates with (possibly very weak) near-infrared emission can now be monitored directly, as has been done successfully with photosensitization by dye (1) and superoxide anion disproportionation (3). The present work used the liquid nitrogen-cooled photovoltaic intrinsic germanium detector, which is described elsewhere (3).

In 1966 Morris and Hager (11) isolated and characterized the enzyme chloroperoxidase (chloride peroxidase; EC 1.11.1.10), a plant peroxidase with M_r 42,000 containing one ferriprotoporphyrin IX prosthetic group per molecule. In addition to catalyzing the normal enzymatic peroxidation reactions, chloroperoxidase can utilize chloride and bromide ions as donors for enzymatic halogenation; many of the reactions catalyzed by chloroperoxidase are either dependent upon or accelerated by the presence of halide ions (12).

Chloroperoxidase (20 μM in 70 mM KCl with 100 mM potassium phosphate buffer at pH 2.85) was mixed dropwise under argon pressure with hydrogen peroxide (10% solution with 30 mM KCl in 100 mM phosphate buffer at pH 2.85) at approximately 1:2 ratio of peroxidase to hydrogen peroxide solution in an optical cell (inside a box in a dark room). The ionic strength, the pH, the hydrogen peroxide concentration, and the buffer were chosen to accelerate the reaction rate to a maximal value (12).

Fig. 1 is the emission spectrum obtained for the enzymatic reaction in the 1.60- to 1.10-μm range. The scanning time for the spectrum was about 40 min with a slit width of 6 mm for both the entrance and exit slits of a Bausch and Lomb high-intensity monochromator ($f/3.5$) with a grating blazed for 1.00 μm. The most prominent spectral feature observed is an emission band at 1.30 μm with a half-bandwidth at half maximum of 234 cm^{-1}.

In the hypochlorite–peroxide simple chemical generation of singlet oxygen our apparatus records the analogous peak at 1.27 μm (2) with the lead sulfide detector and at 1.29 μm (3) with the germanium photodiode. Thus, the expected characteristic 1.268 μm band (gas phase) of singlet Δ oxygen emission is shifted by the spectrometer-detector characteristics. We can confidently assign the narrow band at 1.30 μm to the (0,0) vibronic band of the $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transition of molecular oxygen. It appears likely from other research (unpublished) that the observed oxygen emission from the enzyme reaction is actually gas-phase emission and thus can show no environmental spectral shift.

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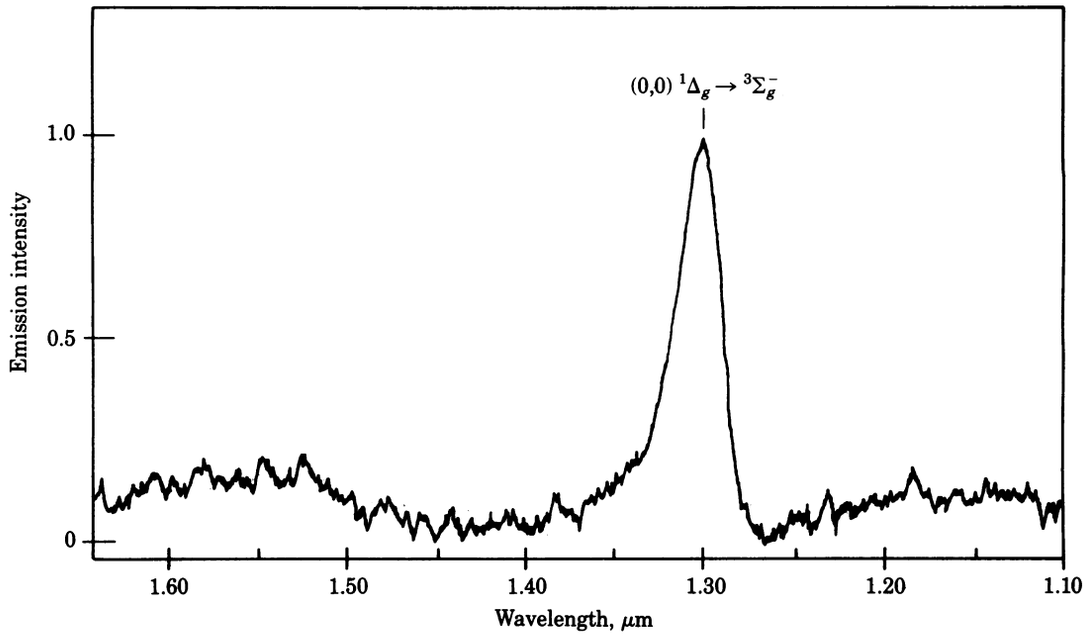

FIG. 1. Near-infrared singlet oxygen chemiluminescence emission spectrum from the enzymatic reaction of chloroperoxidase with hydrogen peroxide and chloride ion as cosubstrates in potassium phosphate buffer at pH 2.85 and 298 K. Shown is the (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission of singlet molecular oxygen (germanium photodiode detector).

On the long-wavelength side of the 1.30 μm band a broader band extends from the edge of the instrumental cut-off at 1.60 μm to 1.50 μm. Another, still weaker band appears from around 1.25 μm to beyond 1.10 μm. We shall not discuss these two broad emission bands in this communication.

Although we do not have an absolute calibration of the photon sensitivity of the detecting system, we have compared the chemiluminescence intensities of the reactions of H_2O_2 (10%) and NaOCl (7%) (3). The enzyme-generated emission intensity is approximately 1/10th the intensity of the (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ band generated chemically for similar mixing rates. The H_2O_2/OCl^- reaction has stoichiometric generation of singlet oxygen, although subsequent reaction and quenching is thought to reduce the measurable emission intensity drastically. Thus, as an approximate comparison indicates, Fig. 1 represents an efficient singlet oxygen generation by the enzyme.

Fig. 1, therefore, represents direct evidence of enzymatic generation of singlet oxygen. The question remains whether this generation is the major reaction pathway. In chloroperoxidase reactions with chloride ion as a cosubstrate, the preferential reaction path is oxidation, not chlorination, as has been shown in competitive reaction studies. For example, a comparison of the rate of methionine oxidation to the rate of 2-chlorodimedone chlorination by the enzyme in the same solution shows that the methionine is completely consumed before the chlorination reaction proceeds (12). Moreover, in the chloroperoxidase reaction singlet oxygen generation must be enzymatic because of the low pH (2.85) of the reaction. The possibility of indirect generation via an enzymatically generated OCl^- diffusing from the active site to react with a remote H_2O_2 molecule is not feasible because the H_2O_2/OCl^- chemical reaction is extremely sluggish at this pH (13).

Similarities between the chemical properties of chloroperoxidase and other heme enzymes are striking; myeloperoxidase, for example, utilizes H_2O_2 and Cl^- as substrates during phagocytosis by polymorphonuclear leukocytes, a microbicidial activity (14). Speculation of the involvement of singlet oxygen in this system has been rejected on the basis of pH considerations (15). Fig. 1 shows that, at least at the active site in the vicinity of an enzymatic Fe center, low pH does not prevent singlet oxygen generation. The third example of a halide ion used as an enzyme cosubstrate is lactoperoxidase, which catalyzes bromination in the presence of H_2O_2 and Br^- (16). Even enzymes such as xanthine oxidase that are superoxide anion sources may be included in the list because singlet oxygen could be generated at the active site as an intermediate step. Singlet oxygen has an enhanced electron affinity compared to the ground state molecule and could abstract an electron from the vicinity. Another suggestive system is cyclooxygenase, which catalyzes Diels–Alder molecular oxygen addition reactions typical of singlet oxygen in prostaglandin synthesis (17). In the light of the present experiment the catalytic activities of peroxidases, oxidases, and oxygenases could be united into a common theme of oxygen activation via generation of the singlet electronically excited states of molecular oxygen.

The present case constitutes a remarkable example of an electronically excited state of a product molecule being produced in a biological reaction. The "dark photochemistry" theme of Cilento (18) is reinforced by this observation. The important biological question of what is the consequence of singlet molecular oxygen production in this reaction remains to be answered. Research on other enzymes (unpublished data) indicates—e.g., in the case of catalase—variations of considerable complexity that are suggestive of enzyme site dependence.

This paper was supported by contract DE-AS-5-78EV05855 between the Division of Biomedical and Environmental Research of the U.S. Department of Energy and Florida State University and a grant from the National Foundation for Cancer Research, Bethesda, Maryland.

1. Khan, A. U. & Kasha, M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6047–6049.
2. Khan, A. U. (1980) *Chem. Phys. Lett.* **72**, 112–114.
3. Khan, A. U. (1981) *J. Am. Chem. Soc.* **103**, 6516–6517.
4. Khan, A. U. (1976) *J. Phys. Chem.* **80**, 2219–2228.
5. Kellogg, E. W., III, & Fridovich, I. (1975) *J. Biol. Chem.* **250**, 8812–8817.
6. Krishnamurty, H. G. & Simpson, F. J. (1970) *J. Biol. Chem.* **245**, 1467–1471.

7. Chan, H. W.-S. (1971) *J. Am. Chem. Soc.* **93,** 4632–4633.
8. Porter, D. J. T. & Ingraham, L. L. (1974) *Biochim. Biophys. Acta* **334,** 97–102.
9. Allen, R. C., Stjernholm, R. L. & Steel, R. H. (1972) *Biochem. Biophys. Res. Commun.* **47,** 679–684.
10. Krinsky, N. I. (1979) in *Singlet Oxygen*, eds. Wasserman, H. H. & Murray, R. W. (Academic, New York), pp. 597–641.
11. Morris, D. R. & Hager, L. P. (1966) *J. Biol. Chem.* **241,** 1763–1768.
12. Libby, D. R., Thomas, J. A., Kaiser, L. W. & Hager, L. P. (1982) *J. Biol. Chem.* **257,** 5030–5037.
13. Kajiwara, T. & Kearns, D. R. (1973) *J. Am. Chem. Soc.* **95,** 5886–5890.
14. Klebanoff, S. J. (1968) *J. Bacteriol.* **95,** 2131–2138.
15. Harrison, J. E., Watson, B. D. & Schultz, J. (1978) *FEBS Lett.* **92,** 327–332.
16. Piatt, J. F., Cheema, A. S. & O'Brien, P. J. (1977) *FEBS Lett.* **74,** 251–254.
17. Samuelsson, B. (1965) *J. Am. Chem. Soc.* **87,** 3011–3013.
18. Cilento, G. (1980) *Acc. Chem. Res.* **13,** 225–230.