DISCOVERY OF ENZYME GENERATION OF $^1\Delta_g$ MOLECULAR OXYGEN: SPECTRA OF (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ IR EMISSION†

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Summary

Enzyme production of $^1\Delta_g$ molecular oxygen in dark reactions is revealed via near-IR emission spectroscopy. Singlet molecular oxygen emission (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ is observed in the enzyme systems chloroperoxidase–H_2O_2–Cl^- and lactoperoxidase–H_2O_2–Br^-. In the catalase–H_2O_2 system a strong emission at 1.64 μm with a bandwidth of 755 cm^{-1} is observed, but is as yet unidentified.

1. Introduction

Enzyme generation of singlet oxygen is now established via IR spectroscopic detection and identification of (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ luminescence emission from the enzyme systems chloroperoxidase–H_2O_2–Cl^- and lactoperoxidase–H_2O_2–Br^- [1, 2]. This is the first direct experimental confirmation of singlet oxygen being produced as a chemical reactant generated in the dark in enzyme systems, although this possibility has been widely explored and critically discussed in the literature [3 - 6]. Furthermore, preliminary reports by Kanofsky on kinetic studies of lactoperoxidase-generated singlet oxygen have appeared recently [7], and an extension of these kinetic studies was reported in the present conference [8]. Also in the enzyme system catalase–H_2O_2 a strong IR emission at 1.64 μm has been discovered, possibly arising from perturbed singlet oxygen emission or the iron–heme coordination complex [2]. These experimental observations were possible because of the recent development of sensitive IR spectroscopic techniques allowing the detection of singlet oxygen in *solution* [9 - 11], a goal which has been pursued since the original discovery of the generation of singlet oxygen as a chemical reagent by Khan and Kasha [12] in 1963.

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2. Experimental details

Figure 1 is a schematic diagram of a new ultrasensitive near-IR spectrometer with a germanium detector and a 1.0 μm blazed grating, operative in the 1.0 - 1.6 μm range. By employing a grating blazed at 2.0 μm, the spectrometer range is extended to 1.75 μm, the long wavelength cut-off dictated by the spectral response of the germanium detector. For experiments with the enzymes chloroperoxidase and lactoperoxidase the 1.0 μm blazed grating was used. For the catalase system the 2.0 μm grating was necessary since the emission is at longer wavelengths.

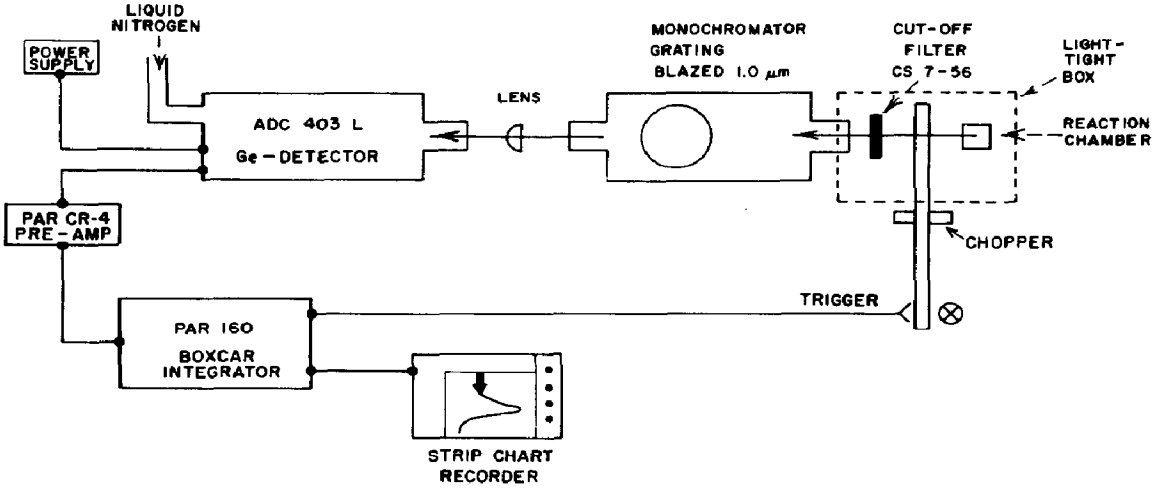

Fig. 1. High sensitivity 1 μm region luminescence spectrometer.

An unusual problem is associated with the use of the photovoltaic germanium detector in high sensitivity IR applications. Single crystals of germanium are excellent detectors of cosmic radiation, and the transit of cosmic radiation through the crystal generates an enormous pulse in the detector. The number of pulses originating from cosmic radiation is small, of the order of two to three per minute, but the amplitude of the signal generated can overwhelm the electronic data handling system, which then ceases to operate. Lead shielding of the detector is not helpful, since the daughter radiation which cascades through the shield is still enormously energetic. In our experiments we use a Princeton Applied Research low noise variable bandpass filter (CR-4), designed to turn off the amplifier momentarily (for about 2 s) when high amplitude pulses arrive. The combination of this low noise amplifier and a PAR 160 boxcar integrator allowed us to obtain the spectra presented here.

Although ours is the near-IR spectrometer that pioneered the use of the IR detectors in singlet oxygen solution spectroscopy [9 - 11], several reports of detection of singlet oxygen emission in solution under special conditions precede us in the literature. Matheson *et al.* [13] observed the double-

molecule simultaneous transition (0,0) $2(^1\Delta_g) \rightarrow 2(^3\Sigma_g^-)$ at 6330 Å from an oxygenated Freon solution pumped by an Nd–YAG laser. The long lifetime of singlet oxygen in this solution (at least 1 ms [14]) allowed them to use a photomultiplier as the detector for this bimolecular process. Krasnovsky [15] also used a photomultiplier at the very edge of its long wavelength sensitivity in a phosphoroscope and observed the photosensitized (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission in CCl_4 (the singlet oxygen lifetime in CCl_4 is about 26 ms [16]).

However, by utilizing detectors with specific IR sensitivity, either the thermoelectrically cooled PbS detector or the liquid-nitrogen-cooled germanium detector, we have spectrally observed photosensitized (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission not only in organic and halogenated solvents but also for the first time in water [9] where its lifetime is approximately 4.2 μs [17]. Additionally the very weak (0,1) vibronic band of the $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transition at 1.58 μm has been observed for the first time in solution [10]. Recently, using the spectrometer of Fig. 1, we have found the (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transition of dissolved oxygen coupled with solvent vibrational transitions in halogenated solvents, providing an unusual case of simultaneous electronic–vibrational transitions involving singlet oxygen and the solvent molecule [18].

3. Results

3.1. Chloroperoxidase

In 1966 Morris and Hager [19] isolated and characterized chloroperoxidase, a plant enzyme, from *Caldariomyces fumago*. Chloroperoxidase is a glycoprotein with $M_r = 42\,000$ containing one ferriprotoporphyrin IX prosthetic group per enzyme. This enzyme exhibits mixed enzymatic behavior [20], catalyzing classical peroxidation reactions, catalyzing the dismutation of H_2O_2, more characteristic of catalase, and catalyzing, with Cl^- and Br^- ions as donors, enzymic halogenation reactions similar to those of the enzymes lactoperoxidase (see ref. 21) and myeloperoxidase [5]. In Fig. 2, adapted from ref. 1, is shown the spectrum of (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ of molecular oxygen at 1.30 μm and a much weaker emission extending from 1.50 μm to longer wavelengths.† The spectrum was obtained by continuously mixing under argon pressure chloroperoxidase (20 μmol ml^{-1}; 70 mM KCl; 100 mM potassium phosphate buffer; pH 2.85) with H_2O_2 (10% solution; 70 mM KCl; 100 mM phosphate buffer; pH 2.85). The luminescence spectrum of the dark reaction on mixing in a light-tight box at room temperature was obtained with the IR spectrometer shown in Fig. 1.

†The monochromator shown in Fig. 1 was assembled by us; a Bausch and Lomb high intensity monochromator (*f*/3.5) was fitted with a 1 μm blazed grating having 600 grooves mm^{-1} and the wavelength was scanned with a clock motor. In this spectrometer the 1.268 μm (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission of molecular oxygen from the H_2O_2–OCl^- reaction appears at 1.290 μm with a ±0.010 μm reproducibility.

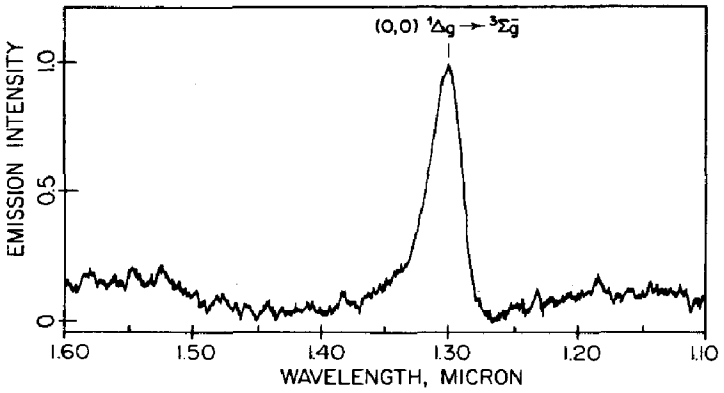

Fig. 2. IR luminescence emission generated in the chloroperoxidase-catalyzed decomposition of H_2O_2 in the presence of Cl^- ion at room temperature showing the (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission of molecular oxygen at 1.30 μm uncorrected for the instrumental shift of the peak position from 1.268 μm (adapted from ref. 1).

3.2. Lactoperoxidase

In 1925 Thurlow [22] was successful in isolating the milk enzyme lactoperoxidase in a low state of purity. Theorell and coworkers [23, 24] were the first to obtain a highly purified preparation also from milk. Subsequently, lactoperoxidase was identified and isolated from the salivary glands [25]. Lactoperoxidase combined with H_2O_2 and a halide (Br^- or I^-) ion or a pseudohalide (SCN^-) ion has a very potent cytocidal activity, inhibiting the growth of a variety of micro-organisms [26 - 29].

Figure 3, adapted from ref. 2, shows the spectrum of (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ molecular oxygen at 1.28 μm†, obtained by continuous mixing under argon

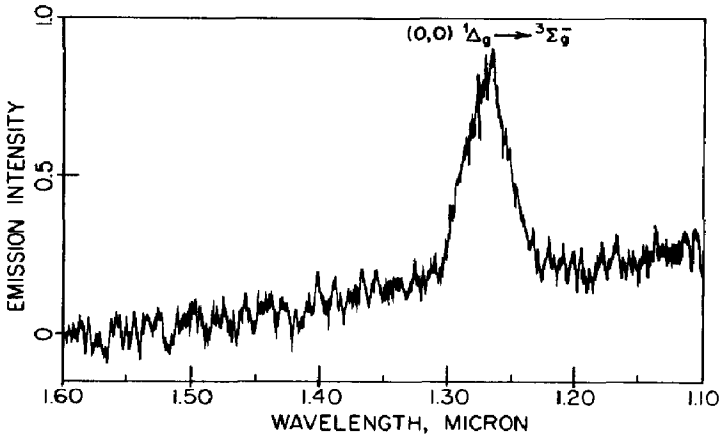

Fig. 3. IR luminescence emission of $^1\Delta_g$ molecular oxygen at room temperature generated in the lactoperoxidase-catalyzed decomposition of H_2O_2 in the presence of Br^- ion showing the (0,0) $^1\Delta_g \rightarrow {}^3\Sigma_g^-$ emission of molecular oxygen at 1.28 μm uncorrected for the instrumental shift of the peak position from 1.268 μm (adapted from ref. 2).

†See footnote to p. 329.

pressure lactoperoxidase (10 μmol ml^{-1}; 10 mM acetate buffer; pH + pD, 4.5; 0.80 M KBr) with H_2O_2 (0.25 mM; 10 mM acetate buffer; pH + pD, 4.5; 0.8 M KBr). The luminescence spectrum was obtained for the dark reaction at room temperature with the IR spectrometer shown in Fig. 1.

3.3. Catalase

In 1811, Thenard observed that both animal and plant tissues possessed the property of decomposing H_2O_2. Jacobson, Bourquelot and Loew eventually showed that the decomposition of H_2O_2 is caused by a specific enzyme, which Loew named catalase (see ref. 30). All aerobic organisms contain catalase, although the concentration of the enzyme varies enormously from organ to organ. In mammals the highest concentrations are in the erythrocytes and the liver [31, 32]. In 1937 Sumner and Dounce [33] isolated the enzyme in a high state of purity, via crystallization from beef liver extract, and extensive physiochemical studies of catalase followed this early biochemical triumph. No detailed X-ray crystal structure has yet been performed on this enzyme, but it is known that catalase is an oligomer containing four tetrahedrally arranged 60 000 dalton subunits, each subunit having an Fe^{3+} heme at the active site. Although the active site geometry is not known in detail, from the kinetic studies of the relative decomposition rate of H_2O_2, CH_3OOH and CH_3CH_2OOH, it is now believed that catalase is a sequestered enzyme with stringent size limitations on substrate access to the active site. Also from kinetic studies, Chance and Herbert [34] have suggested the involvement of two Fe^{3+} active sites in the decomposition of H_2O_2 by the enzyme. Isotopic studies by Jarnagin and Wang [35] show that

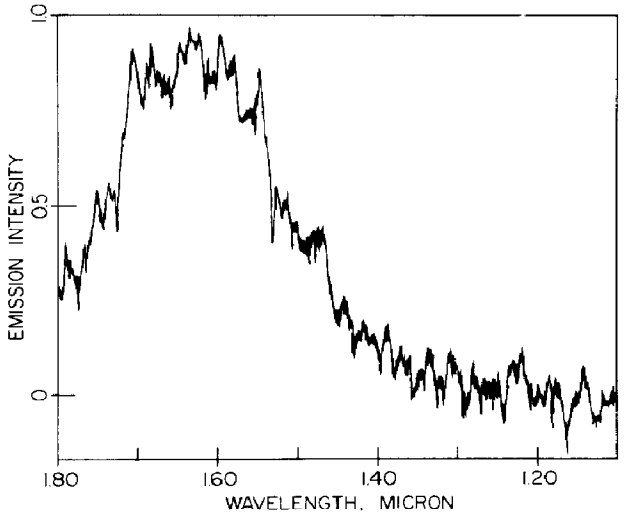

Fig. 4. IR luminescence emission in the catalase-induced decomposition of H_2O_2 at room temperature showing a broad emission peak at 1.60 μm with a bandwidth of 755 cm^{-1} (adapted from ref. 2).

no ^{18}O scrambling occurs in the H_2O_2 decomposition; the molecular oxygen evolved originates from a *single* H_2O_2 molecule.

Figure 4 shows the emission spectrum adapted from ref. 2 of catalase (1.0 mmol ml^{-1}; 0.1 M phosphate buffer; pH 7.6) with H_2O_2 (5% solution; 0.1 M phosphate buffer; pH 7.6). To observe the emission beyond 1.6 μm, the monochromator was fitted with a 2.0 μm blazed grating in the high intensity Bausch and Lomb monochromator in place of the 1.0 μm blazed grating shown in Fig. 1.

4. Discussion

Clearly the peroxidases of Figs. 2 and 3 are generating free singlet oxygen. The substrate and cosubstrate required, H_2O_2 and halide ions, suggest that the method of singlet oxygen generation in these enzyme reactions is similar to that of the classic inorganic H_2O_2–OCl^- reaction [36]. The enzyme reactions are extremely efficient; Kanofsky [8] has found that both the lactoperoxidase and the chloroperoxidase decomposition of H_2O_2 yield singlet oxygen at unit efficiency with respect to H_2O_2 concentration. It appears that the microbicidal activity of lactoperoxidase can be directly attributed to singlet oxygen generation; this is consistent with previous studies of phagocytosis and singlet oxygen chemical activity [5].

Like catalase, chloroperoxidase also catalyzes the dismutation of H_2O_2. The spectral similarity between the broad band onsetting at 1.50 μm and extending to longer wavelengths in Fig. 2 and the emission from the catalase reaction in Fig. 4 is suggestive of a common source or mechanism. However, in the catalase system no free singlet oxygen is being released to the surroundings, probably because the oxygen molecule, if it is being generated in an electronically excited singlet state, cannot exit the tight enzyme cavity without being quenched.

Exactly 20 years transpired between the spectroscopic discovery of the chemical generation of singlet oxygen in 1963 [12] and the enzymatic generation of singlet oxygen in 1983 [1, 2]. In these two decades a virtual explosion of research in the chemistry and physics of singlet oxygen has occurred with remarkable progress in our understanding of oxidation mechanisms of organic molecules, both chemical and photochemical. However, the non-specificity of the available techniques for detecting singlet oxygen in solution presented a barrier to further progress; this was particularly true for dark reactions of singlet oxygen in biological systems [3, 4]. Now that sensitive IR techniques, which can provide unambiguous identification of this excited species are available, many systems where singlet oxygen intermediacy has been suggested by indirect chemical evidence can be re-examined with confidence. Such systems include phagocytosis [5, 37], biosynthesis in the arachadonic acid "cascade" mediated by cyclooxygenase and lipooxygenase [38, 39] as well as the oxidase and peroxidase enzymatic reactions.

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