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Spectroscopic studies of chemiluminescence by Mn^{++}-activated rubisco: Singlet oxygen is entirely absent and the emission spectrum differs between forms of rubisco.

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Introduction

Near-infrared chemiluminescence is emitted when Mn^{++}-activated rubisco is catalysing its oxygenase reaction (Mogel & McFadden, 1990; Lilley et al 1993; Cox et al 1999). Neither the physiological cofactor, Mg^{++}, nor any other metal ion has been found to support observable luminescence. An incomplete spectrum of the emitted light, with a maximum near 800 nm, was consistent with the characteristics of Mn^{++} luminescence and showed no signs of the peaks at 633 and 703 nm that are diagnostic of the dimolecular decay of singlet O_2 (Lilley et al 1993). This contradicts the suggestion that singlet O_2 might have a role in the luminescence Mogel & McFadden (1990). Here we performed a more direct spectroscopic test for singlet O_2 involvement, seeking to observe the much more intense emission at 1268 nm associated with its monomolecular transition to the triplet O_2 ground state. This can be observed in aqueous conditions when singlet O_2 is generated enzymically (Khan 1984). Prompted by observations that the luminescence yield appeared to differ between rubiscos from different sources (Cox et al 1999), we also determined whether the spectral distribution of the radiation was similarly affected by measuring a more complete spectrum of the chemiluminescence with a very sensitive spectrograph.

Methods

Rubisco preparations. Rubisco from spinach was purified by anion exchange chromatography and from tobacco by crystallisation. The rbcM gene from *Rhodospirillum rubrum* encoding an N-terminal histidine tag was expressed in *E.coli* and the recombinant protein purified by Ni-NTA agarose chromatography (Qiagen).

Broadband survey of visible and infrared emission. Total luminescence emitted during oxygenation was monitored simultaneously with a red-sensitive, high-gain photomultiplier (Hamamatsu R943-02, sensitive to wavelengths from 300 to 800 nm) and a cooled germanium detector (Applied Detector Corp. 403L, most sensitive to

wavelengths between 1100 to 1700 nm, but with low level sensitivity to wavelengths as short as 700 nm) positioned on opposite sides of the source (Fig. 1). The source was either a quartz cuvette containing enzyme reaction mixtures or a tungsten lamp for calibration. Filters were interposed between the source and the detectors to select various ranges of wavelengths. Spinach rubisco (197 μg/ml) was preincubated for 10 min in 24.4 mM tris-HCl, 0.19 mM EDTA, 1.9 mM $MnCl_2$, 3.9 mM $NaHCO_3$ in H_2O or ^2H_2O (pH/pD 8.2). Luminescence was started by the addition of D-ribulose-1,5-bisphosphate (RuBP) to 193 μM. Singlet O_2 was generated by lactoperoxidase-catalysed decomposition of H_2O_2 in the presence of Br^- (Kanofsky 1983). Lactoperoxidase (0.1 mg) dissolved in 1 ml of 20 mM acetic acid-NaOH (pH 4.5) was added to the cuvette, followed by 0.1 ml of 200 mM NaBr. After thorough mixing, luminescence was started by adding 0.1 ml of 200 mM H_2O_2 onto the top of the solution without additional mixing.

Near-infrared spectrum. An f4 0.5m spectrograph was constructed using a back-thinned, charge-coupled detector and low-dispersion grating blazed at 800nm. This system could accumulate an entire spectrum in a few seconds (eg. 650 - 890 nm). The spectra were calibrated and corrected for the wavelength dependence of the sensitivity by reference to blackbody radiation from a tungsten filament of known temperature (determined by an optical pyrometer, Leeds and Northrup 8627).

Results and Discussion:

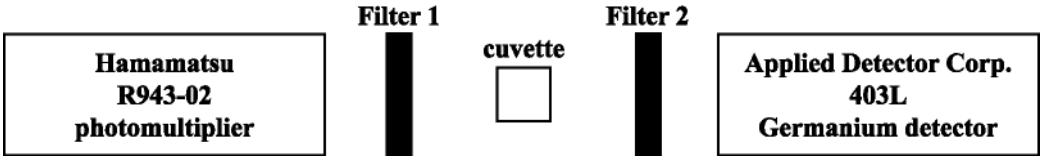

Figure 1. Setup for detecting luminescence.

Table 1. Detector outputs for a range of experimental conditions and filter sets

Photomultiplier output (nA)	Filter 1	Source	Filter 2	Ge detector output (mV)
0.6	nil	Rubisco in H_2O	nil	0.6
0.55	nil	Rubisco in H_2O	<1000 nm cutoff	0.05
0.6	nil	Rubisco in H_2O	<1200 nm cutoff	0
1.2	nil	Rubisco in 98% ^2H_2O	nil	1.2
1.1	nil	Rubisco in 98% ^2H_2O	<1100 nm cutoff	0
0	<650 nm cutoff	Lactoperoxidase in H_2O	<1200 nm cutoff	>1,000
0.13	762 nm interference	Calibration lamp	762 nm interference	10

No emission beyond 1100 nm.

Rubisco chemiluminescence was detected by both the photomultiplier and the Ge detector without filters. Using filters, no radiation was detected by the Ge detector above 1100 nm with H_2O or ^2H_2O as solvent. As a positive control, a strong signal decaying over 5 s was measured at wavelengths longer than 1200 nm by the Ge detector when singlet O_2 was generated by lactoperoxidase. In this case, no radiation at wavelengths longer than 650 nm was detected by the photomultiplier.

The relative sensitivity of the two detectors at 762 nm was estimated from their responses to light from the same source. From this and the sensitivity of the Ge detector at 1268 nm relative to 762 nm radiation (manufacturer's specifications), an upper limit can be calculated for any radiation emitted by the rubisco samples at wavelengths longer than 1100 nm. Thus we can conclude that the >1100 nm-emission is less than 10^{-6} of that detected by the photomultiplier at shorter wavelengths. Absence of the strong 1268-nm emission associated with the monomolecular decay of singlet O_2 eliminates the possibility that singlet O_2 contributes to rubisco chemiluminescence.

Near-infrared spectra. The emission spectra for Mn^{++}-activated spinach rubisco were determined in water and ^2H_2O, with successive scans until the emission ceased due to depletion of the RuBP substrate (Fig. 2).

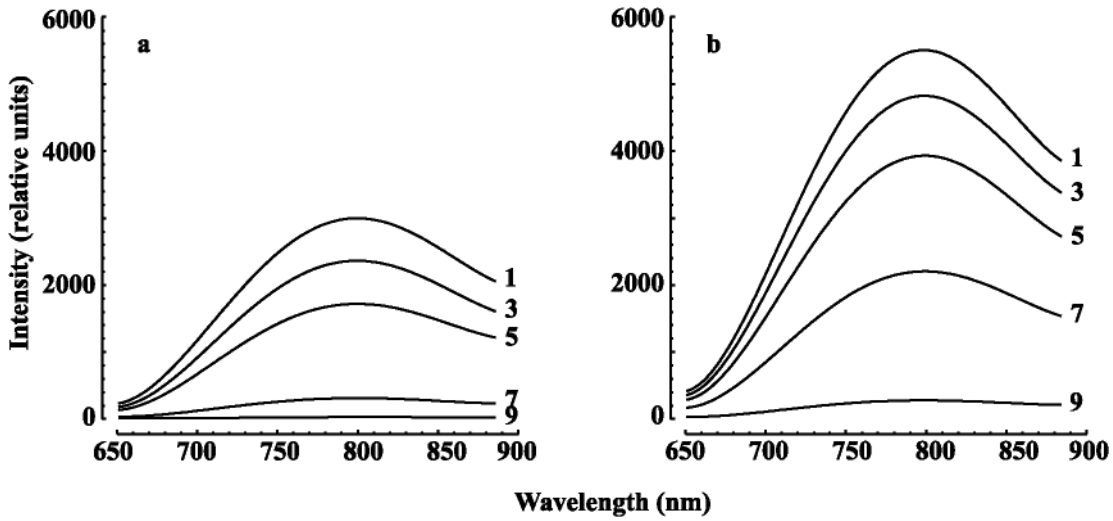

Figure 2. Emission spectra of chemiluminescence by spinach rubisco in (a) H_2O and (b) 96.5% ^2H_2O. Reaction mixture (493 µg rubisco /ml) prepared as in Methods. The spectra were accumulated over consecutive 20 s intervals (every second spectrum shown) until the luminescence ceased upon exhaustion of RuBP. Each spectrum was compiled from 1340 data points (not shown) and the line represents the 4th order polynomial fit to the data.

The rubisco activity, estimated from time to depletion of RuBP, was similar in H_2O and ^2H_2O (0.19 and 0.17 mol RuBP consumed [mol active sites s]$^{-1}$, respectively). Although the intensity of chemiluminescence was approximately doubled in the presence of 96.5% ^2H_2O, the spectral peak averages (799 and 800 nm) and distributions were similar for both solvents.

The spectra of chemiluminescence emitted by tobacco wild-type and L335V mutant rubiscos (Fig. 3) were similar. However, the spectrum for the L_2 rubisco from *R. rubrum* differed substantially from the spectra of the L_8S_8 enzymes of spinach and tobacco. The *R. rubrum* enzyme exhibited peak intensity at 760 nm.

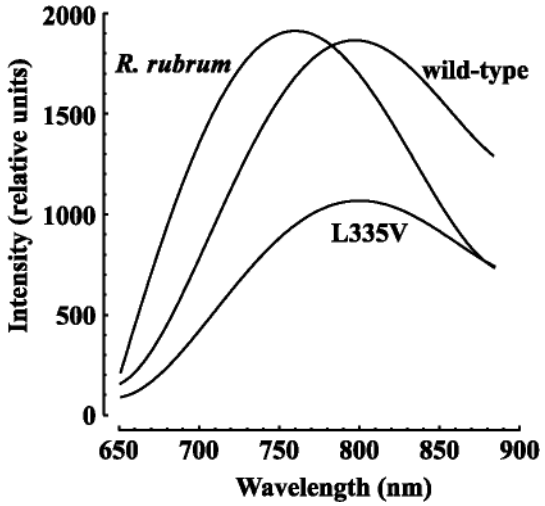

Figure 3 Emission spectra of chemiluminescence by rubisco in H_2O at 25°C. Tobacco wild-type and L335V mutant enzymes (6.21 mg/ml and 11.1 mg/ml respectively) were activated in 25 mM Tris-HCl, 0.2 mM EDTA, 2 mM $MnCl_2$, 10 mM $NaHCO_3$ (pH 8.1). The assay mixture (1.0 ml) contained 500 µg (wild-type) or 887 µg (L335V) of rubisco, 25 mM Tris-HCl, 0.1 mM EDTA, 2 mM $MnCl_2$, 0.8 mM $NaHCO_3$ (pH 8.1) and the oxygenase reaction was started by the addition of RuBP (to 772 µM). *R. rubrum* enzyme was activated in 23 mM Tris-HCl, 0.1 mM EDTA, 1.8 mM dithiothreitol, 2 mM $MnCl_2$, 40 mM $NaHCO_3$ (pH 8.1). The assay mixture (1.0 ml) contained 1.73 mg of rubisco, 24 mM Tris-HCl, 0.1 mM EDTA, 1.1 mM dithiothreitol, 2 mM $MnCl_2$, 21.7 mM $NaHCO_3$ (pH 8.1) and the oxygenase reaction was started by the addition of RuBP (to 772 µM). The *R. rubrum* intensity was multiplied by a factor of 8.

Conclusions.

These results show that singlet O_2 does not contribute to rubisco chemiluminescence. A small change in structure in the active site region of tobacco rubisco (Leu-335 → Val) has no influence on the spectral distribution of rubisco chemiluminescence. In contrast, the L_2 enzyme from *R. rubrum* has a markedly different spectrum, which may arise from subtle differences in the coordination sphere of the Mn^{++} ion at the active site.

Acknowledgements

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