**RESEARCH NOTE**

**OXYGEN DIFFUSION THROUGH HORSE RADISH PEROXIDASE**

**JUAN E. BRUNET**†, CAROLINA JULLIAN and DAVID M. JAMESON

1 Instituto de Química, Universidad Católica de Valparaíso, Casilla 4059 Valparaíso, Chile and 2 Department of Biochemistry and Biophysics, John A. Burns School of Medicine, University of Hawaii at Manoa, 1960 East-West Road, Honolulu, HI 96822, USA

(Received 8 August 1989; accepted 6 September 1989)

**Abstract**—The quenching by molecular oxygen of the fluorescence from a protoporphyrin IX adduct of horseradish peroxidase has been investigated using both intensity and time-resolved techniques. The bimolecular quenching rate constant determined for this process, as evaluated by the conventional Stern-Volmer analysis, was $2 \times 10^7 M^{-1} s^{-1}$, among the lowest observed for protein systems. This result suggests that the heme binding site in horseradish peroxidase is relatively inaccessible to oxygen, which may account for the observation of room temperature phosphorescence in aerated solutions from enzymatically created triplet states.

**INTRODUCTION**

Horseradish peroxidase (HRP, EC 1.11.1.7), a member of the group of plant peroxidases which catalyze the oxidation and peroxidation of a variety of organic and inorganic compounds, is a monomeric glycoprotein of molecular weight 44 000, containing one non-covalently bound heme moiety. The reaction of indole-3 acetic acid and isobutylaldehyde (IBAL) with oxygen, catalyzed by HRP, gives rise to chemiluminescence (Cilento, 1980, 1982; Cilento and Adam, 1988). It has been shown that the HRP catalyzed aerobic oxidation of IBAL generates acetone in the electronically excited triplet state (Duran et al., 1977; Oliveira et al., 1978; Bechara et al., 1979). Rivas-Suarez and Cilento (1980) have established that the acetone triplet state is generated within the enzyme. Oxygen, however, is known to be an extremely efficient quencher of oxygen diffusion in HRP. This emission by dissolved oxygen. In this report we describe fluorescence quenching data on the iron free HRP(desFe) system which address the issue of oxygen diffusion in HRP.
A small modification to this cell suggested by Eftink and Ghiron (1989), namely a device to facilitate gentle bubbling of gas through the solution, greatly enhanced the efficiency of the gas equilibration process which permitted a complete quenching run to be carried out in about 1 h. Pressure of the gas over the solution was determined with a Roylyn precision gauge (Roylyn Rueher Precision, Santa Ana, CA). The sample was illuminated only during the fluorescence measurement period that lasted a few minutes at each oxygen pressure. The lack of porphyrin photoproduct after release of the oxygen pressure was verified as previously described (Valat et al., 1988).

RESULTS AND DISCUSSION

In the absence of elevated oxygen pressures the fluorescence lifetime of the porphyrin emission of HRP(desFe) was a single exponential decay of 16.9 ns as previously reported (Jullian et al., 1989). The effect of elevated oxygen concentrations on fluorescence intensity, as well as the phase and modulation lifetimes, was determined. A Stern-Volmer plot of the intensity and lifetime results (at 10 MHz modulation frequency) is shown in Fig. 1.

Using the conventional Stern-Volmer analysis one obtains a bimolecular quenching constant on the order of $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. This value is noteworthy because it is among the lowest reported for oxygen diffusion in proteins (Lakowicz and Weber, 1973a,b; Eftink and Jameson, 1982; Barbay and Feitelson, 1989). Figure 1 also shows intensity quenching data previously obtained for the oxygen quenching of hemoglobin(desFe) (Jameson et al., 1984b). In the HRP(desFe) system, the low extent of quenching and the lack of significant lifetime heterogeneity in the quenched samples precluded the application of the model which was applied to the myoglobin and hemoglobin systems (Gratton et al., 1984).

Bechara et al. (1979) have reported a Stern-Volmer type analysis of the enhancement of the chemiluminescence signal from the excited acetone as the oxygen is utilized in the reaction and estimated that the bimolecular quenching constant/lifetime product was on the order of 5000 M$^{-1}$. Using our value for the bimolecular quenching rate constant, we thus estimate that the lifetime of the excited acetone would be in the range of 25 ms. This lifetime agrees quite well with the reported value of 22 µs for the lifetime of triplet acetone in deaerated water (Porter et al., 1971). This apparent agreement assumes, however, that the quenching efficiency of the triplet acetone state by oxygen is near unity, which may not be the case. For example, the quenching rate constants by oxygen of protein indole triplet states are an order of magnitude smaller than those of the singlet state (Ghiron et al., 1988). Nonetheless, this result supports the idea of greatly reduced oxygen diffusion to the active site in HRP, relative to other protein systems which have been studied, thus facilitating the observation of the phosphorescence of the excited triplet state of the enzymatic generated acetone. We have previously observed (unpublished observations) that HRP and HRP(desFe) are significantly more resistant to denaturation by guanidinium hydrochloride (GuHCl) or urea than is myoglobin. In the HRP case, GuHCl concentrations of 5.5–6 M are required to remove the heme moiety as compared to around 2–2.5 M (Schechter and Epstein, 1968) for myoglobin. The low diffusional rate for oxygen to the heme pocket in HRP(desFe) is thus consistent with the fact that the porphyrin moiety is tightly bound in a rigid environment.

Acknowledgements—We are grateful to Ms. Vickey Thomas for technical assistance. This work was supported by National Science Foundation—SDC grant INT-8603629. Robert A. Welch Foundation grant 1-986 (D.M.J.) and FONDECTY-Chile grant 697/88 (J.E.B.). D.M.J. is an Established Investigator of the American Heart Association.

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