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Intrinsic tryptophan phosphorescence as a marker of conformation and oxygen diffusion in purified cytochrome oxidase

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Cytochrome oxidase exhibits phosphorescence from tryptophan in aqueous solution in the absence of oxygen. The lifetime for the resting reduced enzyme suspended in Tween-20 is around 30 ms at pH 8. The lifetime is longest between pH 7 and 8 and decreases with lowering of pH. Oxygen quenches the phosphorescence with a Stern-Volmer quenching constant of $\sim 5 \times 10^7$ $M^{-1} \cdot s^{-1}$ at 5°C whereas cytochrome *c* has no effect. We interpret these results to indicate that room temperature tryptophan phosphorescence arises from tryptophan(s) in structured region(s) remote from the hemes and that the protein does not impose a significant barrier for the diffusion of oxygen.

Cytochrome oxidase; Oxygen; Phosphorescence; Tryptophan

1. INTRODUCTION

Cytochrome oxidase is an integral protein of the mitochondrial respiratory chain that catalyzes the reduction of oxygen by cytochrome *c*. In addition, the oxidase in intact mitochondria is implicated in the generation of an H^+ ion gradient that serves as a coupling factor in ATP synthesis [1,2]. To accomplish these feats, the mammalian enzyme contains two hemes and two coppers and a polypeptide aggregate of some 13 subunits with a total molecular weight on the order of 200 000 [3–5]. Although there is evidence concerning the location of the hemes [6,7], the overall structure of oxidase is unknown. Therefore, it is especially desirable to have an intrinsic marker of structure for this complex protein. In this paper we report that tryptophan phosphorescence from purified cytochrome oxidase can be observed.

2. MATERIALS AND METHODS

Horse heart cytochrome *c*, type VI, glucose oxidase, catalase were obtained from Sigma Chemical Co. (St. Louis, MO). Cytochrome oxidase was prepared from beef heart using the non-ionic detergent method [8] according to the King method with slight modifications [9,10]. The oxidase contains 10.5–11 nmol heme *a*/mg of protein. Its specific activity was 16 s^{-1}/mg protein/3 ml at pH 5.7, 23°C, expressed as the oxidation of the reduced oxidase. Heme *a* was determined in oxidase used E^{red}–E^{ox} as 7.6 $cm^{-1} \cdot mM^{-1}$ at 605 nm.

Phosphorescence lifetimes were measured by using the instrument described by Green et al. [11].

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Oxygen was removed from the sample as follows: the buffer was first bubbled with argon and then the oxidase and a coupled enzyme system (80 nM glucose oxidase, 16 catalase and 0.3% glucose) were added. To reduce the heme *a*, dithionite was added at low levels. The optical spectrum of cytochrome oxidase was monitored during our experiments to ascertain the oxidation state of the hemes and also to make sure that dithionite was not added in excess to cause high absorption at the excitation wavelengths.

Oxygen and nitrite quenching was determined by the Stern-Volmer equation [12] modified for lifetimes:

$$\tau_0/\tau = 1 + k_q \tau_0 [Q] \quad (1)$$

where τ_0 is the lifetime in deoxygenated sample, τ is the lifetime at a given oxygen or nitrite concentration and k_q is the bimolecular quenching constant. The oxygen concentration was measured using a water-soluble oxygen-sensitive phosphor, palladium coproporphyrin (Porphyrin Products, Logan, UT) as described in Calhoun et al. [13]. The oxygen concentration present was calculated from the measured lifetime of the probe using a quenching constant of 1×10^9 $M^{-1} \cdot s^{-1}$ at 5°C. Oxygen concentration was varied by injecting the samples with various amounts of air before the experiments.

3. RESULTS

3.1. *Emission of cytochrome oxidase*

In the absence of oxygen purified cytochrome oxidase exhibits weak but detectable phosphorescence at 5°C. The emission maximum was at 450 nm (Fig. 1B) with an excitation in the ultraviolet region (Fig. 1A) where tryptophan absorbs. The spectra shows an apparent red shift relative to the phosphorescence of other tryptophan-containing proteins measured at room temperature, but the strong Soret absorption at ~420 nm produced a trivial absorption artifact. This is substantiated by the observation that the spectra for the oxidized and reduced enzymes shifted with the shift of the Soret maximum (spectra not shown). The decay of

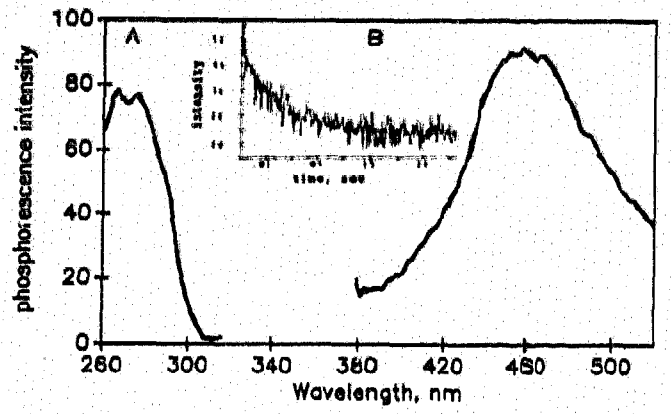

Fig. 1. Phosphorescence excitation (A) and emission (B) spectra of deoxygenated cytochrome oxidase at 5°C. The samples contained 4.3 μM cytochrome oxidase in 10 mM phosphate, 0.2% Tween-20, pH 8.0. Excitation spectrum was recorded using 460 nm as emission wavelength; emission spectrum was measured using 280 nm light beam for excitation. Oxygen was removed from the samples as described in section 2. For both spectra delay time of 0.5 m and gate time 2 ms was applied. Inset: phosphorescence decay.

phosphorescence was fitted by an exponential function (Fig. 1, inset). The emission was also measured at 15, 20 and 30°C. The intensity decreased significantly with increasing temperatures.

The phosphorescence lifetime showed variability using different preparations and different detergents for the measurements. Our data showed good reproducibility when the samples are stored in liquid N_2, thawed just prior to the experiments and dialyzed against 10 nM phosphate, 0.2% Tween-20, pH 8. Samples solubilized in other detergents, such as Triton X-100 and Na-cholate, showed variations in lifetimes. The effect of different detergents on the emission of cytochrome oxidase requires further investigation.

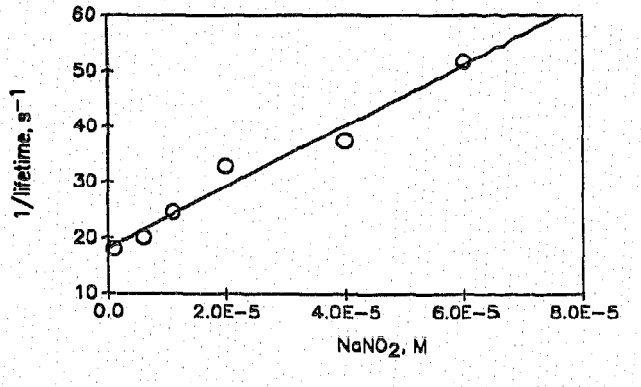

Fig. 2. Quenching of phosphorescence lifetime as a function of nitrite concentration. Stern-Volmer plot of phosphorescence quenching of reduced cytochrome oxidase by $NaNO_2$. Conditions given in legend of Fig. 1.

The quenchability of tryptophan is a function of its location [13]. To estimate its location, nitrite was used as a quencher. The Stern-Volmer quenching profile was linear (Fig. 2) and the quenching constant was $5.2 \times 10^5\ M^{-1} \cdot s^{-1}$. Based upon the dependence of quenching with distance for known quenchers [15], this quenching constant indicates that the emitting tryptophan is buried in the protein, with the closest accessible edge being around 2–3 Å from the surface.

3.2. *Emission as a function of changes in the protein.*

The emission depended upon the redox state of the protein (Table I). In the oxidized sample, the lifetime was 16.4 ms; in the fully reduced sample, the lifetime was 32.4 ms. When the hemes were partially reduced, an intermediate lifetime was observed. The decay data are not accurate enough to resolve whether these decay profiles are the sum of exponentials or indeed single exponential.

The phosphorescence lifetime was very dependent upon pH. The lifetime was maximal at pH 8, being significantly lower at lower pH s (Fig. 3A).

Addition of horse heart cytochrome *c* in stoichiometric ratio and in excess up to 4:1 in low salt (conditions as in the legend of Fig. 1) or high NaCl concentrations led to no significant change in the phosphorescence lifetime. At low ionic strength cytochrome *c* binds to the oxidase, whereas at high ionic strength it dissociates from the oxidase. The emission lifetime showed a dependence upon the salt concentration. There was a moderate (~15%) increase in phosphorescence lifetime up to 0.2 M NaCl concentration, then a decrease at 0.5 M (Fig. 3B).

Oxygen quenches the phosphorescence of the tryptophan in oxidase. The Stern-Volmer quenching plot showed linear dependence upon $[O_2]$ (Fig. 4). The oxygen quenching constants were found to vary between 4 and $7 \times 10^7 M^{-1} \cdot s^{-1}$ in 9 independent experiments.

Table I

Lifetimes of tryptophan in cytochrome oxidase as a function of oxidation

Concentration of cyt. oxidase (μM)		Phosphorescence lifetimes (ms)	
Oxidized	Reduced	Trp	Pd-coproporphyrin
14.3	-	16.4	0.96
6.1	8.2	22.4	0.96
1.4	12.9	26.8	0.94
0.7	13.4	32.4	0.96

Measurements were carried out at 5°C in 10 mM phosphate, 0.2% Tween-20, pH 8. The cytochrome oxidase was reduced by sequential additions of Na-dithionite and the concentrations were determined from the absorption spectra of the samples using molar extinction coefficients $E_{443} = 107\ mM^{-1} \cdot cm^{-1}$ and $E_{421} = 83\ mM^{-1} \cdot cm^{-1}$ for the oxidized and reduced form, respectively. Pd-coprophorphyrin was used to monitor the oxygen concentration in the samples according to Calhoun et al. [13].

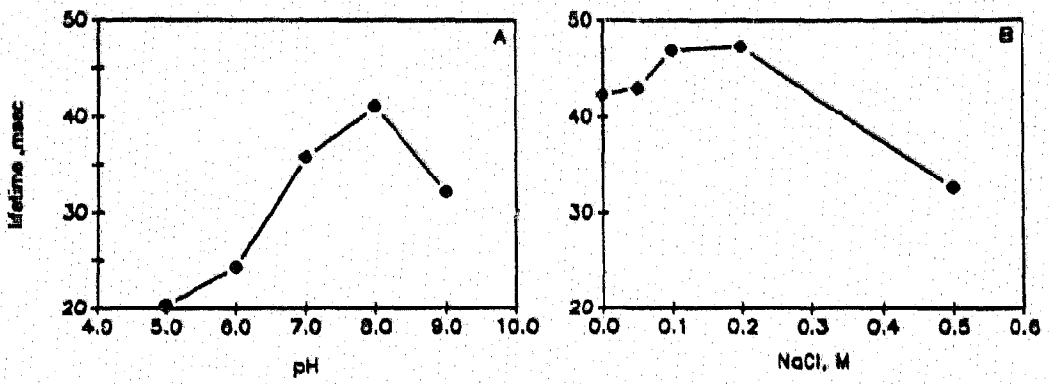

Fig. 3. The effect of pH (A) and ionic strength (B) on the phosphorescence lifetime of cytochrome oxidase. Conditions given in legend of Fig. 1.

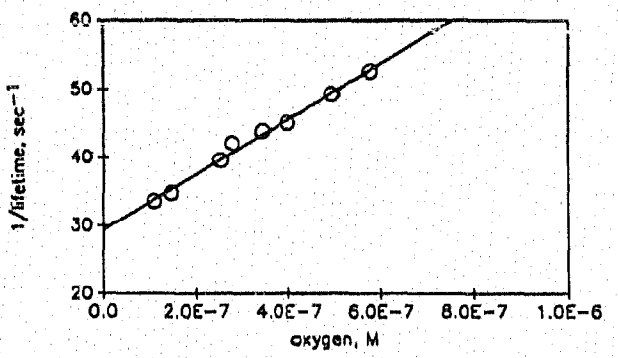

Fig. 4. Phosphorescence quenching of reduced cytochrome oxidase by oxygen at 5°C. Stern-Volmer plot of phosphorescence quenching of reduced cytochrome oxidase by oxygen in 10 mM phosphate, 0.2% Tween-20, pH 8. The oxygen concentrations were determined according to Green et al. [11], and the samples were prepared as described in section 2.

4. DISCUSSION

With the recent recognition that phosphorescence from most proteins can be observed at room temperature, phosphorescence is increasingly being used to study proteins [16,17]. In this paper we show for the first time that cytochrome oxidase also exhibits tryptophan phosphorescence at ambient temperatures.

There are about 50 tryptophans in the oxidase [18,19]. Therefore, assignment of the emitting tryptophan(s) would be a daunting task. However, some indication of the tryptophan location can be obtained based on its spectral characteristics. The triplet state arises from the singlet state and so processes that quench fluorescence will also limit the observation of phosphorescence. Because tryptophan fluorescence emission overlaps the heme absorption spectrum there is dipolar Forster energy transfer from excited singlet state tryptophan to heme. For this reason neither fluorescence nor phosphorescence can be expected for tryptophans close to the heme centers. This distance is around 40–50 Å [20], and therefore tryptophans around the redox centers themselves will not be detected. Hill et al. [21] made a study of the oxidase fluorescence. Using quenching experiments they found that the emitting tryptophans could be localized in the interior of the protein, away from the cytochrome *c* binding domain. We also found that the observed phosphorescence is insensitive to the binding of cytochrome *c* indicating that it also arises from tryptophan(s) away from the cytochrome *c* binding site and the heme centers of oxidase. Phosphorescence measurements are more selective than fluorescence since only those tryptophans that are buried and in a relatively restricted environment will exhibit long-lived phosphorescence [17,22,23].

Determination of oxygen penetrability is especially interesting for this protein which is able to use oxygen down to levels less than μM. No data was previously available for oxygen diffusion in oxidase. We found an average diffusion constant of 5×10^7 $M^{-1} \cdot s^{-1}$, a value somewhat less than for a fully exposed tryptophan which is usually around 10^9 $M^{-1} \cdot s^{-1}$ for phosphorescence quenching at room temperature [13]. A question is whether the diffusion of oxygen through the protein can ever become rate-limiting. Greenwood and associates in studying the reaction of oxidase with oxygen found a value of 3–6 10^7 $M^{-1} \cdot s^{-1}$ [24,25]. Therefore we concur with these authors that the limiting rate for reduction may be the diffusion of oxygen.

There are indications that the tryptophan phosphorescence is sensitive to the structure of the oxidase, but how these changes relate to function is not clear. pH has a dramatic effect on the phosphorescence yield with the maximum occurring at pH 8, where activity is low and the lifetime decreases with lowering the pH, the range where the activity increases [26–28]. Phosphorescence lifetimes also were affected by the redox changes in the oxidase. The reduced enzyme had detectibly longer lifetime than the oxidized enzyme.

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REFERENCES

[1] Mitchell, P. (1979) Science 206, 1148–1159.
[2] Wikstrom, M. and Casey, R.P. (1985) J. Inorg. Biochem. 23, 327–334.
[3] Freedman, J.A. and Chan, S.H.P. (1984) J. Bioenerg. Biomembr. 16, 75–100.
[4] Hatefi, Y. (1985) Annu. Rev. Biochem. 1015–1069.
[5] Capaldi, R.A. (1990) Annu. Rev. Biochem. 59, 569–596.
[6] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[7] Ludwig, B. (1987) FEMS Microbiol. Rev. 46, 41–56.
[8] Sun, F.F. and Jacobs, E.E. (1967) Biochim. Biophys. Acta 143, 633–641.
[9] Kuboyama, M., Yong, F.C. and King, T.E. (1972) J. Biol. Chem. 247, 6375–6383.
[10] Hartzell, C.R., Beinert, H., van Gelder, B.F. and King, T.E. (1978) Methods Enzymol. 53, 54–66.
[11] Green, T.J., Wilson, D.F., Vanderkooi, J.M. and DeFeo, S.P. (1988) Anal. Biochem. 174, 73–79.
[12] Stern, O. and Volmer, M. (1919) Phys. Z. 20, 183–188.
[13] Calhoun, D.B., Englander, S.W., Wright, W.W. and Vanderkooi, J.M. (1988) Biochemistry 27, 8466–8474.
[14] Vanderkooi, J.M., Maniara, G., Green, T.J. and Wilson, D.F. (1987) J. Biol. Chem. 262, 5476–5482.
[15] Vanderkooi, J.M., Englander, S.W., Papp, S., Wright, W.W. and Owen, C.S. (1990) Proc. Natl. Acad. Sci. USA 87, 5099–5103.
[16] Vanderkooi, J.M., Calhoun, D.B. and Englander, S.W. (1987) Science 236, 568–569.
[17] Papp, S. and Vanderkooi, J.M. (1989) Photochem. Photobiol. 49, 775–784.
[18] Matsubara, H., Orii, Y. and Okunuki, K. (1965) Biochim. Biophys. Acta 97, 61–67.
[19] Buse, G., Meinecke, L. and Bruch, B. (1985) J. Inorg. Biochem. 23, 149–153.
[20] Teale, F.W.J. and Weber, G. (1959) Biochem. J. 72, 15.
[21] Hill, B.C., Horowitz, P.M. and Robinson, N.C. (1986) Biochemistry 25, 2287–2292.
[22] Strambini, G.B. and Gonnelli, E. (1985) Chem. Phys. Lett. 115, 196–200.
[23] Strambini, G.B. and Gonnelli, E. (1990) Biochemistry 29, 196–203.
[24] Gibson, Q.H. and Greenwood, C. (1963) Biochem. J. 86, 541–554.
[25] Hill, B.C. and Greenwood, C. (1984) Biochem. J. 218, 913–921.
[26] Wainio, W.W., Eichel, B. and Gould, A. (1960) J. Biol. Chem. 235, 1521–1525.
[27] Wilms, J., Van Rijn, L.M.L. and Van Gelder, B.F. (1980) Biochim. Biophys. Acta 593, 17–23.
[28] Thornstrom, P., Soussi, B., Arvidsson, G. and Malmstrom, B.G. (1984) Chem. Scripta 24, 230–237.