SINGLET OXYGEN GENERATION BY THE LIPOXIDASE SYSTEM*

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Summary: The rate of cytochrome c bleaching by the lipoxidase system is increased in deuterium oxide buffers. This result is consistent with the view that the lipoxidase system produces singlet oxygen. This species does not seem to be responsible for the self-catalyzed destruction of lipoxidase.

The belief that non-radiative excited electronic states may be generated in some biological processes is becoming widespread (1,2). One of the excited species most likely to be generated is singlet oxygen ($^1\Delta g$). In this species, which is 22.5 Kcal above the ground state ($^3\Sigma g^-$) the two unpaired electrons of the degenerate orbital occupy a single orbital:

Singlet oxygen, $^1O_2$, can be generated from superoxide ions(3)

$$O_2^- + O_2^- \xrightarrow{H^+} {}^1O_2 + H_2O_2$$

$$O_2^- + {}^{\bullet}OH \longrightarrow {}^1O_2 + OH^-$$

and also from the disproportioning of peroxy radicals (4):

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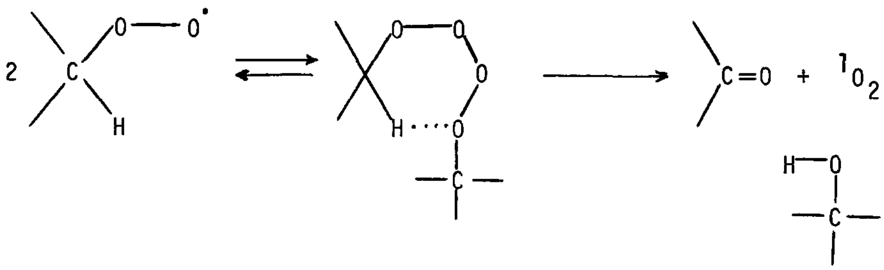

It is often assumed that the very weak emissions observed in some biological systems result from a "leakage" and as such behave as a tracer of electronic energy generation. Finazzi-Agrò et al (5) observed chemiluminescence in the lipoxidase-linoleate system and ascribed it to singlet oxygen. This species was also considered responsible for the bleaching of cytochrome c when the latter was added to the system. Actually, $^1O_2$ is a very reactive species and as such also oxidizes pyrrole derivatives (6).

Since the lifetime of $^1O_2$ is increased about ten times in D_2O relative to H_2O (7), one might expect a large increase in the rate of cytochrome c bleaching under conditions that most of the excited oxygen decays without reaction. This provides a test for the conclusion drawn by Finazzi-Agrò et al (5) which, in turn, might shed light on the vexed question of $^1O_2$ formation by the lipoxidase system (8-11). Such a method does not have the inconvenience of tests based on the quenching effect of foreign substances, such as sodium azide and β-carotene (7).

Materials and Methods. Linoleic acid, 99% pure, from Sigma Chemical Co., was converted to the ammonium salt with ammonium hydroxide. Soybean lipoxidase, Type I, and horse heart cytochrome c, Type II. were from Sigma. Deuterium oxide, was either from Stohler Isotope Chemicals (99,8%) or from Sigma (99,7%).

The enzymatic system contained lipoxidase (10^{-6}M), linoleate (6×10^{-3}M) and cytochrome c (3.0×10^{-5} - 6.0×10^{-4}M) in 0.1M

Tris-HCl buffer. Spectral readings were taken on a Zeiss DMR-21 Recording Spectrophotometer with cells of 1.0 cm optical path at 35° and at 26°C.

To follow the formation of conjugated hydroperoxides as a function of time (no cytochrome added) the solutions in H_2O and D_2O buffers were shaken in a Warburg apparatus. Aliquots were taken at different times and diluted (1:10) with ethanol (12). Spectra were taken in 1 mm cells. From the spectrum the absorbance at 234 mμ was recorded.

Results and Discussion

At 35°C the rate of cytochrome c bleaching by the lipoxidase system is increased 4-5 times in going from water buffer (pH 9.0) to deuterium oxide buffer (pD = 8.6; see ref. 13 and 14). A representative example appears in Fig. 1. At pD = 9.0 the reaction is slightly slower than at pD = 8.6. At 26° the deuterium effect was smaller (about 2).

The initial rate of cytochrome c bleaching increased linearly

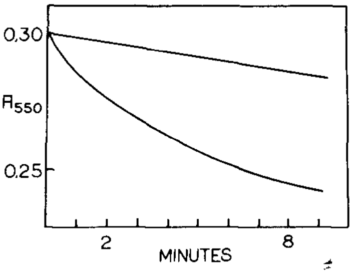

FIG. 1. The bleaching of cytochrome c (6×10^{-5}M) by the linoleate (6×10^{-3}M)-lipoxidase (1×10^{-6}M) system in 0.1M Tris-HCl water buffer, at pH 9.0 (upper curve) and in deuterium oxide buffer (pD = 8.6). T = 35°C. Optical path = 1.0 cm.

with the cytochrome concentration in the range investigated $3.0 \times 10^{-5} - 6.0 \times 10^{-4}$M.

It is unlikely that the increase of reaction velocity in D_2O is due to a denaturation of the cytochrome because D_2O has usually a stabilising effect (15). On the other hand, such a stabilising effect if exerted upon the lipoxidase, would protect it from self--inactivation (11) and therefore might explain the increased bleaching in D_2O. This interpretation is ruled out on two grounds. First, the deuterium effect is observed especially upon the initial velocity. Second, the same curve is obtained in H_2O and D_2O when the formation of conjugated hydroperoxides (A_{234}) is followed as a function of time (Fig. 2).

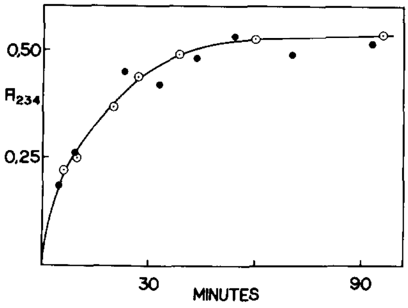

FIG. 2. The formation of conjugated hydroperoxides as a function of time in the linoleate-lipoxidase system, in water buffer at pH 9.0 (full circles) and in deuterium oxide buffer (pD = 8.6) T = 26°C. For details see Materials and Methods.

One possible interpretation of our results is that the D_2O effect is due indeed to the formation of $^1O_2$. Simple calculations then suggest that singlet oxygen is likely to be an important product of the reaction. This in turn makes unlikely that $^1O_2$ originates mainly from the disproportioning of peroxy radicals (16).

More likely, $^1O_2$ would be formed from an intermediate of the main reaction. Important on this regard is that Smith and Lands (11) have proposed a high energy planar-oriented tetroxide and a pereppoxide intermediate "energetically equivalent to (and perhaps in equilibrium with) singlet state oxygen". According to them these reactive intermediates might also react with other compounds. The large deuterium effect we have observed suggests that $^1O_2$ may actually be formed. However since the effect is smaller than by a factor of ten, it seems necessary to assume that $^1O_2$ reacts with cytochrome c in the very act of its formation. The alternative is that in water only 35-45% of the bleaching is due to $^1O_2$, 55-65% being due to the reaction with linoleate hydroperoxide (17).

The "D_2O tool" has hitherto been applied only to photochemical processes (18, 19). Our results also indicate that $^1O_2$ is not responsible for the inactivation of lipoxidase. Work now in progress suggests that $^1O_2$ may also be responsible for the destruction of cytochrome P-450 heme during lipid peroxidation (20).

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