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THE RED ABSORBING FLAVIN SPECIES IN THE REACTION OF BACTERIAL LUCIFERASE WITH $FMNH_2$ AND O_2[1]

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The reaction of luciferase-bound $FMNH_2$ is known to result in the formation of a long-lived intermediate in the bioluminescent reaction (1). This intermediate was isolated and characterized as the luciferase-peroxyflavin (2), whose structure was later shown to be the flavin 4a-substituted peroxy-adduct (3). In the earlier work this peroxy intermediate had been shown to exhibit a single peak at about 370 nm, a shoulder at about 460 nm, the absorption tailing off around 500 nm, with none above 520 nm.

In more recent publications, however (4,5), it has been reported that the reaction of the luciferase-bound reduced flavin mononucleotide with O_2 also results in the appearance

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of absorption in the 570-610 nm region. Although the spectrum appeared similar to that of the neutral semiquinone (6), no ESR signal was detected in preliminary experiments. Kinetic data suggested that this red absorbing species was related to, possibly in rapid equilibrium with, luciferase intermediates (such as the peroxyflavin) in the pathway leading to light emission.

The present work was undertaken in order to study the formation, properties and possible role in bioluminescence of the material absorbing in the 600 nm region, and to resolve some of the questions raised by the observations described above.

Since no absorbance in the red was noted in the preparations of peroxyflavin earlier isolated and characterized (2, 7,8), we undertook the preparation of the peroxy intermediate

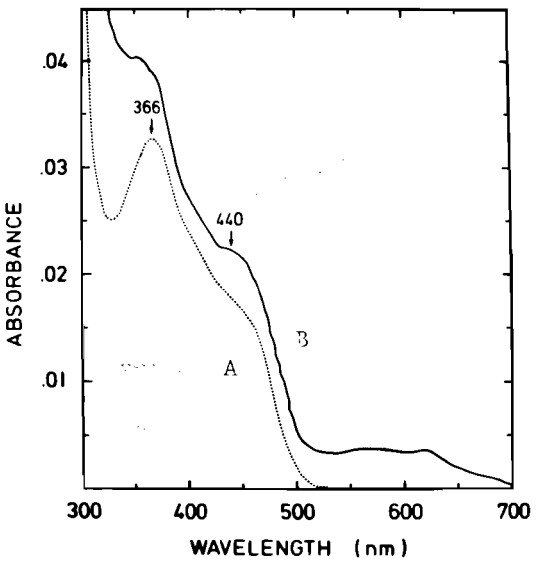

FIGURE 1. Absorption spectra taken at 0° of the Sephadex G-25 purified luciferase-flavin intermediates formed by reaction of luciferase-FMNH$_2$ with oxygen at 0° (A) when the material reduced with excess dithionite was applied directly to the column and (B) when the material was fully oxidized by exposure to air for 3 minutes prior to application to the column. The first (A) appears to be virtually all in the peroxyflavin form, while the second (B) is a mixture of that with the luciferase neutral flavin semiquinone.

following the gel filtration procedure of Hastings and Becvar (9). The result was clear (Figure 1, Curve A); peroxyflavin with no red absorbance was formed.

The essential step of the procedure required to obtain a preparation lacking the blue species seemed to be the reduction of the luciferase-flavin complex with excess dithionite and its application to the Sephadex column without prior reoxidation. In this way the luciferase-bound flavin presumably encountered molecular oxygen on the column only after, or in the course of, its separation from small molecules: flavins, dithionite, H_2O_2, and other products. As a test of this idea, we made a similar preparation but reoxidized with the air in the test tube at 0°C three minutes prior to subjecting the material to Sephadex chromatography. Again, the result was clear (Figure 1, Curve B); a similar amount of peroxyflavin was formed but there was, in addition, significant absorption in the 570-610 nm region. The difference spectrum between traces A and B resembles the spectrum for the purified luciferase-neutral semiquinone (see Figure 2); the absence of a trough in trace B at 330 nm is attributed to the significant contribution that the absorption of the semiquinone makes in this region.

The spontaneous decay of the purified luciferase peroxyflavin was followed, as before (8), by the increase in absorption at 440 nm. The half-life at 2° was about 55 minutes similar to previously reported values under similar conditions. A most significant feature of this experiment was the fact that during the decay there was no development of absorbance in the 570-610 nm region. Thus, under these conditions, no appreciable conversion of the peroxyflavin to the blue species occurred.

As was illustrated in Figure 1, trace B, the oxidation of the reduced flavin-luciferase complex by oxygen resulted in the simultaneous formation of both the peroxy and semiquinone luciferase intermediates. In aerated buffer at 0°C the subsequent decay of the peroxy compound is more rapid (half-life, 50 min) than that of the semiquinone (half-life, 20 hr; Figure 3 inset). This means that the aged preparations should have only the latter species, which has been found to be so.

Such an aged preparation was subjected to chromatography on a Sephadex G-25 at 2°C. Since the affinity of oxidized FMN for luciferase is low (8,10), the majority of the protein-bound flavin species eluted is the luciferase semiquinone. The spectrum of this Sephadex purified material (Figure 2) is fully characteristic of the neutral flavin semiquinone (6). In the same (aerobic) buffer it decayed isosbestically to FMN, the resulting redox equivalents presumably being taken up by oxygen. Based on the amount of FMN finally formed, and

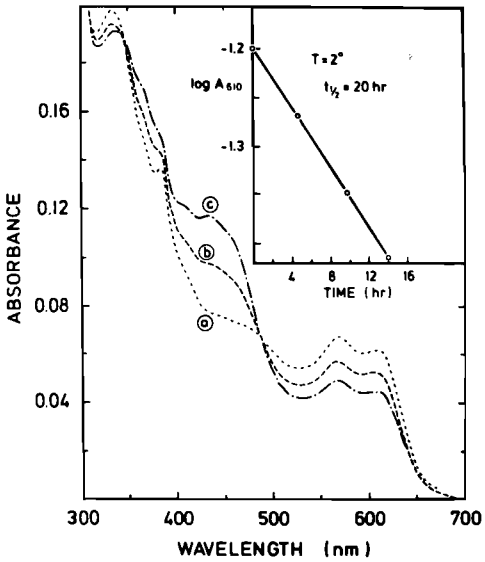

FIGURE 2. Absorption spectra of the luciferase neutral flavin semiquinone (a) a few minutes after elution from G-25 Sephadex and at two later times (b,c) in the course of its decay to FMN and luciferase. The kinetics of the decay are shown in the inset. To 0.5 mg of luciferase in 0.5 ml was added 0.5 ml of 1 mM $FMNH_2$ (photoreduced) 10 mM EDTA, 0.1 M phosphate buffer, pH 7, under low oxygen tension. After repeated photo reduction and reoxidation, the material was allowed to stand at 0° for 1 hour prior to chromatography on Sephadex G-25.

assuming that all of the Sephadex purified material was originally in the neutral semiquinone form, the millimolar extinction coefficient for the latter at 610 nm was calculated to be about 4.8.

As shown in the inset of Figure 2, the luciferase semiquinone decayed slowly at 2°C, with a half-lifetime of about 20 hours. Moreover, the decay under these conditions exhibited a high temperature coefficient with an activation energy of about 46 kilocalories. This suggests that the protein may have to change conformation in order to allow either dissociation of the semiquinone or its accessibility to an oxidizing species.

A sample of such a preparation of the blue species was frozen in liquid nitrogen; its ESR signal was measured and found to be characteristic of the neutral flavin radical. A

millimolar extinction coefficient of about 4.5 was estimated by reference to a 5-ethyl-riboflavin radical standard.

The luciferase neutral semiquinone radical was also formed by titration with dithionite. Dithionite was added step-wise to a mixture of luciferase (0.65 mM) and FMN (0.15 mM). One equivalent of dithionite was required for the full reduction of the flavin; at half reduction the maximum amount of the blue flavin semiquinone was formed. A millimolar extinction coefficient of 4.2 was estimated.

Based on these results it appeared that the flavin semiquinone would be formed directly by reaction of the reduced and oxidized species. This model was verified by mixing equimolar amounts of FMN and $FMNH_2$ with luciferase at 11° in the absence of oxygen. An appreciable amount of the semiquinone was formed within the first minute; this was followed by a slower increase to an equilibrium value. Under these conditions (absence of oxygen) the semiquinone was stable over a period of 36 hours at temperatures between 0° and 26°, the equilibrium between the semiquinone and the other forms being highly temperature dependent. More than twice as much semiquinone (absorption at 610 nm) was present at 0° as at 26°.

The mechanism of the oxidation of aldehyde by the luciferase peroxyflavin intermediate to produce an excited state is a main question to be addressed; the possible involvement of a blue intermediate had prompted interest, proposals, and speculation (11, 12, 13). The fact that one can obtain preparations of the peroxyflavin lacking appreciable quantities of the radical blue species suggests that the latter is not in fact involved, at least directly, in the bioluminescent reaction.

The luciferase peroxyflavin reacts with long chain aldehydes, such as decanal and dodecanal (14), to give light with a half decay time of only a few minutes at 0° (1,5). Thus the blue species should decay more rapidly in the presence of aldehyde if it is capable of forming the luciferase-peroxyflavin. In fact, in the presence of aldehyde, the blue species was formed equally well at 0° and decayed even more slowly than in the absence of aldehyde. Thus there is no indication to support the suggestion that the neutral flavin semiquinone radical is formed by or is in equilibrium with the luciferase peroxyflavin species as such.

As would be expected, luciferase complexed with the flavin radical is not active for light emission. It is also not capable of reacting with reduced flavin to give light emission. But its decay (Figure 2, inset) is mirrored by an increase in luciferase activity, as assayed by its reaction with $FMNH_2$ and decanal.

REFERENCES

1. Hastings, J.W., and Q.H. Gibson, *J. Biol. Chem. 238,* 2537 (1963).
2. Hastings, J.W., C. Balny, C. Le Peuch, and P. Douzou, *Proc. Nat. Acad. Sci. 70,* 3468 (1973).
3. Ghisla, S., J.W. Hastings, V. Favaudon, and J.M. Lhoste, *Proc. Nat. Sci. 75,* 5860 (1978).
4. Presswood, R.P., and J.W. Hastings, *Biochem. Biophys. Res. Comm. 82,* 990 (1978).
5. Presswood, R.P., and J.W. Hastings, *Photochem. Photobiol. 30,* 93 (1979).
6. Massey, V., and G. Palmer, *Biochemistry 5,* 3181 (1966).
7. Hastings, J.W., and C. Balny, *J. Biol. Chem. 250,* 7288 (1975).
8. Becvar, J.E., S.-C. Tu, and J.W. Hastings, *Biochemistry 17,* 1807 (1978).
9. Hastings, J.W., and J.E. Becvar, *Methods in Enzymology, 57,* 194 (1978).
10. Baldwin, T.O., M.Z. Nicoli, J.E. Becvar, and J.W. Hastings, *J. Biol. Chem. 250,* 2763 (1975).
11. Mager, H.I., and R. Addink, *Tetrahedron Lett. 37,* 3545 (1979).
12. Kosower, E.M., *Biochem. Biophys. Res. Comm. 92,* 356 (1980).
13. Wessiak, A., G.E. Trout, and P. Hemmerich, *Tetrahedron Lett. 21,* 739 (1980).
14. Hastings, J.W., K. Weber, J. Friedland, A. Eberhard, G.W. Mitchell, and A. Gunsalus, *Biochemistry 8,* 4681 (1969).