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ENERGY TRANSFER FROM ENZYMICALLY GENERATED TRIPLET CARBONYL COMPOUNDS TO THE FLUORESCENT STATE OF FLAVINS

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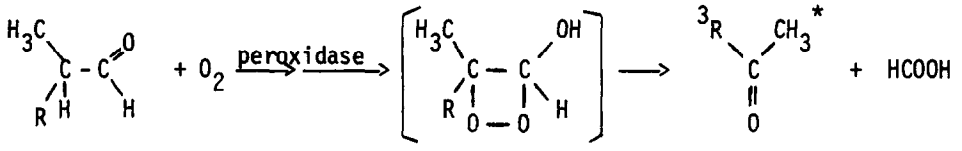
SUMMARY: Enzymically generated triplet carbonyl compounds transfer energy to the fluorescent state of flavins as shown by the suppression of the carbonyl chemiphosphorescence and concomitant appearance of the flavin fluorescence. A Stern-Volmer analysis including the effect of the collisional quenching by a diene indicates that the transfer occurs by a long range process.

The present results open the way to "photobiology without light".

INTRODUCTION

The generation of electronically excited triplet carbonyl compounds by enzymic (peroxidase-oxidase) systems has been firmly established in these laboratories (1-4). The reaction is:



The examples which have been studied in detail are those in which R is H or CH_3 (1-4). The triplet species is generated within the enzyme and thus is considerably protected from collisional deactivation by oxygen. In the case of triplet acetone this is demonstrated by (i) acetone phosphorescence emission (ii) a lifetime longer than 1×10^{-5} sec. (iii) formation of photo-products e.g. isopropanol (iv) quenching by dienes, for instance sorbic acid, with a K_q of 1.2×10^8 M^{-1} sec^{-1} which is much smaller than by diffusion control in solution (7×10^9 M^{-1} sec^{-1}).

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An analogy is provided by those proteins which phosphoresce at room temperature in aerated aqueous solution (5,6).

The enzyme shielded triplet carbonyl compounds can transfer energy efficiently to the excited singlet state of 9,10-dibromoanthracene-2-sulfonate (DBAS, sodium salt)[§] as shown by the appearance of a strong fluorescence emission (1,2), the spectrum being that of the acceptor (3,4). Triplet-singlet energy transfer is spin forbidden (7) but in this case the transfer is facilitated by the heavy atom perturbation effect (8-12) of bromine atoms; thus the non-halogenated acceptor does not fluoresce when added to the system (1-4).

Clearly it would be of great interest to demonstrate energy transfer to photobiologically important and naturally occurring compounds, because this would open the way for "photo biology without light" (13-15). Even without heavy atoms in the acceptor it is expected that the spin forbidden nature of triplet-singlet energy transfer is compensated by the long lifetime of the donor (7); energy transfer can indeed occur efficiently even at long distances provided that the acceptor absorbs strongly in the region of the donor phosphorescence (7,16).

Flavins absorb strongly in the region where aliphatic carbonyl compounds phosphoresce (λ_{max} ~435 nm). In this communication we report energy transfer from enzymically generated triplet acetone and acetaldehyde to flavins.

MATERIAL AND METHODS

Horseradish peroxidase (HRP, Type VI), FMN and FAD (> 96% pure, by ultraviolet (17), fluorescence (18) and paper

[§]Abbreviations used: DBAS, 9,10-dibromoanthracene-2-sulfonate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; HRP, horseradish peroxidase.

chromatography (19) analysis, no detectable FMN impurities appeared) were from Sigma. Isobutanal from Merck was purified by distillation under nitrogen. Riboflavin was from Merck.

The standard reaction mixture was prepared as follows: 10 µl of an aqueous solution of 5 x 10^{-4}M solution of HRP was added to a mixture of 1 ml of 0.1M pyrophosphate buffer, pH 7.4, and 2.5 ml of 1.0M phosphate buffer, pH 7.4, kept at 40°C. The reaction was started by addition of 0.1 ml of 2.2M solution of isobutanal in ethanol. This reaction mixture was 2.0 µM in HRP; 84.6mM in substrate, 0.5M in ethanol and had a total volume of 2.6ml. The chemiluminescence spectrum was taken immediatly after the addition of substrate. For this and also for fluorescence measurements a Perkin-Elmer fluorescence spectrophotometer MPF-4 was used.

Stock 10 mM solutions of the flavins in water were prepared. For riboflavin one drop of 1.0N NaOH was added. The stock solutions were kept in the dark and used within 12 hours.

RESULTS AND DISCUSSION

When either 2 x 10^{-5}M riboflavin, FMN or FAD was added to the triplet acetone generating system, the acetone chemiphosphorescence disappeared and the flavin fluorescence spectrum was evident (Fig. 1). In the three cases a Stern-Volmer plot of the suppression of the acetone chemiphosphorescence is linear (Fig.2). The slope, that is $k_q\tau$, is 4.5 x 10^4 M^{-1} for riboflavin, 5.0 x 10^4 M^{-1} for FMN and 7.5 x 10^4 M^{-1} for FAD. These values are much larger than the value of 1.2 x 10^3 M^{-1} obtained for sorbic acid (to be published), for which the quenching act must surely require intimate donor-acceptor contact (20). They are, however, similar to the value, of 7.5 x 10^4 M^{-1} obtained for the transfer to DBAS (4), an acceptor for which quenching mechanisms such a "long range" triplet-singlet energy transfer that do not require intimate contact are operative (3). This clearly indicates that the transfer to flavins is not impeded and is therefore a "long-range" process not requiring intimate donor-acceptor contacts.

Values of $k\tau$ can also be obtained from the intercept/slope ratio of a double reciprocal plot of the effect of the acceptor

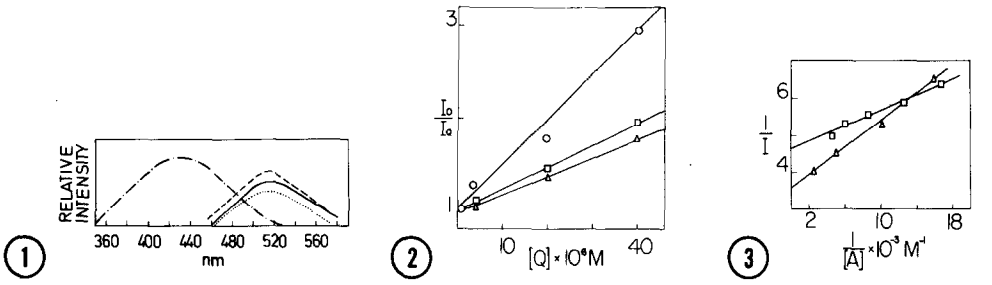

Fig. 1 - Chemiluminescence spectrum of the system isobutanal/HRP/O_2 in the presence of 2 x 10^{-5}M flavin; ——— riboflavin; ---- FMN; ....FAD. The shorter wavelength spectrum, taken in the absence of flavin, originates from triplet acetone generated within the enzyme; this spectrum coincides with the phosphorescence spectrum of acetone.

Fig. 2 - Stern-Volmer plot for the quenching of acetone chemiphosphorescence by riboflavin (-Δ-Δ-); FMN (-□-□-) and FAD (-o-o-). The readings were taken at 435 nm where acetone phosphoresces maximally.

Fig. 3 - Double reciprocal plot of the effect of flavin concentration upon the flavin sensitized chemiluminescence; -Δ-Δ-Δ-, riboflavin; -□-□-□-, FMN.

concentration upon the acceptor fluorescence intensity (21). Fig. 3 shows this plot. The k_T values, 2.0 x 10^4 M^{-1} for riboflavin and 4.8 x 10^4 M^{-1} for FMN agree with those obtained from Fig. 2. In the case of FAD, the flavin emission was independent of the coenzyme concentration. This anomalous behavior of FAD is presumably due to a saturation with the enzyme with this flavin producing conformational changes which occur immediatly after binding of FAD with the enzyme. Perhaps the observed emission (Fig. 1) comes from these few molecules tightly bound to the enzyme (22,23).

Transfer from enzymically generated triplet acetaldehyde to flavins could also be demonstrated albeit only with a photon counter (Hamamatsu TV C-767).

For a possible "in vivo" occurence of the energy transfer process herein reported, it is interesting that, potentially, triplet acetone may arise a peroxidase-oxidase reaction from isobutyric acid, from isobutyryl-CoA or from α-ketoisocaproic acid. Excited linear aldehydes may arise from the higher homologue (4) or from the acid form of the latter (24).

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