DINOFLAGELLATE LUCIFERIN IS STRUCTURALLY RELATED TO CHLOROPHYLL

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1. Introduction

Bioluminescence in dinoflagellates is the result of the oxidation of dinoflagellate luciferin by molecular oxygen, catalyzed by dinoflagellate luciferase [1,2]:

$$\text{Dinoflagellate luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Products} + \text{Light } (\lambda_{max}\ 474\ \text{nm})$$

Among the several genera of dinoflagellates thus far examined, the luciferins and luciferases are cross-reactive [3–5], thus indicating identity or close similarity in their luciferins. Recent studies have suggested that the cross-reactive luciferin of *Pyrocystis lunula,* a non-motile, bladder-like, open ocean dinoflagellate, is an open chain polypyrrole, possibly similar to a bile pigment [6,7].

We report here that luciferin of *Pyrocystis* is a novel bile pigment structurally related to chlorophyll *a* or *c*, distinctly differing from ordinary bile pigments which are derived from, and, therefore, structurally related to heme. Thus the structure of *Pyrocystis* luciferin is similar, though not identical, to the structure of the fluorescent substance 'F' of euphausiid shrimps [8] which has been until now the only known example of naturally occurring bile pigment that is structurally related to chlorophyll.

2. Experimental

Studies reported here were carried out with material isolated from cells of *Pyrocystis lunula* harvested from ~1000 liters of culture, grown over a 3 year period [9]. Purification of luciferin was carried out as in [7], yielding ~10 mg purified luciferin (assuming a molar extinction coefficient in water of 2.8×10^4 at 390 nm and M_r of 600). This was distributed into 10 tubes, evaporated to dryness, and stored under argon at −80°C until used.

Chlorophyll *a* was obtained from Sigma Chemical Co. (St Louis, MO) and pyrochlorophyll *a* produced by boiling chlorophyll *a* in pyridine for 24 h [10]. Bilirubin was purchased from Serva Chemical (Heidelberg) and $^{18}O_2$ (99.1 atom %) and $H_2{}^{18}O$ (90–91 atom %) from Prochem (Summit, NJ). ITLC Type SA silicic acid sheets for thin-layer chromatography (TLC) came from Gelman Instrument Co. (Ann Arbor MI). Ethyl ether and methanol were distilled before use.

Chromic acid oxidation of luciferin and reference compounds (bilirubin, chlorophyll *a* and pyrochlorophyll *a*), and the identification of oxidation products were carried out as in [8], by the method in [11,12]. Two kinds of oxidizing solutions were employed: 1% CrO_3 solution containing 1% $KHSO_4$ (pH 1.2) and 1% CrO_3 in 2 N H_2SO_4. The oxidation products were extracted with ethyl ether, then chromatographed on ITLC Type SA sheets, using 2 solvent systems: CH_2Cl_2/ethyl acetate (10:1, v/v) and CH_2Cl_2/ethyl acetate/ethanol/acetic acid (200:10:5:0.5, by vol.). Identification of oxidation products was done by the mobilities of the spots visualized with Cl_2-benzidine (for maleimides and succinimides) or with 2,4-dinitrophenylhydrazine (for pyrrole aldehydes) [12], in comparison with the oxidation products of the reference compounds. Methylvinylmaleimide was easily recognizable by its blue fluorescence on TLC sheets. The identities of the products were further confirmed by mass spectrometry of the eluate of the spots.

To prepare the blue oxidation product of luciferin,

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an ethanolic solution of luciferin was diluted with 10 vol. acetone, and then slowly titrated with 0.1% I_2 in ethanol, monitoring the absorbance at 630 nm. The reaction was complete when there was no more increase in absorbance with added I_2. The product was chromatographed on ITLC Type SA sheets using acetone/methanol (8:3, v/v), to purify the blue compound (R_F 0.35).

3. Results and discussion

Chromic acid oxidation of *Pyrocystis* luciferin yielded hematinic acid (I), a fused ring aldehyde (II), methylvinylmaleimide (III), and methylethylmaleimide (IV) (fig.1, table 1). Compounds II–IV were obtained when the oxidation was carried out in 1% $KHSO_4$ (pH 1.2) at room temperature, but compound I was obtained only after heating at 90°C in 2 N H_2SO_4. These 4 oxidation products obtained from luciferin were the same as those obtained from pyrochlorophyll *a* except for one minor difference, i.e., the difference of one double bond between hematinic acid (I) derived from luciferin and 3,4-dihydrohematinic acid derived from pyrochlorophyll *a*.

Titration of the luciferin solution with I_2 changed its color from pale greenish-yellow (fig.2A) to dark blue. A blue compound was isolated from the reaction mixture by TLC. The absorption spectrum of the blue compound showed an absorption maximum at 635 nm (in methanol), and indicated the absence of the Soret band around 400 nm (fig.2B), suggesting that the blue compound, probably as well as luciferin itself, is a bile pigment-type compound and not a compound of porphyrin-type.

Sequence analysis of the 4 pyrroles represented by

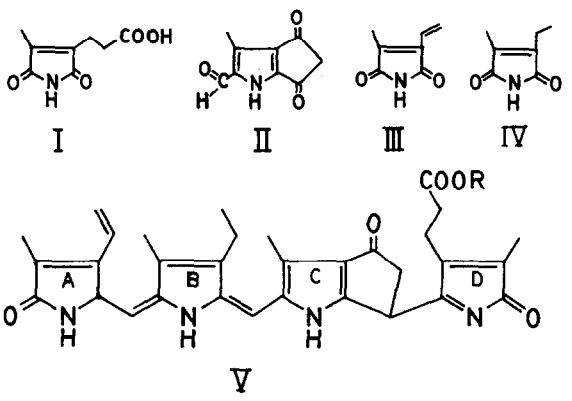

Fig.1. Structures of the CrO_3-oxidation products of dinoflagellate luciferin (I–IV) and a possible partial structure of dinoflagellate luciferin (V).

Table 1
Chromatographic and mass spectrometric properties of the CrO_3-oxidation products of dinoflagellate luciferin

Oxidation product	R_F value[a]	Molecular ion of 0–16 oxidation product (*m/e*)	Molecular ions of 0–18 oxidation product (*m/e*) (Relative abundance)[b]
I	0.36	165[c]	[d]
II[e]	0.24	177	183(100), 181(61), 179(12), 177(3)
III	0.69	137	139(100), 137(9)[f]
IV	0.65	139	143(100), 141(24), 139(9)

[a] TLC with CH_2Cl_2/ethyl acetate/ethanol/acetic acid (200:10:5:0.5) for I, and CH_2Cl_2/ethyl acetate (10:1) for other compounds
[b] Oxidation was carried out in 90–91 atom% $H_2{}^{18}O$ under 99.1 atom% $^{18}O_2$
[c] This is a M-18 ion; the molecular ion was not detectable
[d] No meaningful result was obtained due to a high level of oxygen exchange caused by the conditions of oxidation to produce this compound (2 N H_2SO_4, 90°C)
[e] Unstable on dry TLC sheets: Mass spectrometry of 0–18 oxidation product was performed with the ether extract of the oxidation product of luciferin, without separating by TLC
[f] Identical data were obtained when bilirubin was used instead of luciferin

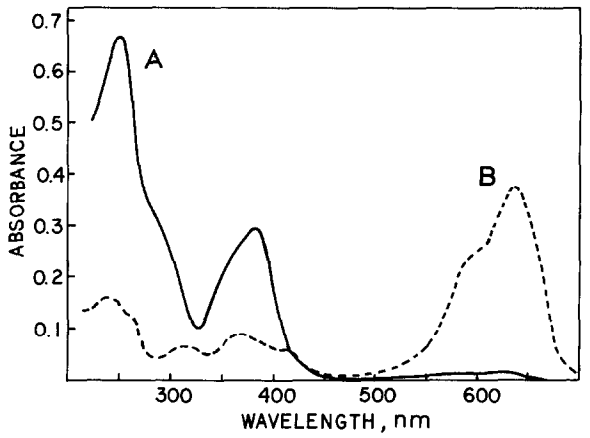

Fig.2. Absorption spectra of: slightly autooxidized dinoflagellate luciferin in 50% ethanol (A), and blue oxidation product of luciferin in methanol (B).

compounds I–IV, was carried out by CrO_3 oxidation of luciferin in the presence of $^{18}O_2$ and $H_2{}^{18}O$ (see [8] for experimental details). Under these conditions, maleimides resulting from the oxidation of the outer 2 pyrroles should show a mass increase of 2 corresponding to the incorporation of one ^{18}O, whereas maleimides or aldehydes resulting from the oxidation of the inner 2 pyrroles should show a mass increase of 4 corresponding to the incorporation of 2 atoms of ^{18}O. In both cases this assumes that none of the oxygen atoms already present are exchangeable. The results of this analysis are shown in table 1. Pyrrole rings of products II and IV must be in the center because they yielded products whose mass had increased by 4. The additional species with a mass increase of 6 noted for II is probably a result of oxygen exchange between the medium $H_2{}^{18}O$ and the keto-oxygen already present in the molecule. Since the mass of compound III increased by only 2 mass units, it must be on the outside, and by process of elimination, I must also be on the outside, although in a derivatized form in the native molecule (see below). The only remaining ambiguity concerns the ordering of the center 2 pyrroles. On the assumption that luciferin is derived from chlorophyll, however, they can be ordered and a tentative structure (V) assigned for the backbone of the molecule (fig.1). Either chlorophyll *a* or chlorophyll *c* could be considered as the likely parent molecule. Dinoflagellates contain both chlorophylls, and either molecule could give the same backbone (V) following only a few enzymatic steps. Based on the evidence that I (free acid) was obtained by the CrO_3-oxidation of luciferin in 2 N H_2SO_4 at 90°C but not in 1% $KHSO_4$ (pH 1.2), luciferin must contain a group which is bound to the carboxyl group of I, probably by an ester bond, as shown by R in structure V. Preliminary efforts at obtaining a molecular ion peak for V by the field desorption mass spectrometry have so far been unsuccessful, so the nature of this R group must await further study.

Structure V is not the same as the structure proposed for the backbone of the fluorescent substance 'F' of euphausiids [8], the major difference apparently arising when putative chlorophyll precursors are split at different points in the ring. Thus, the backbone structure of dinoflagellate luciferin can be formed from pyrochlorophyll *a* or *c* by an oxidative cleavage between ring A and ring D, whereas substance F can be formed from pyrochlorophyll *b* by cleaving between ring A and ring B. The difference in structure between dinoflagellate luciferin and substance F is consistent with other observations:

(1) Luciferin did not yield the yellow dipyrrole compound produced upon NaOH/ethanol degradation of F (see [8]);
(2) Cross-reactivity between luciferin and F was not complete [6];
(3) Absorption spectra, though very similar, are not superimposable [6].

Acknowledgements

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