The Fluorescence of the Chromophore of the Green Fluorescent Protein of *Aequorea* and *Renilla*

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A possible explanation for the lack of fluorescence in the isolated chromophore of the green fluorescent protein found in bioluminescent coelenterates has been provided by the synthesis of model compounds and a more satisfactory structure is suggested.

Luminescent coelenterates such as the jellyfish *Aequorea*,[1] the hydroid *Obelia*,[2] and the sea pansy *Renilla*[3] alter or enhance light emission from their tissues by means of transfer of electronic energy from the excited product of the oxidation of a luciferin (coelenterazine) to a green fluorescent protein (GFP). This protein contains a chromophore responsible for the fluorescence whose structure has not been satisfactorily determined. Shimomura[4] has isolated the chromophore from *Aequorea* and assigned it structure (**1**), with R^1 and R^2 amino acid residues of the protein chain. The assignment was based on the detection of the amino acids glycine, valine, glutamic acid, phenylalanine, and tyrosine on hydrolysis of the hydrogenated compound, and the isolation of *p*-hydroxybenzaldehyde on hydrolysis of the chromophore itself. Confirmation was claimed by an acceptable but rather less than rigorous synthesis of a closely related model compound (**1**, $R^1 = CH_2CO_2H$, $R^2 = Pr^n$).[4] The purpose of this communication is to suggest that the unsaturation in the chromophore (or its model) is insufficient to explain the spectroscopic properties, and that at least one other double bond is necessary.

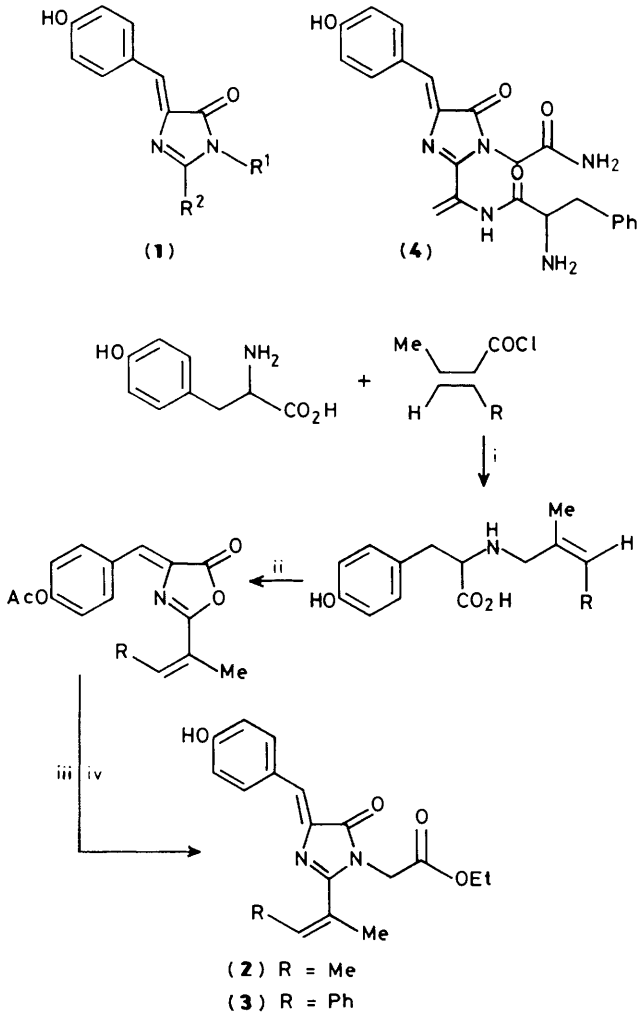
Previous investigators[3] have assumed that the discrepancy between the absorption spectra of the intact protein (and its chromophore), and that of the synthetic model could be explained by the special protein environment. It has also been assumed that the reversible loss of fluorescence on denaturation could be ascribed to this cause. However it has been our experience that almost all of the *qualitative* changes observed on the binding of small potentially fluorescent molecules to protein can be obtained by readily accessible means such as alterations in pH or solvent. The persistent failure to reproduce the fluorescence indicates some structural revision. We have synthesised a compound (**1**, $R^1 = CH_2CONH_2$, R^2 = Me) chromophorically identical to that made by Shimomura, supporting the previous structure, and have examined various environments in an attempt to obtain fluorescence.[5] The compound (**1**) is non-fluorescent in all solvents and conditions including that of covalent linking to a variety of proteins. No procedure has given the slightest hint that the highly characteristic fluorescence is obtainable from this structure.

We have therefore assumed that the structure is incomplete and that the denaturation and digestion required to free the chromophore from the protein has destroyed a significant feature. The presence of at least one dehydro-amino acid, a similar requirement for the synthesis[6] and biosynthesis of the closely related luciferin found in the previously mentioned organisms and the fact that the native chromophore absorbs at longer wavelength than the isolated chromophore suggests that further unsaturation may be present.

Although it is not yet possible to define the exact nature of the additional double bond, the dramatic nature of the change in fluorescence in our new model compound convinces us that the hypothesis is reasonable. In view of the extreme difficulty in acquiring suffcient protein with which to determine the structure of the chromophore, we believe that the suggestions made in this communication will direct the spectroscopic examination and proper handling of intact protein most effectively.

We have synthesised (**2**) and (**3**) by the routes shown (Scheme 1).† Both compounds showed green fluorescence in solution under u.v. light with a quantum yield of about 3%.

† All new compounds had n.m.r., u.v., and i.r. spectra in accordance with the structures given, and satisfactory microanalyses and mass spectra were obtained.



Scheme 1. *Reagents*: i, $NaBO_3$, $Na_2S_2O_4$, dimethoxyethane; ii, pyridine hydrobromide, Br_3, Ac_2O, pyrdine; iii, glycine ethyl ester hydrochloride, pyridine; iv, dilute NaOH.

There are differences in the spectra of the chromophores of the various coelenterates (*e.g. Renilla* and *Aequorea*) which require considerable interpretation (although they are considered to be identical in structure)[3] and only comparison with *Aequorea* will be made here. The u.v. spectrum of (**2**) (λ_{max} 248 and 384 nm) and that of the native *Aequorea* chromophore (λ_{max} 280 and 393 nm) in their neutral form are similar. The shift in alkaline solution is also comparable, although the spectrum of (**2**) does not shift to quite the same extent [258, 445 nm and 280, 470 nm for (**2**) and native chromophore respectively]. The differences in the positions of the absorption maxima reflect the simplicity of this preliminary model.

Our model is not designed to predict the exact structure of the native chromophore, but rather to indicate the shortcomings of the current structure. Nevertheless, we intend to synthesise a compound such as (**4**), on the basis of our present findings. In this structure we find the suggested double bond in the dehydro-amino acid formed from serine by dehydration. The explanation of the ready, reversible[7] loss of fluorescence and undetectability of the serine residue on digestion of the protein may be found in facile isomerisation and hydrolysis (to pyruvic acid rather than an amino acid) respectively at this reactive site. This suggestion is at present illustrative only, but should guide further investigations.

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