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Mechanism of the Redox Reaction of the *Aequorea* Green Fluorescent Protein (GFP)

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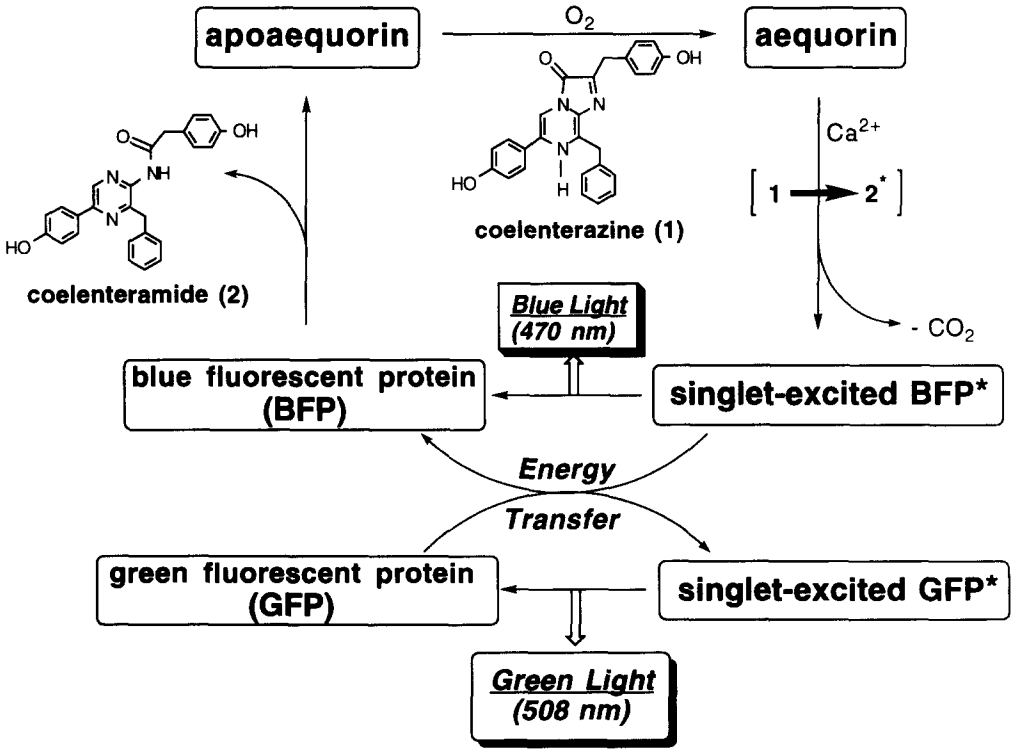
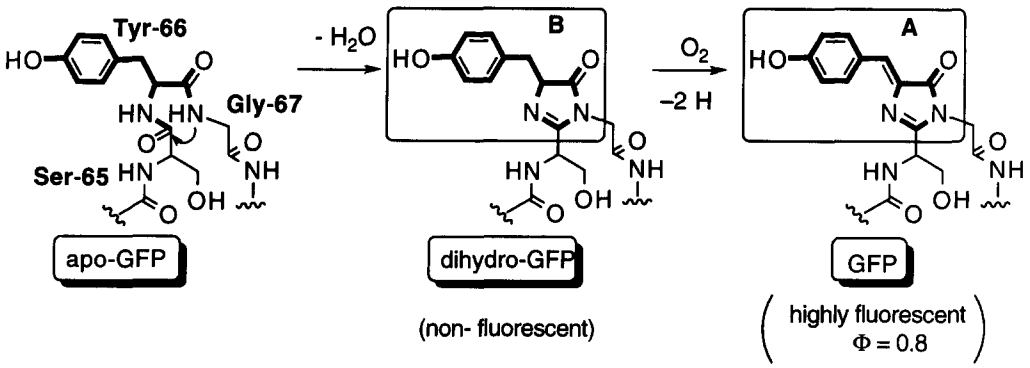
Abstract: A model compound, 4-(4-hydroxyphenyl)methylideneimidazol-5-one, undergoes a reversible redox reaction identical to that of the *Aequorea* green fluorescent protein (GFP), strongly suggesting that the GFP chromophore is derived via the autoxidation of a nonfluorescent dihydro precursor in dihydro-GFP. © 1997 Elsevier Science Ltd.

On mechanical or electrical stimulation, the bioluminescent jellyfish *Aequorea victoria* emits a greenish light (λmax 508 nm) from the margin of its umbrella.[1] The bioluminescence system involves two closely associated proteins, a calcium-binding photoprotein aequorin[2-4] and a green fluorescent protein (GFP) (Scheme **1**).[5-7] Aequorin is made up of apoaequorin (apoprotein), molecular oxygen, and coelenterazine (**1**).



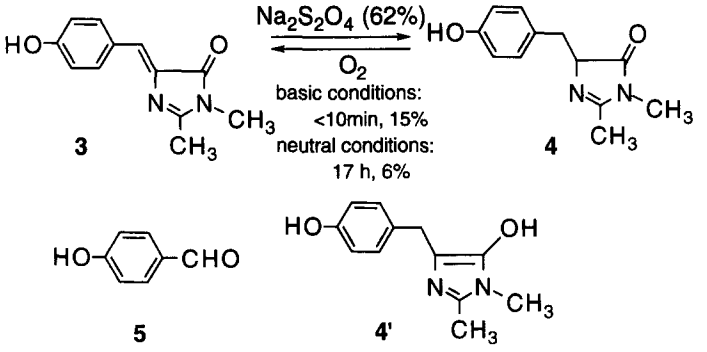
Scheme **1**. Bioluminescence System of the Jellyfish, *Aequorea victoria*



Scheme 2. Biogenesis of Chromophore **A** in GFP

When calcium ions bind to aequorin, **1** is oxidized by the bound oxygen to form electronically excited coelenteramide (**2***) of the excited state blue fluorescent protein (BFP*). Thus, BFP* is a molecular complex of apoaequorin and **2***.[8,9] In vitro, BFP* emits *blue* light (λmax 470 nm), while in the live jellyfish a resonance energy transfer takes place from **2*** to the GFP chromophore, generating the excited state of GFP (GFP*) from which *green* light (λmax 508 nm) is emitted.[5,6,10] GFP has a fluorescence quantum yield, $\Phi \cong 0.8$, when irradiated at 366 nm,[6] and is made up of 238 amino acid residues in a single polypeptide chain.[11-13] The chromophore consists of a 4-(4-hydroxyphenyl)methylideneimidazol-5-one system,[14-16] depicted as formula **A** in Scheme **2**, and the true light emitter of GFP* is the phenolate anion of **A**.[16] Heterologous expression of the cDNA has fostered widespread use of GFP as a reporter in gene expression, cell lineage, protein localization, and protein trafficking studies.[17] Recent crystallographic[18] and chemical studies[16,19] have provided details of the three dimensional structure of GFP and the chemical nature of the chromophore. The GFP chromophore is formed by a posttranslational, intramolecular, autocatalytic dehydration within the peptide main chain, followed by dehydrogenation as shown in Scheme **2**.[14-16] This unusual posttranslational modification occurring in the peptide main chain of the nascent apoprotein (apo-GFP) has never been reported previously in nature. Although recent studies have indicated that oxygen is required for the formation of the GFP chromophore,[13,20] the precise biosynthetic process is still unclear. The fluorescent properties of GFP has been found to disappear on treatment with various reducing reagents and to recover spontaneously on exposure to air.[13] This reversible phenomenon strongly suggested that chromophore **A** might be reduced to the dihydro form, a 4-(4-hydroxyphenyl)methylimidazol-5-one system (**B**), and then oxidized to **A** in air, as shown in Scheme **2**.

We report herein that the GFP redox reaction can be reproduced using a synthetic model compound **3** (Scheme **3**). Compound **3**[21]was prepared as previously reported.[16] Reaction of **3** with sodium dithionite (20 equiv)[13] in a mixture of EtOAc and H_2O, in the presence of $NaHCO_3$ (10 equiv), gave the air-sensitive dihydro compound **4**[22] in 62% yield after recrystallization from ether. Figure **1** shows the UV specta of **3** and **4**. The NMR spectrum of **4** indicated that a small amount of **4** was present as the enol form **4'**[23] in the $CDCl_3$ solution (ca. 15%). Compound **4** was readily oxidized into **3** by molecular oxygen. Thus, exposure of an EtOH solution of **4** to oxygen at room temperature for 17 h gave **3** in 6% yield along with *p*-hydroxybenzaldehyde (**5**) in 15% yield after purification by TLC. Under the basic conditions (4:1 EtOH/1 M NaOH), the oxidation



Scheme 3. Redox Reaction of the Model Compound 3

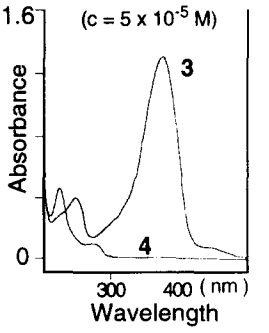
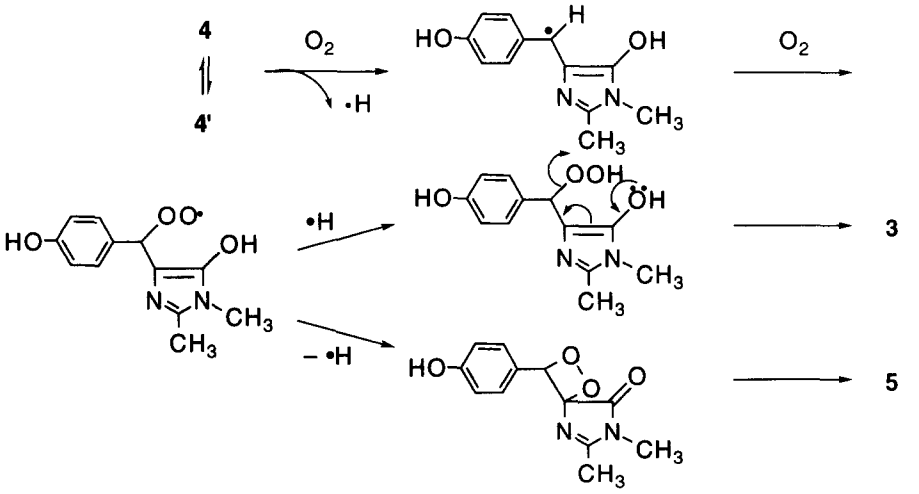


Figure 1. UV Spectra of 3 and 4 in EtOH.

of 4 by molecular oxygen proceeded more rapidly and was completed within 10 min to give 3 (15%) and 5 (15%) after purification by TLC. These reactions may proceed via radical process. A plausible reaction mechanism which accounts for the formation of 3 and 5 from 4 is shown in Scheme 4.

Based on these results, it appears that the GFP chromophore is derived via the autoxidation of nonfluorescent dihydro-GFP. Thus, the chromophore **A** would be formed through autoxidation of dihydro **B** in dihydro-GFP generated by posttranslational autocatalytic dehydration of -Ser^{65}-Tyr^{66}-Gly^{67}- in the primary structure. It is also conceivable that the environment around **B** in dihydro-GFP may play a role in the efficient autoxidation of dihydro-GFP into GFP. It seems worth noting that **3** and the close analogue of the GFP chromophore, ethyl 4-(4-hydroxyphenyl)methylidene-2-methyl-5-oxo-1-imidazolacetate, showed no fluorescent properties in liquid solutions, but exhibited a marked bluish-green fluorescence *in ethanol glass at 77 K*, suggesting that the GFP chromophore may exist in a restricted state in its peptide environment.[16]



Scheme 4. Plausible Mechanism for the Formation of 3 and 5 from 4

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21. **3**: yellow needles; mp 238 °C (dec) ($CHCl_3$-MeOH); UV (EtOH) λmax 372 nm (ε 25400); IR (KBr) 3350-2500, 1680, 1640, 1600, 1580, 1445 cm^{-1}; ^1H NMR (500 MHZ, DMSO-d_6) δ 2.23 (3 H, s), 3.07 (3 H, s), 6.81-6.83 (2 H, m), 6.87 (1 H, s), 8.05-8.07 (2 H, m), 10.04 (1 H, s; OH); EIMS (70 eV) *m/z* (relative intensity) 216 (M^+, 31), 56 (100). HREIMS: found *m/z* 216.0938 (M^+); calcd for $C_{12}H_{12}N_2O_2$ 216.0899.
22. **4**: colorless needles; mp 123-125 °C (ether) ; UV (EtOH) λmax 224 (9100), 276 nm (ε 2400); IR (KBr) 3700-2900, 1735, 1640, 1515 cm^{-1}; ^1H NMR (270 MHZ, $CDCl_3$) δ 2.15 (3 H, d, J = 2.0 Hz; 3 H, s by the addition of D_2O), 2.81 (1 H, dd, J = 7.9, 13.8 Hz; 1 H, d, J = 13.8 Hz by the adition of D_2O), 2.95 (3 H, s), 3.33 (1 H, dd, J = 13.8, 4.3 Hz; 1 H, d, J = 13.8 Hz by the addition of D_2O), 4.21 (1 H, m; disappeared by the addition of D_2O), 5.74 (1 H, br, OH), 6.59 (2 H, m), 7.02 (2 H, m); EIMS (70 eV) *m/z* (relative intensity) 218 (M^+, 24), 112 (83), 107 (100), 56 (19). HREIMS: found *m/z* 218.1067 (M^+); calcd for $C_{12}H_{14}N_2O_2$ 218.1056.
23. **4'**: ^1H NMR (270 MHZ, $CDCl_3$) δ 1.99 (3 H, s), 2.17 (3 H, s), 2.95. (2 H, s), 4.51 (1 H, br, OH), 6.26 (1 H, br, OH), 6.66 (2 H, m), 7.06 (2 H, m).

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