Tautomeric Equilibrium of Fluorescein in Solution: Ab Initio Calculations

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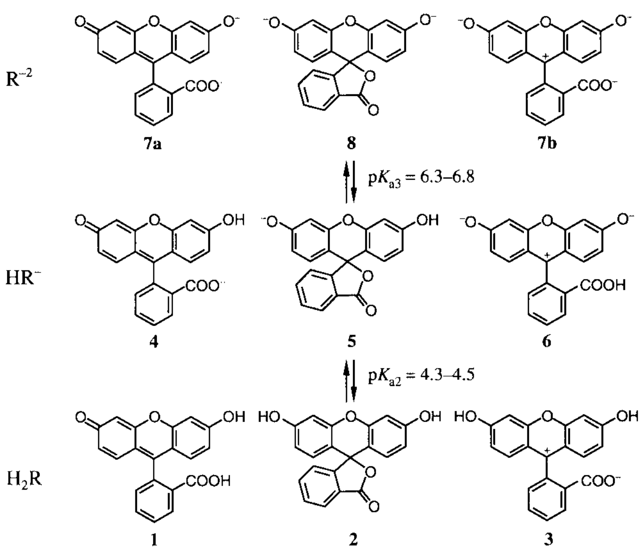
The tautomeric equilibrium of fluorescein was investigated by calculating the relative free energies of various tautomers at neutral, anionic, and dianionic states both in the gas phase and in solution (water or DMSO) using an ab initio quantum mechanical method (B3LYP/6-31++G**) in combination with the Poisson–Boltzmann continuum solvation approach.

Fluorescein, 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (**1**), since the first synthesis in 1871,[1] has been widely used as a fluorescent derivatization reagent for various substances including proteins and nucleotides for biological assays.[2] Its behavior in solution, however, is very complicated. In aqueous solution it can exist in neutral (**1**–**3**), anionic (**4**–**6**), or dianionic (**7**–**8**) forms (Scheme 1), making its absorption and fluorescence properties strongly pH-dependent.[2] Moreover, each protolytic species can exist as a mixture of multiple tautomers: (1) tautomers with a quinoid structure (red fluorescein **1**, **4**, and **7a**), (2) tautomers with a zwitterionic character (yellow fluorescein **3**, **6**, and **7b**), and (3) colorless, non-fluorescent lactone tautomers (**2**, **5**, and **8**). Furthermore, these tautomeric and protolytic equilibria strongly depend on solvent environments. Mchedlov-Petrossyan and coworkers[3–5] and Choi[6] reported an anomalous behavior of fluorescein, the inversion of the order of pK_a values (or at least the collapse into a single value; p$K_{a2} \geq$ pK_{a3}) in a DMSO–water mixed solvent or other organic environments.

Since great effort has been invested in designing new derivatives of fluorescein,[2] it would be advantageous to predict the properties of fluorescein and its derivatives from theory. Theoretical studies to date, however, have been conducted on a limited number of tautomers or with a semi-empirical quantum mechanical method.[7–9] In this work we investigated the tautomeric equilibrium of fluorescein of each charge state (dianionic, anionic, or neutral) in various environments (gas phase, aqueous solution, or DMSO solution) by ab initio (density functional theory) quantum mechanical calculations.



Scheme 1. Tautomeric and protolytic equilibria of fluorescein.

The standard free energy of each species in solution (with respect to the reference state where all the nuclei and electrons are completely separated from one another at 0 K in the gas phase) was calculated as the sum of the gas-phase free energy and the free energy of solvation:

$$\Delta G^0_{\mathrm{aq}} = \Delta G^0_{\mathrm{g}} + \Delta G^0_{\mathrm{solv}} \qquad (1)$$

The standard free energy in the gas phase was calculated as

$$\Delta G^0_{\mathrm{g}} = \Delta E_0 + \Delta(\mathrm{ZPE}) + \Delta G_{0\rightarrow 298} \qquad (2)$$

The total energy of the solute at 0 K (ΔE_0) was calculated at the B3LYP/6-31++G** level after re-optimization starting from the B3LYP/6-31G** geometry. The zero-point energy (Δ(ZPE)) and the free energy change from 0 K to 298 K ($\Delta G_{0\rightarrow 298}$) were calculated from the vibration frequencies obtained at the B3LYP/6-31G** level. The translational and rotational free energy contribution was also calculated in the ideal gas approximation. The standard free energy of solvation (ΔG_{solv}) was calculated using the Poisson–Boltzmann continuum solvation approach[10–12] at the boundary between a low-dielectric cavity of solute ($\varepsilon_{\mathrm{solute}} = 1$) and a high-dielectric continuum of solvent ($\varepsilon_{\mathrm{water}} = 80$, $\varepsilon_{\mathrm{DMSO}} = 47$) with atomic charges of the solute determined at the B3LYP/6-31++G** level. The solute/solvent boundary is described by the surface of closest approach as a probe sphere (radius 1.4 Å for water, 2.414 Å for DMSO) is rolled over the van der Waals envelope of the solute. The atomic radii used to build the envelope were taken from the literature:[12] 2.0 Å (sp^2 C), 1.55 Å (sp^2 O), 1.25 Å (H attached to sp^2 C), and 1.15 Å (other H's). All calculations used the Jaguar v4.0 quantum chemistry software.[13,14]

The initial dianionic structure was taken from a crystal structure of fluorescein attached to a protein.[15] The optimized structure resulted in C_s symmetry where the hydroxyxanthenone ring and the benzoic acid ring are perpendicular to each other (Figure 1). These two ring moieties remained almost mutually perpendicular throughout all the charge states (Figure 1), in agreement with the crystal structure of red fluorescein,[16] where the interplanar angle was 82.2–87.1°, and with other calculation results.[8,9] The dianionic fluorescein is best described by the resonance structure between **7a** and **7b** in Scheme 1. The ring structure **8**, where the resonance is localized on two outer benzene rings, was unstable and converted to **7b** during the geometry optimization.

The three different tautomers of anionic fluorescein (**4**, **5**, and **6**) remained stable during the optimization (Table 1(A) and Figure 1). The ring structure **5**, however, was at least 5

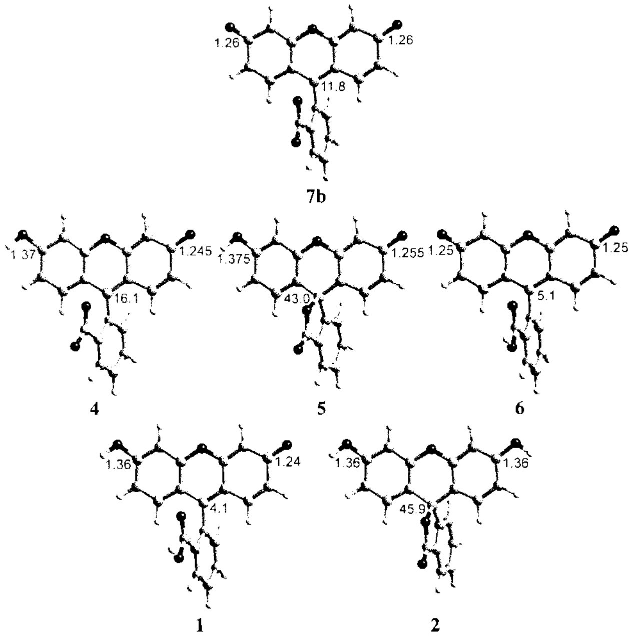


Figure 1. Optimized structures of the tautomers of dianionic (R^{2-}; top), anionic (HR^-; middle), and neutral (H_2R; bottom) fluoresceins. Some C–O distances (1.245–1.375) and inversion angles (4.1–45.9) are shown in angstroms and degrees, respectively.

Table 1. Relative free energies[a] ($\Delta\Delta G^0$; kcal/mol) and populations (f) of anionic and neutral tautomers of fluorescein in various environments (gas phase, DMSO solution, and aqueous solution) at 298 K

		(A) anionic (HR^-)			(B) neutral (H_2R)	
		4	**5**	**6**	**1**	**2**
gas	$\Delta\Delta G^0$	5.1	8.7	0	4.6	0
	f	2×10^{-4}	7×10^{-7}	1.00	4×10^{-4}	1.0
DMSO	$\Delta\Delta G^0$	−0.03	5.4	0	1.5	0
	f	0.51	1×10^{-4}	0.49	0.08	0.92
aq	$\Delta\Delta G^0$	−0.3	5.4	0	0.4	0
	f	0.63	4×10^{-5}	0.37	0.33	0.67

[a] Anions (**4**–**6**) with respect to **6**; neutral species (**1**–**2**) with respect to **2**.

kcal/mol higher in free energy than the others both in the gas phase and in solution. In aqueous solution, the tautomer **4** which has a proton attached to the hydroxyxanthenone ring was calculated to be the most stable tautomer, as proposed from experiments performed by Mchedlov-Petrossyan and coworkers.[3,5] However, contrary to their suggestion that **4** is the only anionic tautomer, another tautomer **6**, which has a proton attached to the carboxyl group, was calculated to have a significant population (more than one-third) in aqueous solution. Its relative population was even higher in a less polar solvent such as DMSO (about half), and it became predominant in the gas phase. The significant population of **6**, especially in nonpolar solvents, seems to stem from the gain in the resonance over the whole hydroxyxanthenone ring at the expense of the solvation of the carboxylate group (COO^-). The enhanced resonance of **6** is demonstrated by its higher planarity (inversion angle of 5° at C10) compared to that of **4** (16°) or **5** (43°). A phenolate-type tautomer such as **6** has been proposed as the dominant anionic form for eosin (tetrabromofluorescein),[5] but only a few studies have suggested multiple tautomers for anionic fluorescein.[17,8]

Only two tautomers, **1** and **2**, of neutral fluorescein were optimized to stable structures (Table 1(B) and Figure 1). The zwitterionic tautomer **3** was converted into the ring tautomer **2** during optimization both in the gas phase and in aqueous solution. The tautomer **3** had been suggested to have a population as much as 22% in aqueous solution,[3] but the same authors reported later that they could not detect any evidence of this form.[5] The ring tautomer **2** having both protons attached to the hydroxyxanthenone oxygens was calculated to be the most stable neutral tautomer both in the gas phase and in solution. The tautomer **2**, which has a resonance over two outer benzene rings, would be preferred over **1**, which has a resonance over only one benzene ring, but **2** would be less soluble than **1** in polar solvents. Indeed, the preference of **2** over **1** was higher in less polar solvents (67% in water, 92% in DMSO, and 99.99% in the gas phase). Indeed, the **1**-to-**2** shift in the tautomeric equilibrium of neutral fluorescein has been reported from experiments in which DMSO was added to the aqueous solution of fluorescein.[3,5]

In summary, in aqueous or DMSO solutions the dianionic fluorescein should exist as a single tautomer (**7a** ↔ **7b** in resonance) but the anionic and neutral fluoresceins should exist as a mixture of two major tautomers, respectively [**4** and **6** (anion); **1** and **2** (neutral)]. The equilibrium would shift towards **6** (anion) and **2** (neutral) in less polar solvents. Their relative populations in each solvent, however, are not yet conclusively determined, and depend on the choice of the parameters employed in the solvation energy calculation, which will be addressed in future studies.

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