Anomalous Acid-Base Equilibria in Biologically Relevant Water Nanopools

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Water plays a crucial role in many principal biological phenomena such as enzymatic catalysis and proton pumping through a membrane protein channel.[1-4] Moreover, in biological systems, water is usually contained in a small pocket of a membrane,[5-8] and such confined water, which is generally called a water nanopool,[8-10] shows peculiar properties differing considerably from the properties of bulk water.[8-16] The confinement effect and the enclosing interfacial surfaces of waterpools are the main factors to determine the properties, such as polarity, viscosity, and H-bonding ability, of water nanopools.[5-13] For example, the dielectric constant of a water nanopool has been reported to be much lower than that of bulk water ($\varepsilon = 78.5$)[17] but similar to that of an alcohol ($\varepsilon = 30\text{-}40$).[18,19] On the other hand, biological processes often take place based on proton relay along a hydrogen (H)-bonded chain,[1-4,20-24] and the dynamics of biological proton relay is determined by the size, the structure, and the motion of a water cluster which is the prime agent in most of biological systems.[3,13-15,24-26] Thus, for better understanding of cellular dynamics, it is necessary to investigate the properties of a biologically relevant water nanopool as a biomimetic system of water confined in a cell membrane. In this regard, water nanopools confined in reverse micelles, which are formed by surfactant molecules having polar headgroups pointing inward and dispersed in hydrocarbon solvents, can be good model systems of biological water.[6,18,19,27]

It is a unique feature of reverse micelles that they can make nonpolar media to solubilize a large amount of water by encapsulating water molecules in their inner polar cores;[28] reverse micelles are surrounded by a layer of surfactant molecules such as Aerosol-OT (sodium 1,4-bis-2-ethylhexyl-sulfosuccinate, AOT) and immersed in a nonpolar solvent, so that nanometer-sized droplets of a polar solvent such as water are formed inside. The polar headgroups of surfactant molecules point inward toward a polar solvent pool, and the alkyl chains of the surfactant molecules point outward toward a nonpolar solvent. About 20 surfactant molecules of AOT form a reverse micelle having a diameter of 3.0 nm above the critical concentration of 1 mM in a hydrocarbon solvent such as *n*-heptane.[29] By adding water to the AOT solution, microemulsions of nanometer-sized water droplets surrounded by AOT molecules are formed, and the size of water nanopools confined in AOT reverse micelles increases as the concentration of water increases. In *n*-heptane, the diameters in nanometers of the waterpools have been report-

ed to be about $0.3w_0$, in which w_0 is the molar ratio of water to AOT.[30]

The catalytic properties of water nanopools depend strongly on the hydration extent of reverse micelles and on the solvation structure of reactants relevant to the polarity of waterpools confined in reverse micelles.[5-8,17,18] The peculiar structure of waterpools contained in reverse micelles, which is in conjunction with the solvation ability and the acid-base activity of waterpools, is of major importance to understand the reactivity of biological water confined in a membrane. In aqueous solutions, while the acidity of water can be adjusted by pH, the prototropic dissociation of solutes would vary with pH. Thus, to know whether solutes have a cationic form or an anionic form at a given pH, the pK_a value of the solute should be determined. Many researchers have reported numerical values of pK_a, which are deduced in empirical ways, in waterpools confined in reverse micelles.[31-33] As already mentioned above, water is the major medium of biological systems, and biological proton relay usually occurs through a H-bonded water chain.[1-4] Accordingly, water molecules act as proton donors or acceptors, and the activity of water plays a key role to determine the rate constant of proton transport.[15,16,34] In addition, biological water which is confined in a membrane shows characteristic properties distinctive from the properties of bulk water.[5-14] Thus, we consider that waterpools confined in reverse micelles are good biomimetic systems to investigate the properties of biological water.

Hydroxyquinolines, having two prototropic groups of acidic enol and basic imine, are extensively explored because they are good experimental models to investigate biological
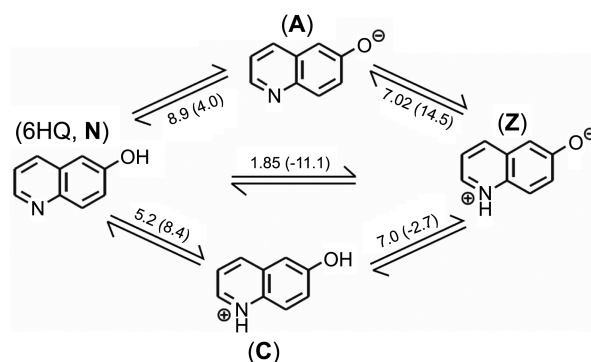

Figure 1. Equilibria among the prototropic species of 6HQ in water. The indicated values of p*K* (p*K**) are taken from Ref. 39.
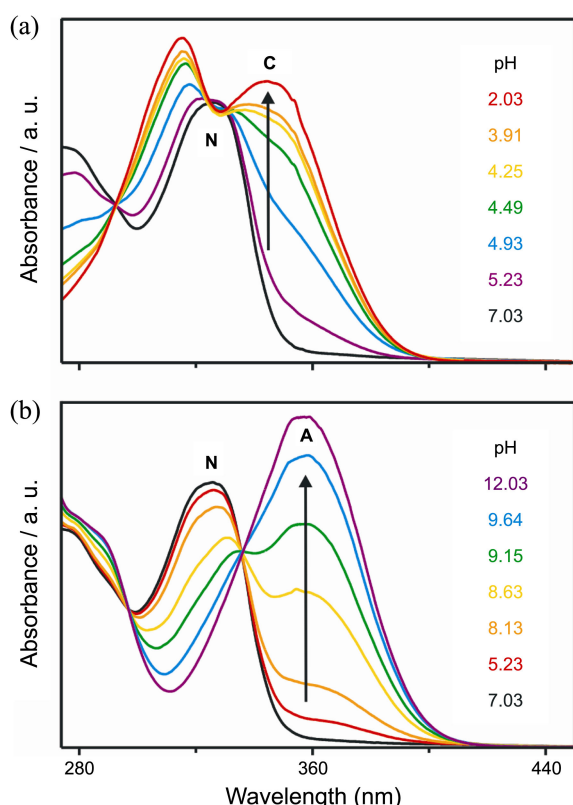
Figure 2. pH-dependent absorption spectra of 6HQ in acidic (a) and basic (b) bulk-water solutions. pH values are indicated inside, and absorption bands of neutral, cationic, and anionic species of 6HQ are marked as **N**, **C**, and **A**, respectively.

proton transport.[13-16,35-44] On one hand, a 7-hydroxyquinoline molecule can form a cyclically H-bonded complex with two protic solvent molecules such as water and alcohols, so that proton relay from the enolic group to the imino group of 7-hydroxyquinoline occurs along a H-bonded solvent chain in a concerted fashion.[15,16,42-44] On the other hand, those two prototropic groups of 6-hydroxyquinoline (6HQ) are too far from each other to form a cyclic complex with solvent molecules, so that proton transfer from the enolic group to the imino group of 6HQ takes place in a stepwise manner *via* accumulating cationic or anionic intermediate species.[36-40] Figure 1 shows equilibria among prototropic species of 6HQ in aqueous solutions: the neutral species (**N**), the anionic species (**A**), the cationic species (**C**), and the zwitterionic species (**Z**).[36,40] Here we report anomalous prototropic equilibria of 6HQ in water nanopools of AOT reverse micelles with variation of water content.

The absorption spectra of 6HQ in water vary remarkably with pH, showing isosbestic points (Figure 2). In a neutral solution, the absorption spectrum of 6HQ has the band maximum at 330 nm, which is attributed to **N**. In acidic conditions, as pH decreases, absorption bands at 315 nm and 350 nm, which are ascribed to **C**, grow up whereas the absorption band of **N** at 330 nm diminishes (Figure 2(a)); this spectral transformation in acidic conditions arises from the protonation of the imino group of 6HQ to form **C**. In basic conditions, as pH increases, the absorption band of **A**

at 358 nm increases to be dominant whereas **N** absorption disappears gradually (Figure 2(b)); this spectral change in basic conditions is caused by the deprotonation of the enolic group of 6HQ to form **A**. The appearance of isosbestic points generally indicates that one species changes into the other species directly. In other words, Figure 2 showing isosbestic points suggests that **N** changes into **C** with decreasing pH in acidic conditions while **N** does into **A** with increasing pH in basic conditions.

In principle, the absorbance ratio of **N** and **C** is proportional to the molar ratio of **N** and **C** while the absorbance ratio of **N** and **A** is to the molar ratio of **N** and **A**. Thus, the spectral changes of 6HQ absorption in acidic and basic conditions are based on the ground-state acid-base equilibria of the imino and the enolic groups, respectively, of 6HQ. Figure 3 shows the titration curves of **C** absorption at 350 nm and **A** absorption at 358 nm, which are fitted to Eq. (1) to obtain the pK_a values of the protonated imino group (iminium group) and the enolic group of 6HQ, respectively.[45,46]

$$Y = \frac{10^{pK_a} \times A_{min} + 10^{pH} \times A_{max}}{10^{pK_a} + 10^{pH}} \quad (1)$$

where Y at given pH is the absorbance of **C** at 350 nm or the absorbance of **A** at 358 nm while A_{min} and A_{max} indicate the smallest and the largest values, respectively, among Y values in the given range of pH. The pK_a values of the iminium and the enolic groups of 6HQ are deduced to be 4.88 and 8.76,
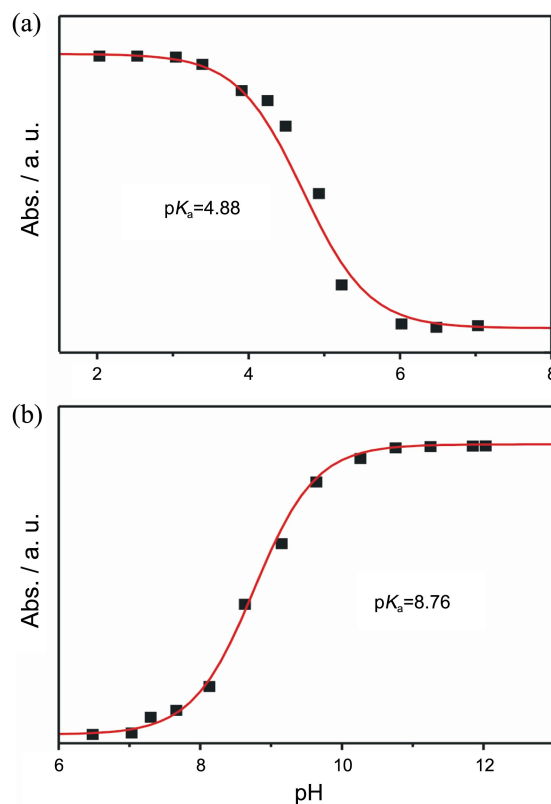

Figure 3. pH-dependent absorbance in bulk water of **C** at 350 nm (a) and **A** at 358 nm (b). Solid lines are the best-fitted curves of Eq. (1) to extract pK_a values indicated inside.

Table 1. Ground-State pK_a of the Prototropic Groups of 6HQ in Water Nanopools of AOT Reverse Micelles

w_0	d^a (nm)	pK_a (C⇌N)	$\Delta[\Delta G°(C⇌N)]^b$ (kJ/mol)	pK_a (N⇌A)	$\Delta[\Delta G°(N⇌A)]^b$ (kJ/mol)
4	1.2	1.63	-18.6	12.4	20.8
12	3.6	3.05	-10.4	11.3	14.5
36	10.8	4.16	-4.1	10.4	9.4
∞^c	∞^c	4.88 (5.18^d)	0	8.76 (8.87^d)	0

^{a}Estimated diameters of water nanopools. ^{b}($\Delta G°$ at given w_0) – ($\Delta G°$ at $w_0 = \infty$). ^{c}Bulk water. $^d pK_a$ values taken from Ref. 39.

respectively (Figure 3), which are similar to the respective pK_a values reported already (Table 1).[39]

Figure 4 shows the absorption spectra of 6HQ in reverse micelles having $w_0 = 4$. In acidic conditions, with the decrement of pH, the **N**-absorption band at 330 nm decreases whereas the **C**-absorption bands at 315 nm and 350 nm grows up gradually (Figure 4(a)). In basic conditions, with the increment of pH, the **N**-absorption band disappears whereas the **A**-absorption band at 358 nm arises newly (Figure 4(b)). Of note is that the absorption spectra of 6HQ in reverse micelles having $w_0 = 36$ (Figure S1) show similar spectral behaviors as the absorption spectra in reverse micelles having $w_0 = 4$ do.

In water nanopools of AOT reverse micelles, the ground-state pK_a values of the prototropic groups of 6HQ are found to depend mostly on the w_0 values of the micelles; the pK_a values of the iminium and the enolic groups of 6HQ in reverse micelles having $w_0 = 4$ are deduced to be 1.63 and

12.41, respectively (Figure 5) while those values in reverse micelles having $w_0 = 36$ are found to be 4.16 and 10.4, respectively (Figure S2). Table 1 shows that as the average diameter of water nanopools is reduced from 10.8 nm at $w_0 = 36$ to 1.2 nm at $w_0 = 4$, the pK_a value of the iminium group decreases by 2.53 whereas the value of the enolic group increases by 2.0. In other words, with decreasing w_0, the K_a value of the C⇌N reaction becomes larger but the value of the N⇌A reaction becomes smaller. This indicates that **N** rather than **C** or **A** is dominant in small water nanopools. On the other hand, we have calculated standard Gibbs-free-energy changes ($\Delta G°$) for the acid-dissociation reactions of both the iminium and the enolic groups, and we have shown the difference of $\Delta G°$ values at given w_0 and at $w_0 = [\Delta(\Delta G°)]$ in Table 1. As the size of a water nanopool gets smaller, $\Delta(\Delta G°)$ for C⇌N decreases remarkably, suggesting that the at low w_0, 6HQ prefers to exist as **N** rather than **C**. In contrast, $\Delta(\Delta G°)$ for N⇌A increases substantially as the diameter of a water nanopool gets smaller, indicating that
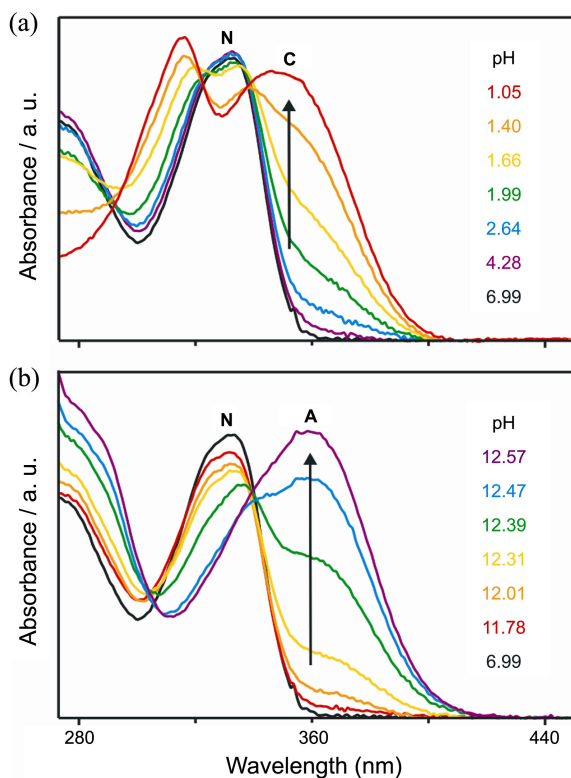

Figure 4. pH-dependent absorption spectra of 6HQ in acidic (a) and basic (b) water nanopools of AOT reverse micelles having $w_0 = 4$. pH values are indicated inside, and absorption bands of neutral, cationic, and anionic species of 6HQ are marked as **N**, **C**, and **A**, respectively.
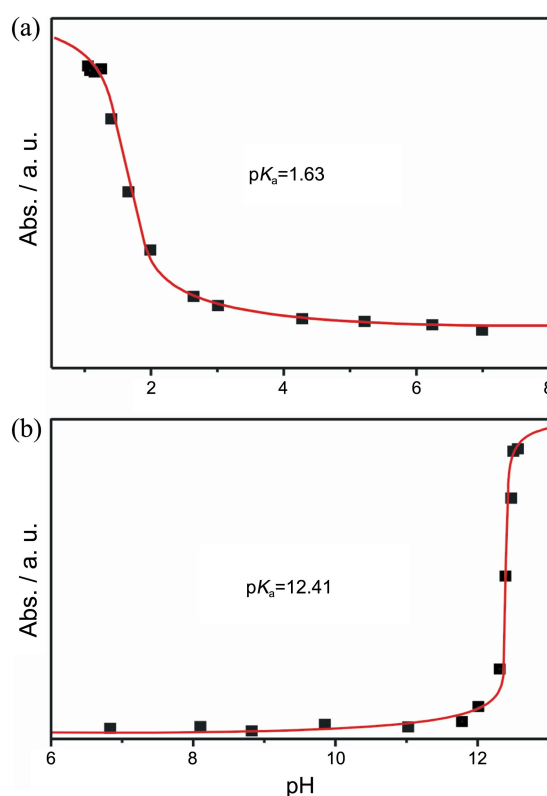

Figure 5. pH-dependent absorbance, in AOT reverse micelles having $w_0 = 4$, of **C** at 350 nm (a) and **A** at 358 nm (b). Solid lines are the best-fitted curves of Eq. (1) to extract pK_a values indicated inside.

6HQ prefers to exist as **N** rather than **A** in small waterpools. Thus, with the decreasing pore size of waterpools, both the protonation of the imino group and the deprotonation of the enolic group hardly take place. We attribute this to the polarity decrement of waterpools as w_0 decreases. As already mentioned above, the confinement effect and the enclosing interfacial surfaces of water nanopools are the major factors to determine the properties, such as H-bonding ability, polarity, and viscosity, of waterpools.[5-13] Water nanopools of AOT reverse micelles have been suggested to be alcohol-like in polarity; water confined in reverse micelles has a relative dielectric constant (ε) of 30-40, which is very close to ε of methanol (33) and much smaller than ε of bulk water (78).[17-19] Thus, the micropolarity of water near 6HQ in reverse micelles is substantially low compared with the polarity of bulk water.[9,10,47] Consequently, as the pore size of water nanopools becomes smaller, the polarity of water nanopools confined in reverse micelles decreases, so that the charged species of 6HQ, *i.e.* **C** and **A**, become energetically unstable to exist in the ground state.

In summary, anomalous prototropic equilibria of 6HQ in biologically relevant water nanopools of AOT reverse micelles have been observed. Waterpools confined in reverse micelles show alcohol-like polarity, and the polarity of water nano-pools decreases as the average diameter of waterpools becomes smaller. As a result, the charged species of 6HQ, *i.e.* **C** and **A**, becomes energetically unfavorable to exist in the ground state, so that the pH range where **N** is dominant expands extensively with the decreasing size of waterpools. Our results can give significant information to understand the properties of biological water confined in a membrane.

Experimental

6HQ (98%), purchased from Sigma-Aldrich, was used after being purified *via* column chromatography and vacuum sublimation. AOT (> 99%) was used as purchased from Sigma-Aldrich, while *n*-heptane (anhydrous, ≥ 99%), purchased from Sigma-Aldrich, was stored over molecular sieves of 4 Å prior to use. Aqueous solutions of 0.2 mM 6HQ were prepared as reported.[9] pH was adjusted by adding a dilute HCl or NaOH aqueous solution to triply distilled water, and then a requisite amount of pH-adjusted water was added to the AOT solution to control w_0. Absorption spectra were obtained using a UV/vis spectrometer (Scinco, S-3100) while pH was measured using a pH meter (Fisher Scientific, AR15) calibrated with three buffers of pH 4, 7, and 10. All the measurements were carried out at room temperature.

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