Partitioning of 4-nitrophenol in aerosol-OT reverse micelles

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Received (in Cambridge, UK) 9th March 1999, Accepted 13th July 1999

Partitioning of 4-nitrophenol in a reverse micellar system consisting of aerosol-OT (AOT)–H_2O–isooctane was monitored spectrophotometrically. At pH 10.0, the ionized form 4-nitrophenolate in the water pool absorbs visible light with a maximum peak at 402 nm. However, that partitioned into the interface region is not ionized due to interactions with the negatively charged polar head of the surfactant. The partitioning depends on the water content of the system. In some intermediate [H_2O]/[AOT] molar ratio values, two absorption peaks were clearly observed, which can be utilized in the partition coefficient estimation. The partitioning also depends on the buffer used. While partitioning of 4-nitrophenol into the interface is observed in carbonate buffer, the partitioning disappeared in 2-amino-2-methylpropanol buffer presumably due to displacement of 4-nitrophenol from the interface region into the water pool. This displacement is not a salt effect but is due to the amino group of 2-amino-2-methylpropanol, because *tert*-butylamine, rather than isobutanol, induced the replacement. When the surfactant concentration was increased, while keeping the system water content constant, the absorption peak at 402 nm increased with a concomitant decrease in the A_{310} peak, which demonstrated the affinity of the non-ionized 4-nitrophenol with the surfactant. Multiple apparent pK_a values of 4-nitrophenol were observed in the AOT reverse micellar system. We propose a model of the AOT reverse micelles with a gradient micro-polarity in the water pool that results in a continuous influence on the ionization of 4-nitrophenol in the water pool of the system.

Introduction

Reverse micelles, which are formed upon dissolution of a surfactant in an organic solvent, provide an artificial system that mimics the membranous biological system.[1] Self-micellar- and internal RNA-replications have been demonstrated in reverse micellar systems, which provide an experimental approach to a minimal cell.[2] We have used the reverse micelles as a model system to mimic a detoxification enzyme glutathione transferase (EC 2.5.1.18). Our results demonstrated that the cationic surfactant cetyltrimethylammonium bromide (CTAB) or cetylbenzyldimethylammonium chloride (CBAC) reverse micelles provide ideal model systems mimicking the active center of glutathione transferase.[3] We proposed that stabilization of the Meisenheimer complex by the positively charged polar heads, on-edge quadrupole interactions of the aromatic ring, and a hydroxy group are involved in the reverse micelles' enhanced nucleophilic aromatic substitution.[3] We have also described the enzyme-catalyzed hydrolysis of 4-nitrophenyl phosphate by human placental alkaline phosphatase (EC 3.1.3.1) in a reverse micellar system prepared by dissolving an anionic surfactant AOT† [sodium bis(2-ethylhexyl) sulfosuccinate] in isooctane (2,2,4-trimethylpentane).[4] The ionization of the leaving group, 4-nitrophenol, depends on the inclusive volume of the reverse micelles. The reverse micellar system thus provides a convenient system to examine the catalytic mechanism of alkaline phosphatase.[4–6] However, partitioning of 4-nitrophenol between the water pool and the interface raises additional considerations in the data interpretation.[4]

4-Nitrophenyl phosphate is one of the most convenient substrates used in the alkaline phosphatase assay. 4-Nitrophenol is the hydrolytic product of the enzymatic reaction. Measurement of the enzyme activity is based on the ionized 4-nitrophenolate, which absorbs visible light at 402 nm. Mammalian alkaline phosphatases are membrane-anchored proteins and reverse micellar systems are utilized as a tool to study the kinetic properties of the enzyme with the assumption that these systems mimic the physiological conditions.[4–6] Partitioning of 4-nitrophenol between the water pool and the interface results in different ionization states of the hydrolyzed 4-nitrophenol and thereby causes a solvatochromatic effect. Thus it is important to characterize the partition equilibrium between 4-nitrophenol and 4-nitrophenolate in order to fully appreciate the enzyme activity under various experimental conditions.[7] Among the practical applications of reverse micelles is utilization of the partitioning of proteins between different phases in reverse micelles for isolating enzymes.[8] A full understanding of the partitioning of a substance among various pseudo-phases of reverse micelles should be beneficial to future practical applications.

In this article, various factors that influence the partitioning of 4-nitrophenol in AOT reverse micelles were examined. We proposed a model of the reverse micelles that contains a continuous gradient microenvironment in the water pool. This model explained the molecular basis of the displacement of 4-nitrophenol from the interface region into the water pool.

Experimental

Materials

4-Nitrophenol, 2-amino-2-methylpropanol, bis-tris propane {1,3-bis[tris(hydroxymethyl)methylamino]propane}, and AOT (aerosol-OT) were purchased from Sigma-Aldrich (St. Louis, Missouri). The purity of AOT was examined previously and the product was used without further purification.[9] Isooctane was from Mallinckrodt Baker (Paris, Kentucky). *tert*-Butylamine (2-amino-2-methylpropane) and isobutanol (2-methylpropan-1-ol) were from Riedel-deHaën (Seelze-Hanover, Germany). Other chemicals used were all of reagent grade and were obtained from Merck (Darmstadt, Germany) as described previously.[9,10]

† The abbreviations used are: AOT, aerosol-OT [sodium bis(2-ethylhexyl) sulfosuccinate]; CTAB, cetyltrimethylammonium bromide; Triton X-100, *tert*-octylphenoxypolyethoxyethanol; ω_o, the molar ratio of water concentration to AOT concentration, *i.e.*, [H_2O]/[AOT].

Preparation of AOT reverse micelles in systems of various degrees of hydration

AOT reverse micellar stock solution (0.2 M) was prepared by dissolving AOT (8.89 g, 20 mmol) in isooctane to give 100 ml.

For preparing the working solution, AOT concentration was maintained constant at 187 mM. The $[H_2O]$/[surfactant] ratio was adjusted by varying the amount of water which changes the dimensions of the reverse micelles. Aqueous solution was introduced into the system by the injection technique.[11] The water droplet was solubilised by mechanical agitation (vortex).

Ionization of 4-nitrophenol in AOT reverse micelles

The absorption spectrum of 4-nitrophenol (67 μM) in AOT–isooctane reverse micelles at various water contents ($[H_2O]$/[AOT] = 10–19) was scanned from 500 to 250 nm. An absorption coefficient of 18.3×10^3 M^{-1} cm^{-1} was used for the absorption peak at 402 nm, which represented the ionized 4-nitrophenolate.[12] The molar absorption coefficient of non-ionized 4-nitrophenol was determined in acetic acid (100 mM, pH 3.2) as 9.5×10^3 M^{-1} cm^{-1} at 310 nm, which is identical to the value for 4-nitrophenyl phosphate at all pH values.[12] The total volume of the system was used in all calculations.[11]

The apparent pH of the reverse micellar solution was determined by directly immersing a glass electrode in the transparent reverse micellar solution. The apparent pH of the reverse micellar solution was the same as that of the aqueous solution, which indicates that the pH of the water pool is close to that of the initial buffer system, at least for the carbonate buffer, in which both forms of the buffer cannot associate to the negatively charged isooctane–water interface. This agrees with the generally accepted conception of the pH of a reverse micellar system at $[H_2O]$/[surfactant] > 10–15.[1] However, the apparent pK_a value of 2-amino-2-methylpropanol will be dependent on the water content of the system.[13] The intensity of the absorption band of 4-nitrophenol in reverse micelles at various water contents of the system was calculated according to the Henderson–Hasselbach equation (1).

$$pH = pK_a - \log([\text{4-nitrophenol}]/[\text{4-nitrophenolate}]) \quad (1)$$

Results

Partitioning of 4-nitrophenol in the AOT–isooctane reverse micelles

4-Nitrophenol has a pK_a value of 7.14 in the aqueous solution. However, in reverse micelles, the 4-nitrophenol that partitioned into the interface region has a pK_a value of 11.5.[14] The UV-visible spectrum of ionized 4-nitrophenolate in the aqueous carbonate buffer solution (pH 10.0) shows a maximum peak at 402 nm. Injecting the same solution into AOT–isooctane reverse micelles caused the absorption coefficient of the solution at 402 nm to decrease with a new absorption peak appearing at 310 nm, because of the non-ionized 4-nitrophenol.[4] We have measured the partitioning of 4-nitrophenol at pH 10.0 in a binary system consisting of isooctane and water. No partitioning with the organic medium was observed. Thus, a negligible amount of 4-nitrophenol was partitioned into the organic phase. In the first approximation, the partition coefficient of a compound in a simple biphasic system consisting of water and organic solvent can be used as an estimate for the partition coefficient of the compound between the aqueous and organic phases in the multiphasic reverse micellar system.[15]

When the spectrum of 4-nitrophenol was examined with increasing water content of the system (represented by the molar ratio of $[H_2O]$/[AOT]), which increases the micropolarity of the water pool, there was a concomitant batho-

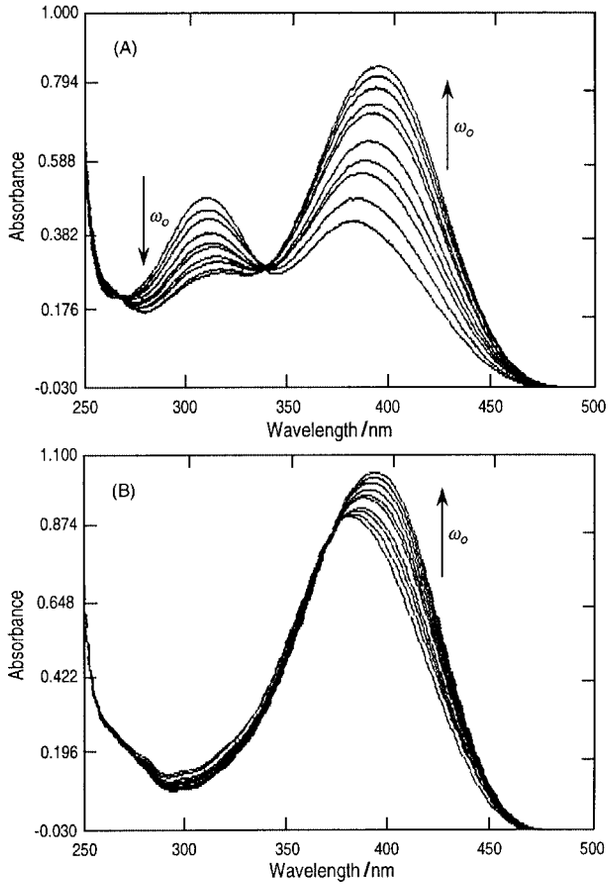

Fig. 1 Absorption spectra of 4-nitrophenol in an AOT reverse micellar system with different buffers. The absorption spectra of 4-nitrophenol (67 μM) in AOT reverse micelles (187 mM) containing $NaHCO_3$–Na_2CO_3 buffer (5.3 mM, pH 10.0) (A) or 2-amino-2-methylpropanol buffer (5.3 mM, pH 10.0) (B) were monitored between 250–500 nm. In the order indicated by arrows, the ω_o ($[H_2O]$/[AOT]) value of the system was 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19, respectively.

chromic shift of both the A_{402} and the A_{310} peaks; but an opposite dependence on ω_o of the absorbance intensity of these two peaks, hyperchromicity at A_{402} and hypochromicity at A_{310} with an isosbestic point at 345 nm, was observed (Fig. 1A). These results indicate an equilibrium of two species in the system. For the results shown in Fig. 1A, the $[H^+]$ at the AOT/water interface should be high due to H^+ exchange with Na^+.

The above partitioning phenomenon was less pronounced when the buffer was changed to 2-amino-2-methylpropanol at the same pH value (Fig. 1B). There is, however, also a batho-/hyper-chromic shift of the A_{402} peak at increasing water content of the reverse micellar system. In the results shown in Fig. 1B, it is the protonated form of 2-amino-2-methylpropanol, instead of H^+, which exchanges with Na^+ at the interface. The absorption maximum of 4-nitrophenolate ion shifts from 402 nm in water to 380 nm in reverse micelles, indicating that the microenvironment that senses the absorbing probes is quite different. Furthermore, the absorbance reading at $\omega_o = 19$ in Fig. 1B is higher than in Fig. 1A indicating that there is a greater amount of 4-nitrophenolate ion in the experimental conditions of Fig. 1B. This latter effect is further enhanced in the presence of the cationic surfactant CTAB in the interface (Fig. 2). In this case, partitioning also occurs but now it is the 4-nitrophenolate ion which distributes between water and the CTAB interface. The reasons for this are that 4-nitrophenolate ion binds to the cationic interface and H^+ is excluded from the positively charged interface. The absorbance in Fig. 2A is higher as compared with the scan at $\omega_o = 15$ in Fig. 1A. The effect of the neutral charged surfactant Triton X-100 is in between (Fig. 2B).

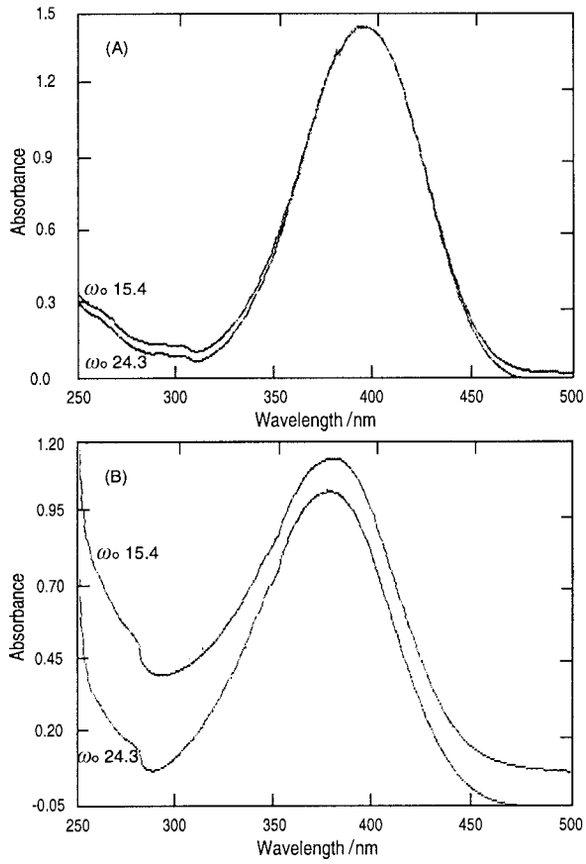

Fig. 2 Absorption spectra of 4-nitrophenol in CTAB or Triton X-100 reverse micellar system. The experimental conditions were the same as described in Fig. 1(A), except that 4-nitrophenol was dissolved in 187 mM CTAB-reverse micelles (A) or in Triton X-100-reverse micelles (B). For simplicity, only those spectra with ω_o 15.4 and 24.3 are shown.

Effect of ionic strength or surfactant concentration on the partitioning of 4-nitrophenol in AOT reverse micelles

The above results indicate that the partitioning of 4-nitrophenol in AOT reverse micelles depends on the buffer system used. Since the ionic strength of the carbonate buffer is much larger than that of the 2-amino-2-methylpropanol buffer, which might cause an effect on the microenvironment of the system and hence the partitioning of 4-nitrophenol,[16] we then examined the influence of ionic strength of the buffer system on the variation in partitioning. Similar and slightly hypochromic shifts in the visible peak of the absorption spectra were obtained with different amounts of carbonate buffer (Fig. 3, curves a) or the same amount of 2-amino-2-methylpropanol buffer but with various amounts of NaCl to give ionic strengths corresponding to those for the carbonate buffer (Fig. 3, curves b), but no change in the overall spectrum was observed. In our other experiment, where the ionic strength of the solution was fixed by adding NaCl, a 4-fold difference in carbonate buffer concentration at constant ω_o of 11 changes the partitioning by 6% as calculated from the absorption intensity differences. These results rule out the possibility that ionic strength is the main cause for the partitioning of 4-nitrophenol in AOT reverse micelles. It is the buffer composition that contributes to the spectral changes.

When the water and surfactant concentrations were increased simultaneously at a constant ratio (constant ω_o), the number of reverse micellar particles increases but the inclusive volume of each particle remains constant.[17] We observed an increased partitioning of the 4-nitrophenol into the interface region at higher AOT concentration (Fig. 4), which demonstrates the affinity of 4-nitrophenol with the interface of the system.[17] At $\omega_o = 10$, the partitioning seems to reach a plateau

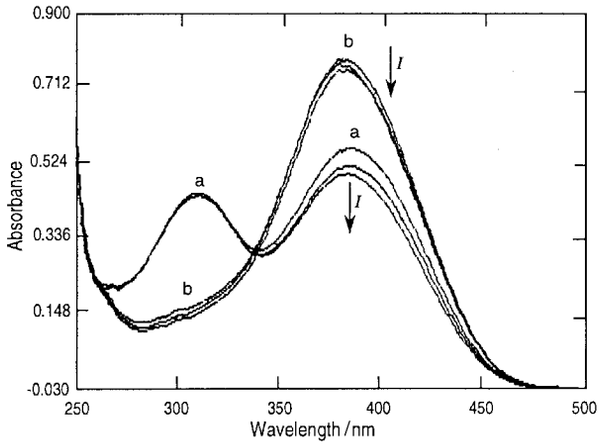

Fig. 3 Effect of ionic strength on the absorption spectra of 4-nitrophenol in AOT reverse micellar system. The experimental conditions were the same as described in Fig. 1, except that ω_o was fixed at 11. In the curves of (a) group, the carbonate buffer (pH 10.0) was used at 2.2, 3.33, and 6.67 mM, with ionic strength of the solution corresponding to 1, 1.5 and 3, respectively. In the curves of (b) group, the same amount of 2-amino-2-methylpropanol buffer (6.67 mM, pH 10.0) was used but various amounts of NaCl were added to give ionic strength of the solution corresponding to 1, 1.5 and 3, as in curves of (a). Similar results were obtained at $\omega_o = 19$.

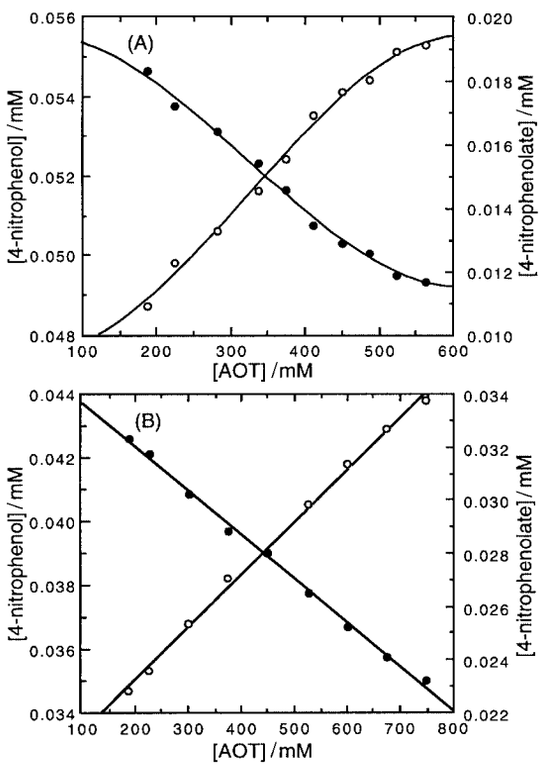

Fig. 4 Effect of surfactant concentration on the partitioning of 4-nitrophenol in AOT reverse micellar system. The experimental conditions were the same as described in Fig. 1, except that AOT and water concentrations were increased proportionally to maintain a constant $[H_2O]/[AOT]$ ratio; $\omega_o = 10$ in (A), and $\omega_o = 15$ in (B). Open circles: 4-nitrophenol concentration calculated from A_{310}; solid circles: 4-nitrophenolate ion concentration calculated from A_{402}.

at AOT concentration larger than 500 mM. On the other hand, in larger reverse micelles ($\omega_o = 15$), the partitioning phenomenon proceeded in a wide range of AOT concentrations. Neither the maximum at A_{310} nor the A_{402} peak was shifted at different AOT concentrations.

Displacement of 4-nitrophenol from the interface to the water pool region of AOT reverse micelles

The partitioning of 4-nitrophenol from the interface region to

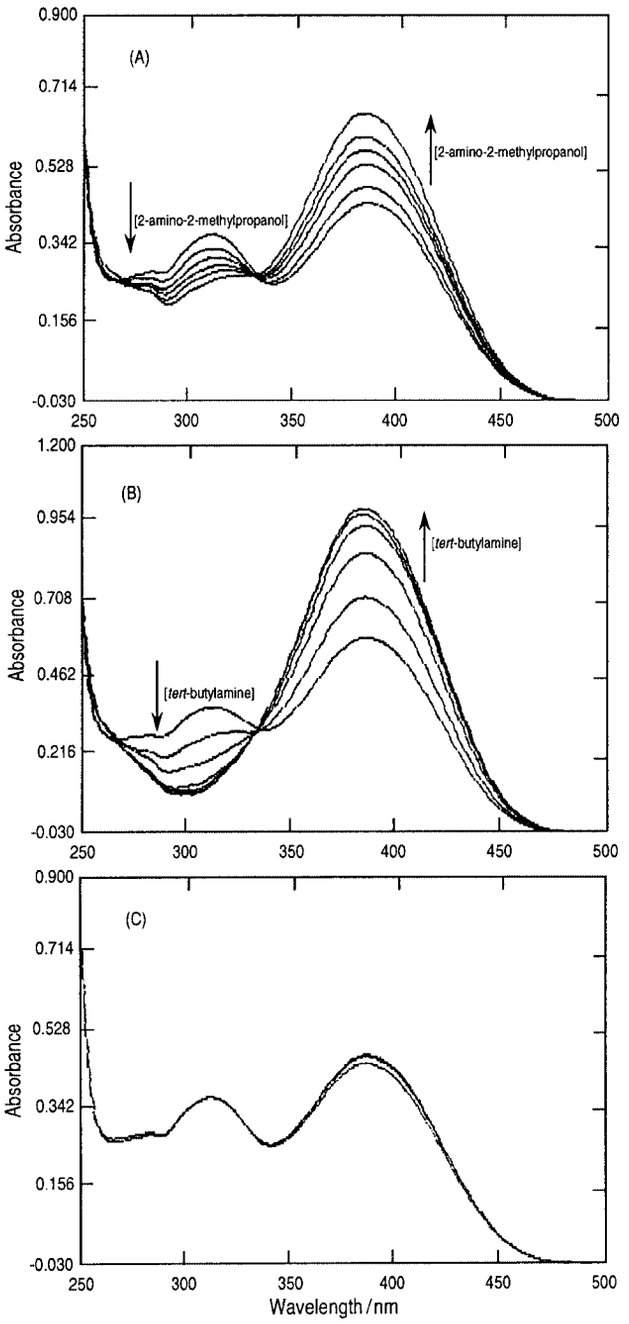

Fig. 5 Absorption spectra of 4-nitrophenol in AOT reverse micellar systems with carbonate buffer in the presence of 2-amino-2-methylpropanol, *tert*-butylamine (2-amino-2-methylpropane), or isobutanol (2-methylpropan-1-ol). Experimental conditions were the same as described in Fig. 1(A), except that the water content of the system was fixed at $\omega_o = 11$. (A) In the order indicated by arrows, the 2-amino-2-methylpropanol concentration was 3.3, 2.6, 2.0, 1.33, 0.66 and 0 mM, respectively. (B) In the order indicated by arrows, the *tert*-butylamine concentration was 3.3, 2.6, 2.0, 1.33, 0.66, or 0 mM, respectively. (C) The isobutanol concentration used was 3.3, 2.6, or 2.0 mM.

the water pool in $NaHCO_3$–Na_2CO_3-buffered AOT reverse micelles was further analyzed in the presence of 2-amino-2-methylpropanol, *tert*-butylamine, or isobutanol. Fig. 5A shows that at constant water content of the system, increasing 2-amino-2-methylpropanol concentration decreases the partitioning, *i.e.*, more 4-nitrophenol has been displaced into the water pool in which it was ionized at basic pH value. *tert*-Butylamine induced more partitioning than 2-amino-2-methylpropanol (Fig. 5B). There is about a 2-fold difference between *tert*-butylamine and 2-amino-2-methylpropanol in the efficiency of displacement (Fig. 5B). The partition coefficient of 4-nitrophenol ([4-nitrophenolate]/[4-nitrophenol]) is 1.47 in

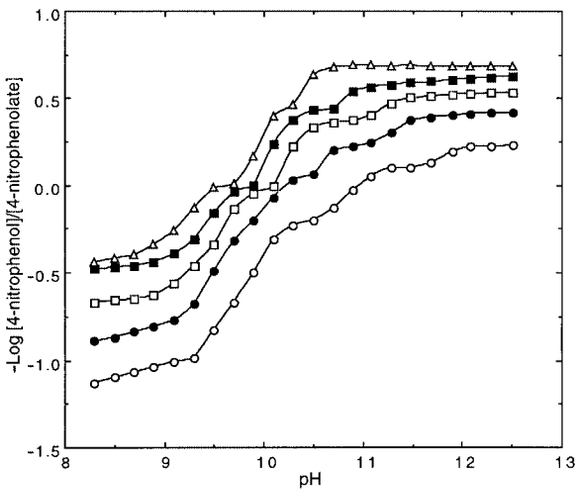

Fig. 6 Ionization of 4-nitrophenol in AOT reverse micellar system at different water contents of the system. The ionization of 4-nitrophenol was examined at several fixed water contents of the system by varying the pH value of the solution with carbonate buffer (5.3 mM). Open circles: $\omega_o = 10$; solid circles: $\omega_o = 12$; open squares: $\omega_o = 15$; solid squares: $\omega_o = 17$; and open triangles: $\omega_o = 19$.

2-amino-2-methylpropanol (3.3 mM), but 3.54 in *tert*-butylamine (3.3 mM). However, isobutanol ($pK_a \sim 18$) did not induce the same displacement (Fig. 5C). Obviously, it is the amino group rather than the hydroxy group that induces the displacement. This may be attributed to the salt formation between 4-nitrophenolate ion and the amino group.

Ionization of 4-nitrophenol in AOT reverse micelles

We observed that the apparent pK_a value of the phenolic -OH increased by 2–3 pH units in a reverse micellar system (Fig. 6). The multiphasic changes in the apparent pK_a values clearly demonstrate that 4-nitrophenol exists in multiple populations distributed in different locations of the reverse micelles and the observed pK_a values represent the result of these distributions.

We proceeded to modulate the occupancy of these multiple populations by adjusting the water content of the system. At smaller $[H_2O]/[AOT]$ ratios, the inclusive volume of the reverse micelles decreases and more 4-nitrophenol molecules are partitioned into the interface. Thus, the apparent pK_a value of 4-nitrophenol was high. This is true for all pK_a values of 4-nitrophenol in the system. For the carbonate buffer in the pH range shown in Fig. 6, the pK_a value of the water core is not detected. In our other experiments with the bis-tris propane buffer, which has a different operating pH range ($pK_1 = 6.8$, $pK_2 = 9.0$), the pK_a value of 4-nitrophenol in the water core region was found to be about 7.8–8.0 at $\omega_o = 10$–15, similar to the value determined by Menger and Saito.[14]

Discussion

We have studied the spectra of 4-nitrophenol in the presence of two buffers as a function of ω_o. The spectral changes that have been analysed are a result of the combination of distribution equilibria and acid–base equilibria. The topic dealt with here on 4-nitrophenol in the AOT–isooctane–water system is especially significant because this phenol is a common leaving group in studies of acyl transfer and many other reactions. Our results clearly indicate that the pK_a values of 4-nitrophenol are sensitive to the buffer used and to the water content in the reverse micellar system. These results support the fact that organic dyes have only limited application in measuring the acidity of the water pool of reverse micelles; because the location of an organic compound in the reverse micelles contributes to its final spectrum.[18] Any dye cannot have a defined pK_a value at the interface. The apparent pK_a value of 4-nitrophenol, like that of

other compounds, depends on the relative extension of both water and AOT interface, that is, of those phases between which the dye distributes. Thus at each AOT concentration (or ω_o value) there will exist a different pK_a value. On the other hand, some of these organic compounds provide an excellent probe to investigate the micro-polarity of the reverse micelles.[19] The macrohomogenous transparent reverse micellar system is composed of at least three microheterogenous phases, *i.e.*, an organic phase plus an interface and water pool pseudophases. Partitioning of the dissolved compound among these phases results in the compound being in contact with different micro-environments with different properties that might either affect the reactivity[20] or induce a solvatochromic effect[19] on the dissolved compound. These factors should be considered in the interpretation of the experimental results.

Partitioning of 4-nitrophenol in AOT–heptane reverse micelles has been reported by Menger and Saito,[14] who suggested that 4-nitrophenol distributes in reverse micelles in two populations. That located in the water pool has a pK_a value of 7.6–7.9, close to that in the bulk aqueous solution, while that located in the interface has a pK_a value of 11.5. Ionization of 4-nitrophenol in the interface is impaired because embedding 4-nitrophenol into the interfacial phase of the system connects the phenolic hydroxy group to the anionic sulfonates of AOT. The above hypothesis was confirmed in the present study of 4-nitrophenol in CTAB or Triton X-100 reverse micelles with only one absorption peak shown at 402 nm. Partitioning still exists in these reverse micellar systems. However, owing to the cationic or non-ionic nature of the polar head, the 4-nitrophenol partitioned into the interface was still in the ionized form.

Menger and Saito[14] have observed displacement of 4-nitrophenol from the interface into the water core of the AOT reverse micelles by the imidazole buffer. We further narrow down that a single amino group is enough to induce this displacement. Since a hydroxy group did not induce the displacement, ionic interactions, rather than a hydrogen bond, are probably the major reason for the displacement.

Our novel finding includes demonstrating that the multiple ionization of 4-nitrophenol depends on its location in the AOT reverse micelles (Fig. 6). However, as shown in Fig. 6, there is not a sharp variation of the function log ([4-nitrophenol]/[4-nitrophenolate]) with pH. We can only determine the apparent pK_a value at each ω_o value as being equal to the pH value corresponding to the function. The value of this function obviously increases as the water content decreases and 4-nitrophenol is less acidic in an organic solvent. The classification of 4-nitrophenol in the water pool into two populations as originally proposed by Menger and Saito[14] may be over-simplified. In Menger's treatment, the distribution is between only two locations, free water and the interfacial region, four equilibrium constants govern the behaviour.

Here we propose a new model for the reverse micelles that further delineates the properties of the water molecules in the water pool of the reverse micellar system. We interpret the multiple ionization of 4-nitrophenol as a gradient polarity from the water core to the interface region. Our model shown in Fig. 7 includes clustered surfactant molecules, which surround a water pool. The water molecules in the pool, however, experience different micro-polarities due to the polar head influence of the surfactant. This influence should have an inverse relationship with the distance (measured by the inclusive volume of the reverse micellar system). Putting an ionisable compound into the water pool will subject the compound to different micro-polarities depending on the location. The interaction of the negative charge of the AOT polar head with the 4-nitrophenol renders ionization less favorable which results in more non-ionized 4-nitrophenol molecule near the interface region. We propose that within the water pool, the microenvironments are in a gradient according to their distance from the polar head

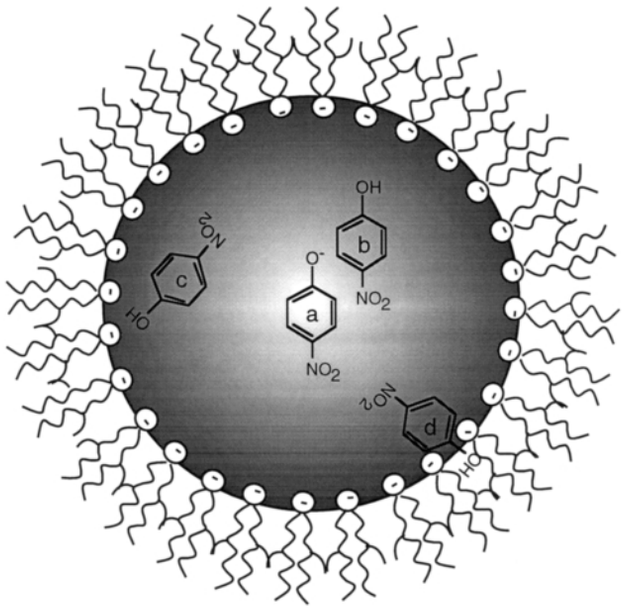

Fig. 7 A schematic model showing 4-nitrophenol entrapped in AOT reverse micelles. The polar head of the AOT molecule is drawn into the water pool (gray area) according to Martinek *et al.*[17] The gradient shade expresses differential influence of the polar head of the surfactant molecule on the water molecules. The number of 4-nitrophenol molecules in the water pool does not represent the average number of 4-nitrophenol in a reverse micellar particle. In practice, since the surfactant/4-nitrophenol concentration ratio is over a thousand-fold, it is more likely that each reverse micellar particle contains only one 4-nitrophenol molecule, albeit it could be distributed in some different regions according to the water content of the system.

as shown in Fig. 7. Since the variations in observed absorption maxima are a consequence of the distribution between the different environments, a larger number of environments will mean that further pairs of distribution and acid–base equilibrium constants will be required.

Conclusions

4-Nitrophenol has an affinity with the surfactant AOT in carbonate buffer. Binding of 4-nitrophenol with the anionic surfactant polar head hinders ionization resulting in elevation of the pK_a value of the phenolic -OH group, which occurs in a gradient manner with the most basic -OH at the interface region. Binding of 4-nitrophenol with AOT can be affected by the 2-amino-2-methylpropanol buffer, which perturbs the partitioning of 4-nitrophenol between the water pool and the interface. The perturbation of 4-nitrophenol partitioning in AOT reverse micelles in 2-amino-2-methylpropanol buffer is due to the amino group of the buffer molecule.

Acknowledgement

This work was supported by the National Science Council, Republic of China (Frontier Science Grant NSC 87-2312-B016-008).

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