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Sodium Cholate Aggregation and Chiral Recognition of the Probe Molecule (R,S) 1,1'-binaphthyl-2,2' diylhydrogenphosphate (BNDHP) Observed by ¹H and ³¹P NMR Spectroscopy

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Abstract

Bile salt micelles can be employed as a pseudo-stationary phase in micellar electrokinetic capillary (MEKC) separations of chiral analytes. To improve MEKC separations of chiral analytes, a molecular level understanding of micelle aggregation in the presence of analyte is needed. Here, aggregation of sodium cholate has been observed by exploiting the presence of a model analyte molecule. The ³¹P and ¹H nuclear magnetic resonance spectroscopy (NMR) chemical shifts of (R,S) 1,1'-binaphthyl-2,2'-diylhydrogenphosphate (R,S-BNDHP), a model analyte in chiral MEKC separations, are demonstrated to be very sensitive to the aggregation state of the bile salt sodium cholate. In addition to probing micellar aggregation, the NMR spectral resolution of enantiomeric species is also strongly correlated with chiral separations in MEKC. In this work, the aggregation of sodium cholate in basic solutions (pH = 12) has been observed over the concentration range 0-100 mM. The primary cmc was found to be 14 ± 1 mM for basic solutions of sodium cholate. In addition a primitive aggregate is clearly observed to form at 7 ± 1 mM sodium cholate. The data also show pseudo-cmc behavior for secondary aggregation observed in the regime of 50-60 mM cholate. Finally, the H5-H7 edge of BNDHP is shown to be sensitive to chirally selective interactions with primary cholate micelles.

I. Introduction

Bile acids are rigidly planar steroidal compounds that are involved in a number of biologically significant functions including dietary lipid emulsification and solubilization.^{1, 2} Derived from cholesterol, bile acids (a.k.a. bile salts since they are often obtained as sodium salts) are composed of a saturated ring system with alpha oriented polar hydroxyl groups (Figure 1). Many hydrophobic and pharmacologically active compounds can associate with bile acid aggregates^{3, 4} and membrane structures in general.^{5, 6} Bile acids are characterized by hydroxyl groups that lie beneath the equator of the planar steroid skeleton. As shown in Figure 1, cholate is an example of a trihydroxy bile acid. Due to the directionality of their hydroxyl groups, bile acids possess both hydrophilic and hydrophobic “sides”, and are therefore amphipathic molecules. Monomers of bile acids aggregate as a function of concentration, however despite considerable progress fundamental questions remain on the structure and formation of these aggregates.^{1, 4, 7-12 13} Diverse techniques have been brought to bear on bile salt aggregation,

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yet no consistent values for critical micelle concentrations or aggregation numbers have emerged.

In addition to their physiological importance, bile acids such as cholate form aggregate micellar structures that are employed as a pseudo-stationary phase for chiral recognition of analytes in separation methods such as capillary electrophoresis (CE). This process of micellar electrokinetic capillary chromatography (MEKC) was initially developed to separate neutral analytes⁹⁻¹⁰, but is also capable of chiral separations when the pseudo-stationary phase has chiral selectivity.¹¹ Indeed, the use of bile salts as a chiral selector in MEKC has been shown to be particularly useful for analyte molecules that have planar sub-structures.^{11,12,14,20} Despite the use of bile salts to facilitate chiral separations in MEKC, the structural and mechanistic details of the interactions of chiral analytes with bile salt aggregates remain unclear.

(R,S)- 1,1'-binaphthyl-2,2'-diylhydrogenphosphate (R,S BNDHP) are rigid chiral molecules (Figure 1) that have been employed as model chiral analytes in high performance liquid chromatography and/or capillary electrophoresis techniques for chiral separations.¹⁴⁻¹⁸ The majority of chiral molecules possess a stereogenic center; in contrast, BNDHP exists as atropisomers with restricted rotation around the 1-1' bond between naphthyl rings, indicated by an asterisk (*) in Figure 1. In BNDHP two hydrophobic naphthyl groups are bridged by a phosphate which can be viewed as a hydrophilic capping group.

Optimizing MEKC separations in the absence of a molecular explanation for how bile acid aggregates mediate chiral selection currently requires exhaustive systematic trials over a large number of experimental variables. Temperature, pH, ionic strength, capillary preconditioning time, and concentrations of all species (bile salts, buffers, additives, analyte) are examples of such variables. Since it is not practical to test all combinations of these parameters, a desirable alternative would be to identify independent analyses which could help predict the performance of chirally selective MEKC separations. More generally, a structural and potentially dynamic explanation for enantiomeric discrimination in cholate mediated interactions would be of greatest value in optimizing the resolution of chiral isomers by MEKC.

The atomic specificity of information yielded by NMR spectroscopy should reveal new information on aggregates of bile salts as well as their interactions with guest molecules.^{13, 19-21} This work reports an NMR investigation of the chiral analyte R,S-BNDHP in the presence of varying concentrations of the sodium salt of cholate in basic (pH 12) solutions. We have found that basic solutions result in higher resolution of (R,S-BNDHP) by MEKC, and herein probe this system with NMR. We demonstrate a direct correspondence between ³¹P NMR chemical shifts and the degree of enantiomeric resolution in MEKC and discuss reasons for mutual trends in MEKC and NMR experimentation. We also describe NMR-based approaches for determining the primary critical micelle concentration (cmc) of cholate in the presence of BNDHP. Broadly, the results support a stepwise aggregation of cholate through primary and secondary stages. In addition, aggregation of monomers is observed below the primary cmc. Secondary aggregation is found to preserve the hydrophobic environment sampled by BNDHP in primary micelles. Finally, ¹H NMR experiments identify localized regions of BNDHP which participate in chiral recognition when bound to the cholate micelle. Overall, NMR has provided new insight on cholate aggregation and on the mechanism of chiral separations by MEKC.

II. Experimental

Sample Preparation and NMR Spectroscopy

Sodium cholate hydrate (98% purity), 1,1'-binaphthyl-2,2'-diylhydrogenphosphate (95% purity), (R)-(-)-1,1'-binaphthyl-2,2'-diylhydrogenphosphate (>98% purity), (S)-(-)-1,1'-binaphthyl-2,2'-diylhydrogenphosphate (97% purity), and deuterium oxide (99.9 atom % D) were purchased from Aldrich. A concentration of 2.5 mM R,S-BNDHP, R-BNDHP, and S-BNDHP was maintained for their respective experiments while the concentration of cholate was varied from 0 mM to 100 mM. A solution of 90% 18 M Ω -cm H₂O and 10% deuterium oxide was used as a solvent. A pH of 12 was achieved by using 1 M NaOH. One dimensional ³¹P NMR spectra were obtained on a Bruker ARX spectrometer operating at 300 MHz for ¹H (Bruker Biospin, Billerica MA). Uncertainties in ³¹P chemical shifts are .01 ppm. One dimensional ¹H NMR spectra were collected on a Bruker Avance 600 MHz spectrometer. ³¹P relaxation spectra were acquired at a Larmor frequency of 162 MHz (9.4 T, 400 MHz for ¹H) on a Varian DirectDrive spectrometer (Varian Inc., Palo Alto CA). We employed the inversion recovery (T₁) and CPMG echo (T₂) pulse sequences and analysis software supplied in the Varian software suite. Uncertainties in ¹H chemical shifts are .001 ppm. A DSS external standard was used to reference ³¹P spectra, utilizing appropriate conversion factors for referencing ³¹P spectra to 85% H₃PO₄.²² For aqueous samples, water suppression obtained by WATERGATE was used to obtain high signal-to-noise ratios for sample resonances.²³ Solid state ³¹P NMR data was collected at a Larmor frequency of 202 MHz (500 MHz for ¹H) on a Chemagnetics/Varian Infinity spectrometer.²⁴ Predicted ³¹P SSNMR spectra were obtained using the WSolids1 software program, and were additionally processed with 500 Hz of Lorentzian broadening.²⁵

MEKC

CE experiments were performed on an Agilent 3D CE (Palo Alto, CA) with 50 μ m i.d. fused silica capillaries (PolyMicro, Phoenix, AZ) having a 32.5 cm effective length, 41.0 cm total length. The capillary was pretreated by flushing (ca. 925 mbar) with 1 M NaOH for a minimum of 5 minutes daily. Prior to individual runs, the capillary was preconditioned as follows: the capillary was sequentially flushed for 3 minutes each with i) 1 M NaOH, ii) 18 M Ω -cm H₂O, and iii) the separation buffer. The analyte sample was introduced into the capillary via a 25 mbar hydrodynamic injection for a total of three seconds (75mbar-s). A separation potential of 10kV was utilized, and online UV absorbance detection at 217nm (8 nm bandwidth) was employed. All experiments were conducted at a column temperature of 25.0 $\pm$ 0.1°C.

Stock solutions of sodium cholate and racemic mixtures and individual conformers of R/S-1,1'-binaphthyl-2,2'-diylhydrogenphosphate were prepared in 18 M Ω -cm H₂O from reagents obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Separation buffers of sodium cholate were adjusted to pH 12 by dropwise addition of 1M sodium hydroxide, prepared from reagents obtained from Fisher Scientific (Fairlawn, NJ, USA), while monitoring the pH with an Accumant pH meter (Fisher Scientific). All chemicals were used without further purification. Each solution was filtered through a 0.45 μ m PTFE membrane filter using a syringe into a 1 mL Agilent Technologies (Palo Alto, CA, USA) polypropylene vial prior to capillary electrophoretic study.

For both MEKC and NMR experimentation, all solutions were prepared within one week of use. Further, NMR spectroscopy showed no appearance of impurities or degradation products over this time frame.

Results

³¹P NMR and MEKC

The compound (R,S)-BNDHP exhibits atropisomerism and is a model analyte for chiral MEKC separations. For MEKC separations involving bile salt micelles such as cholate or deoxycholate, the analyte is assumed to exchange on a very fast timescale such that equilibrium populations of free and micelle-bound analyte can be assumed throughout the MEKC experiment.^{26, 27} It is observed that the S-isomer has a longer retention time than the R-isomer, indicating a longer residence time for the S-isomer in the micelle. Representative electropherograms of pure and racemic samples of (R,S)-BNDHP are given in column I of Figure 2. Both R- and S-BNDHP are significantly retained by cholate micelles, and small changes in the rate of electroosmotic flow give rise to marked changes in the observed migration time for the analytes. However, the electrophoretic mobility of each isomer is constant under any given set of conditions. Chiral resolution is achieved in the presence of sodium cholate concentrations ca. 14 mM or greater, which is consistent with the primary cmc observed by others²⁸⁻³⁴ and herein (*vide infra*). Specifically, MEKC results demonstrate that the bile salt primary micelle structure is necessary for resolving R,S-BNDHP enantiomers using capillary electrophoresis.

Phosphorous NMR exhibits high sensitivity ($\gamma_{31P}/\gamma_{1H} = 0.405$) and is useful in studying biophysical properties and conformational exchange in biomolecules.³⁵⁻⁴¹ For this study, the sole phosphate moiety of BNDHP gives a simple spectral response consisting of just one resonance for each of R- and S-BNDHP. Representative ³¹P NMR spectra are given in columns II and III of Figure 2 for (R,S)-BNDHP in the presence and absence, respectively of sodium cholate at pH 12. First, ³¹P chemical shifts for the R- and S-enantiomers are confirmed to be degenerate in the absence of cholate, regardless of whether they are prepared in separate solutions or as a racemic mixture (Figure 2g-i). In the presence of cholate above the first critical micelle concentration (e.g. > 14 mM), enantioselective association with the cholate micelle results in chiral resolution of R- and S-BNDHP by MEKC. In the presence of 14 mM cholate or greater the ³¹P chemical shift of S-BNDHP is more strongly perturbed than that of R-BNDHP for spectra of single enantiomers (Figure 2e-f) and of the racemate (Figure 2d). The ³¹P chemical shift of the phosphate group is sensitive not only to the environment of the BNDHP molecule, but also to the fraction of micelle-bound BNDHP molecules. The observation of a single resonance demonstrates that BNDHP satisfies the condition for fast exchange with the micelle on the NMR timescale.⁴² Spectra obtained at temperatures of 0-4 °C exhibit one narrow ³¹P peak, so it was not possible to observe two slow exchanging components. Under fast exchange the observed chemical shift is a population weighted sum of the ³¹P chemical shifts of BNDHP in the free and bound states. Then the data in Figure 2 support that differences between R- and S-BNDHP chemical shifts reflect different populations of micelle-bound species (assuming approximately: $\delta_{\text{bound}}^R \approx \delta_{\text{bound}}^S$).

A series of ³¹P NMR spectra as a function of the cholate concentration was obtained for R- and S-BNDHP separately and as racemic mixtures. The resulting ³¹P chemical shifts are given in Figure 3b. All samples were prepared at pH 12 and incorporated 2.5 mM analyte. An inflection point in the change in chemical shift is observed at ca. 14 mM sodium cholate, while the shifts are perturbed beginning at about 7 mM cholate. MEKC experiments were conducted on racemates of R,S-BNDHP, and net mobilities obtained from these data are given in Figure 3a. A general trend toward lower net mobilities with increasing cholate concentration is observed due to the partitioning of the analyte into the micelle. A maximum in the difference of net mobilities is seen ca. 10-20 mM in Figure 3a, although the number of samples in this trial restricts observing this point to high precision. An interesting observation in Figure 3b is that the ³¹P chemical shifts of the R-isomer in the presence of cholate are degenerate for

solutions that are enantiopure or racemic. In contrast, the S-isomer chemical shift is more strongly perturbed in the racemate (open squares) than when enantiopure (filled squares). The presence of R-BNDHP is therefore inferred to enhance the fraction of micelle-bound S-BNDHP. This is likely an allosteric effect in which micelles are able to simultaneously accommodate R and S isomers. The present data do not provide insight into the possible mechanism of this cooperativity. In a broader context, Figure 3 demonstrates a correspondence between ^{31}P chemical shifts and MEKC performance characteristics, specifically the analyte mobilities. Taking **Figures 2-3** together we conclude that ^{31}P shifts are sensitive reporters on the performance of MEKC separations.

^{31}P Relaxation

Spin-spin (T_2) and spin-lattice (T_1) relaxation time constants for the ^{31}P nucleus in (R,S)-BNDHP have also been measured over a series of cholate concentrations. Nuclear spin relaxation depends strongly on molecular reorientation. Both T_1 and T_2 exhibit a strong dependence upon cholate concentration. When (R,S)-BNDHP enantiomers spend time in a bound state with an aggregate of cholate, the BNDHP nuclei, where ^{31}P is the NMR reporter in this case, will undergo relaxation governed by the slower reorientation of the macromolecular assembly. Additionally the exchange process itself can be associated with oscillating magnetic fields and can also drive relaxation, notably T_2 . Furthermore, the T_1/T_2 ratio is very sensitive to the molecular rotational correlation time, and therefore the size and shape of the macromolecule.^{40, 43} We observe in Figure 4a-b changes in T_1 and T_2 for both R,S-BNDHP immediately upon the addition of sodium cholate. Significant changes in the T_1/T_2 ratio (Figure 4c-d) for both R,S-BNDHP are observed beginning at 5 mM sodium cholate. Aggregation of monomers prior to the primary cmc would be consistent with these observations.

In Figure 4a-b, expansions of the T_1 and T_2 regions corresponding to 30-100 mM sodium cholate concentrations indicate a nonsingularity in the relaxation behavior occurring at about 50-60 mM cholate. Monotonic decreases in the ^{31}P BNDHP T_1 and T_2 values abruptly level off at cholate values of 50-60 mM. The T_2 behavior of the R isomer does not appear to show this trend as clearly as the (R,S)- T_1 and S- T_2 measurements, which may be attributable to the fact that the R isomer does not sample the micelle as strongly as the S isomer and may not be as sensitive a reporter on the dynamics of the aggregated state. The S-BNDHP T_1/T_2 values are discontinuous at about 50-60 mM cholate, whereas the R-BNDHP T_1/T_2 values gradually increase above 50-60 mM cholate. Overall, these data show a change in the relaxation behavior of R,S-BNDHP at about 50-60 mM cholate.

Potential additional information that can be obtained from the T_1 and T_2 time constants (or $R_1=T_1^{-1}$, $R_2=T_2^{-1}$ rates) are the reorientation dynamics (e.g. rotational correlation time) of the combined BNDHP/cholate aggregate. In principle, ^{31}P NMR of BNDHP can be a good reporter on dynamics. Due to the phosphate moiety, ^{31}P is not subject to strong dipolar couplings, and is expected to only be very weakly dipolar coupled to the H3 proton in BNDHP. To a good approximation the relaxation can be attributed solely to (i) chemical shift anisotropy (CSA) driven reorientational relaxation and (ii) exchange driven relaxation. We obtained the ^{31}P CSA tensor by a Herzfeld-Berger analysis for a powdered (a.k.a. polycrystalline) sample of R,S-BNDHP⁴⁴ (see supporting information) The tensor components are $\delta_{11}=58$ ppm, $\delta_{22}=35$ ppm, and $\delta_{33}=-103$ ppm. As expected, identical CSA parameters were independently measured for R-BNDHP. The extraction of detailed dynamics is outside the scope of this work, but a preliminary outline of the methods is considered in the discussion.

¹H NMR of R,S-BNDHP

¹H NMR spectra of (R,S)-BNDHP are also useful probes of the aggregation processes and binding interactions between BNDHP and the cholate micelle. In analogy to the ³¹P chemical shift titration experiments in Figure 3, ¹H chemical shifts of BNDHP can be monitored as a function of cholate concentration. Representative ¹H 1D-NMR spectra obtained at 600 MHz are illustrated in Figure 5, which reveal a number of cholate-dependent trends.

Figure 5 demonstrates that the ¹H resonances of R,S-BNDHP are dependent upon cholate concentration, and also exhibit different cholate-dependent trends for the two enantiomers. The ¹H shifts of BNDHP are especially useful since they are confined to the region 7.0 - 8.2 ppm, whereas all cholate chemical shifts (not shown) are entirely in the 0 - 4 ppm region of the spectrum. The ¹H shifts of BNDHP are also well resolved, so that it is possible to obtain distinct cholate dependent changes of individual BNDHP proton chemical shifts in order to gain localized information. Assignments are indicated on Figure 5 and have been previously described²¹.

A finely grained cholate titration was conducted for each enantiomeric species separately; the resulting isotropic chemical shifts for R,S-BNDHP are plotted in Figure 6. The chemical shifts for H4-H8 all follow a similar trend of undergoing a perturbation at 7 mM cholate. In contrast H3 undergoes chemical shift perturbations at 14 mM cholate. For cholate concentrations below a given cmc, all BNDHP molecules must be free in solution and the BNDHP chemical shifts must be constant; the cmc is then given as the onset of a perturbation in the measured parameter, which is often a chemical shift.^{45, 46} Thus, the proton data indicate progressive cmc values of 7 and 14 mM for sodium cholate solutions at pH 12 and with 2.5 mM BNDHP. These data support a model in which protons H4-H8 of R,S-BNDHP are responsible for interacting with aggregates of cholate that form at 7 mM cholate, specifically a model in which binaphthyl groups insert one naphthyl moiety into a hydrophobic planar pocket formed by what we will call here a *primitive aggregate* of cholate, which could be a dimer. Proton H3, however, is expected to be solvent exposed and therefore much less sensitive to premicellar aggregates and reports only on a larger and more stable primary micelle that forms at 14 mM.

The proton chemical shift titrations probe structural details of distinct interactions between cholate micelles and the enantiomers of BNDHP. As with the ³¹P data, the proton chemical shifts of S-BNDHP are more strongly perturbed relative to R-BNDHP. The ³¹P data, however, are limited in providing structural information on the BNDHP-micelle interaction. Evidence of localized interactions of BNDHP with the cholate micelle is observed in the ¹H NMR spectra in Figure 6, where the degree of chemical shift perturbation of the R- and S- isomers is not identical for all BNDHP protons. The differences between R- and S-isomer chemical shifts for all proton sites on BNDHP are given in Figure 7. Protons H5, H6, and H7 are seen to experience the greatest chemical shift differences between the enantiomers ($\delta_R - \delta_S$) in the region of the primary cholate micelle (14-15 mM); however these differences diminish with increasing cholate, suggesting a gradual loss of chirally selective interactions between BNDHP and the host micelles. There are minor or no chemical shift differences between the enantiomers in Figure 7 at 7 mM cholate, suggesting that this pre-micellar primitive cholate aggregate sampled by R,S-BNDHP does not provide chirally selective interactions. These data indicate that H5, H6 and H7 undergo chirally selective interactions with the primary micelles that form at about 14-15 mM cholate.

IV. Discussion

A general framework for discussing bile acid aggregation was presented by Small,⁴⁷ who described two general regimes termed primary and secondary aggregation. Although there are competing models for explaining bile aggregation,^{14-20, 32-41} it appears to be widely agreed

that aggregation is progressive in two stages.^{7, 10, 47-49} Primary aggregation is generally reported to occur at a well defined critical micelle concentration (cmc) for a given set of aqueous conditions. For cholate, cmc values for primary micellization are commonly reported ca. 15 mM using a number of techniques, although there is considerable variation.^{8, 11, 30, 50-56} As bile concentration increases, a secondary aggregation takes place that increases the size of the bile micelles further.^{1, 7, 47} Secondary aggregation is not well characterized for cholate, but is thought by man, including Small,⁴⁷ to be an association of primary micelles into larger structures by further hydrophilic interactions.

For bile salt concentrations below the primary cmc the formation of a primitive aggregate such as a dimer likely occurs as a necessary precursor to the primary micelle.^{30, 47} Direct evidence for dimer formation for bile acids has been elusive,^{7, 8, 57, 58} but recently 2D-NMR NOE/ROE measurements on taurocholate have supported back-to-back hydrophobic dimerization at concentrations below the cmc.¹³ The primary micelle is widely suspected to be formed from a small number of dimers, but plausible models for primary micelles do not need to be restricted to dimers.^{4, 10, 58, 59} Broadly, a primitive aggregate, be it a dimer or otherwise, is characterized by hydrophobic interactions among two or more monomers.⁴⁷ The data here indirectly support a dimer as the primitive cholate aggregate since the planar hydrophobic pocket formed by back-to-back interactions of a cholate dimer would be able to accommodate the planar naphthyl moieties. The hydrophobic interactions in a primitive aggregate are thought to be insufficient to form a persistent micelle, and hydrophilic contacts between multiple primitive units are important for stabilizing the primary micelle.⁵⁸

In this study we examined the aggregation of sodium cholate under basic conditions in the presence of a probe molecule, (R,S)-BNDHP, which is known to undergo exchange with bile salt micelles. We will interpret our findings in the context of a primitive aggregate followed by primary and secondary stages of micelle formation, where we stress that these interpretations are in the presence of a probe molecule and high pH, and may not represent bile acid aggregation under other conditions.

MEKC and ³¹P NMR

A particularly strong correspondence between the mobilities of R,S-BNDHP in MEKC experiments and their respective ³¹P chemical shifts in NMR experiments is demonstrated in **Figures 2-3**. The ³¹P chemical shifts appear to report directly on the separation efficiency of MEKC. For example, below about 14 mM cholate, the R- and S-enantiomers are not resolved by either MEKC electropherograms or by ³¹P NMR chemical shifts. These data agree with the widely reported cmc value of 14-15 mM for aqueous sodium cholate solutions and show that the primary cholate micelle is necessary and sufficient for chirally selective interactions with (R,S)-BNDHP.

The close agreement between MEKC and NMR results is straightforwardly explained since both depend critically on establishing different relative fractions of the enantiomers in the micelle-bound state. The observed BNDHP chemical shifts (δ_{obs}) are population weighted averages of their values in the free (δ_{free}) and micelle-bound (δ_{bound}) environments. Since the MEKC results require that S-BNDHP interact more strongly with the cholate pseudo-stationary phase, we can therefore interpret the ³¹P chemical shifts as reporting on different population distributions of R- and S-BNDHP in the free and micelle-bound states (i.e. MEKC results require that $f_{bound}^R < f_{bound}^S$). The close correspondence between the MEKC and NMR results supports the assumption that the ³¹P phosphate chemical shifts of the micelle-bound R,S-BNDHP are sufficiently similar ($\delta_{bound}^R \approx \delta_{bound}^S$) that the observed differences between the ³¹P chemical shifts of the two enantiomers must be due to different populations in the free and micelle-bound states. It was not possible to obtain direct measurements of the

separate ^{31}P chemical shifts for the free and micelle-bound forms of BNDHP by cooling samples to 4 °C. Also supporting the assumption $\delta_{\text{bound}}^{\text{R}} \approx \delta_{\text{bound}}^{\text{S}}$ is that the hydrophilic phosphate groups of R,S-BNDHP experience similar local environments in their micelle-bound states since they will be exposed on the surface of the micelle particle and experience similar solvation. To be clear, the data do not suggest equality in the ^{31}P chemical shifts for micelle bound R- and S-BNDHP, only sufficient similarity to allow us to ascribe large changes in the total observed chemical shift (δ_{obs}) to changes in fractional populations of free and micelle bound states of BNDHP.

Increasing cholate concentration beyond the primary cmc leads to diminished separation of (R,S)-BNDHP by MEKC and also a slight decrease in the ^{31}P chemical shift separation between the R and S forms, further highlighting the correspondence between MEKC and NMR data. The secondary aggregates that form at high cholate concentrations must therefore be less effective at differentially solubilizing (R,S)-BNDHP enantiomers; these data suggest that the MEKC separations can be optimized by favoring the formation of primary micelles.

The ^{31}P chemical shift difference for (R,S)-BNDHP is greater in racemic mixtures than for enantiopure solutions (Figure 3). The correspondence we have discussed here means that a larger difference in ^{31}P chemical shifts reports on a larger difference in the fractions of bound enantiomers and should lead to better separation in the MEKC experiment. We may expect to see a greater difference in MEKC mobilities of (R,S)-BNDHP for racemates than for separate MEKC experiments on enantiopure R- and S-BNDHP samples. However, this prediction is difficult to experimentally confirm since in the MEKC environment the racemate will exist only transiently in the capillary; that is, the R and S forms will separate inside the capillary early in the MEKC experiment and any cooperative effect of the R-isomer on the fraction of bound S-isomer will be too short-lived to cause an observed change in migration time.

Measuring Cholate cmc's with BNDHP Chemical Shifts

Micellization phenomena for sodium cholate at both 7 mM and 14 mM in the presence of BNDHP are indicated by the ^{31}P and ^1H NMR data reported here. As discussed above, we assume that the observed ^{31}P and ^1H chemical shifts are a weighted sum of the free and bound values so that the cmc is identified as the cholate concentration at which chemical shifts of the probe molecule first deviate from their free values.^{45, 46} That is:

$$\begin{aligned} \text{Case 1: } [\text{Cholate}]_{\text{Total}} &< \text{cmc} \\ \delta_{\text{obs}} &= \delta_{\text{free}} \\ \text{Case 2: } [\text{Cholate}]_{\text{Total}} &\geq \text{cmc} \\ \delta_{\text{obs}} &= f_{\text{free}}\delta_{\text{free}} + f_{\text{mic}}\delta_{\text{bound}} \end{aligned} \quad (1)$$

In this model, BNDHP chemical shifts will be constant until cholate reaches a critical micelle concentration.

The individual R or S ^{31}P NMR data in Figure 3 are approximately constant in the range of 0-7 mM cholate, and decline steeply at about 5-7 mM, showing a preliminary association in this regime. In addition, we discussed above that (R,S)-BNDHP are only resolved above 12-16 mM cholate by either MEKC or NMR, requiring the existence of a cholate aggregate that is capable of performing chiral discrimination among (R,S)-BNDHP and which forms only at this higher concentration. Therefore, the MEKC and ^{31}P NMR data show two micellization phenomena at approximately 5-7 mM and 12-16 mM cholate. The ^{31}P chemical shift data gives approximate concentrations for these aggregation stages and cannot be satisfactorily modeled at this time to yield more precise values.

In contrast, very detailed information on the stages of aggregation is revealed by ^1H NMR. The ^1H chemical shifts of H4-H8 are constant in the range 0-7 mM and steeply change after 7 mM cholate, showing a cmc at this value. The H3 chemical shift is constant over the range 0-14 mM and changes immediately after 14 mM. The data show that a cholate aggregate is sampled by H4-H8 at cholate concentrations of 7 mM or higher and an aggregate forms at 14 mM cholate which is sampled by H3. The H4-H8 data and the H3 data refine the ^{31}P chemical shift results and show that well defined cmc's occur for basic solutions of cholate at 7 mM and 14 mM. If we assert that BNDHP is a reporter on the cholate local environment, then we can analyze BNDHP chemical shifts as a function of cholate concentration using Eqn (1), which is shown in Figure 8 for H3 and H4 of S-BNDHP. The close agreement between the model and the experimental data support that BNDHP is a very accurate reporter on the cholate local environment, and clearly establishes the 7 mM and 14 mM aggregation steps for cholate.

All of H3-H8 in R- and S-BNDHP have been modeled using Eqn (1) and these figures are available as supplemental materials. In summary: H4-H8 all show 7 mM cmc values, H3 shows a 14 mM cmc value, and interestingly H8 exhibits a clear cmc ca. 6-7 mM and a sudden deviation from the model at 14 mM cholate, so that a single cmc is insufficient to model the H8 data in the 0-20 mM cholate concentration range. Modeling the H5-H7 BNDHP ^1H chemical shifts shows large deviations with the model at 50-60 mM, which we assign to the establishment of the secondary micelle. In Figure 8, the H3 and H4 data only subtly deviate from the model at 50-60 mM cholate, showing that the secondary micelle perturbs H5-H7 much more strongly than H3-H4. The role of the secondary micelle in influencing the local environment of H5-H7 will be seen to be important in chiral discrimination of (R,S)-BNDHP.

In surveys of cholate cmc's, many investigators have reported values ca. 5-9 mM or values ca. 10-19 mM.^{8, 11} The disparity in cmc values might be viewed as a continuum of values owing to the considerable differences in experimental conditions and techniques, or could be viewed as bimodal with respect to cmc's observed either above or below 10 mM cholate. We are aware of one prior study that measured both an early (6.2 mM) and a primary (12.8 mM) cmc for solutions of sodium cholate at 298 K in aqueous solution, using light scattering.⁶⁰ Also, we are not aware of any study of cholate aggregation at pH = 12. For the conditions considered here (pH = 12 and the presence of a probe molecule) the 7 mM early cmc which we attribute to a primitive aggregate that may be a dimer, and the 14 mM primary cmc which we attribute to a stable primary micelle consisting of multiple primitive aggregates, are observed. Both aggregation processes are unambiguously established using the exquisite localized sensitivity of H3 to the surface of the primary micelle, and of H5-H7 to the hydrophobic interior of the primitive aggregates. However corroborating support for these values was obtained from ^{31}P NMR and from MEKC experiments as well. While it is possible that different experimental techniques may preferentially detect the early or primary cmc, we propose that the very close proximity of these two aggregation events may also present a significant difficulty in distinguishing between them. That the proximity of these two cmc's could complicate their detection may be supported by a number of reports of 10 mM cmc values for cholate^{8, 11} which would appear to be an average of the early and primary cmc's.

Overall, the results show that BNDHP chemical shifts are sensitive NMR probes for measuring cmc's in cholate in analogy to the use of fluorophores such as pyrene as fluorescent markers of primary aggregation.^{30, 48, 60}

^{31}P NMR T_1 , T_2 Relaxation—The ^{31}P T_1 , T_2 data depend on both molecular reorientation (T_1 , T_2) and on exchange (T_2). We note that in all cases, clear monoexponential fits to the data were obtained. Steep decreases are observed for ^{31}P T_1 , T_2 values in the region of 5-10 mM cholate, while T_1/T_2 increases in this regime, which are all consistent with an increase in the average rotational correlation time for (R,S)-BNDHP. In other words BNDHP must partition

with a cholate aggregate in the 5-10 mM regime, which we postulate above to be a primitive aggregate such as a dimer. In the bound form, the rotational correlation time is larger than for free BNDHP and relaxation will be enhanced for micelle-bound BNDHP molecules. The T_1 and T_2 values continue to decrease as cholate increases to about 20-30 mM, which is consistent with the primary micellization that occurs at 14 mM cholate and higher. Similarly T_1/T_2 values increase up to about 20-30 mM cholate.

The relaxation data support secondary micellization at higher concentrations of cholate. The insets of Figure 4a-b show that both T_1 and T_2 abruptly level off at 50-60 mM cholate, indicating a change in the aggregation state of cholate at this concentration. And particularly for the S-enantiomer there is a discrete increase in the T_1/T_2 ratio at about 60 mM cholate that indicates an increased rotational correlation time and therefore a larger particle. The S-enantiomer samples the micelles more strongly and is expected to be a better probe, however the T_1/T_2 ratio for R-BNDHP shows similar behavior: it is approximately constant in the range of 30-60 mM and then begins to increase. We interpret secondary micellization occurring at about 50-60 mM cholate according to the relaxation data.

The presence of exchange is a complicating factor in interpreting the relaxation data and we have not developed a satisfactory model for these data at this time. The relaxation qualitatively supports the early and primary cmc's determined above by chemical shift analysis, but more work is needed to determine the exchange contribution to relaxation and to develop a model for these data, particularly in samples of 0-30 mM cholate.

Localizing Chiral Discrimination on BNDHP

We observe from Figure 6 that H5, H6 and H7 are the most strongly perturbed BNDHP signals by the cholate micelle, and are furthermore sensitive to the structural basis of chiral selection (Figure 7) since they exhibit the highest values of $\delta(H_R)-\delta(H_S)$ as a function of increasing cholate concentration. We have established also (Figure 2) that the primary micelle which forms at 14 mM is strictly required for successful resolution of (R,S)-BNDHP by MEKC, and so Figure 7 shows that H5-H7 exhibit the greatest values of $\delta(H_R)-\delta(H_S)$ where the primary micelle predominates. In contrast the H3 chemical shift is more weakly perturbed as a function of cholate concentration, and also reports very little chiral discrimination in Figure 7, especially in the vicinity of the primary micelle. The H4 and H8 chemical shifts follow similar trends as H5-H7 but not as strongly. Thus H4-H8 must sample the hydrophobic micellar interior of cholate that is established at 7 mM cholate with H5-H7 being the most sensitive to the hydrophobic site, while H3 is a poor reporter on the hydrophobic binding site but is most sensitive to the surface of the primary micelle. In Figure 6, the H5-H7 chemical shifts remain approximately constant for cholate concentrations of 30-100 mM. The hydrophobic binding pocket sampled by H5-H7 is therefore conserved throughout primary and secondary aggregation. This supports the commonly cited model that secondary aggregation is the association of primary micelles. In contrast, the H3 chemical shift does not show a plateau until about 60-70 mM cholate, indicating a sensitivity to secondary aggregation. Qualitatively, the packing of primary micelles into secondary micelles must replace solvent on primary micellar surfaces with interactions between primary micelles, and this change in the surface of the primary micelle is being reflected in the H3 chemical shift. Both H4 and H8 also weakly show a similar trend of developing distinct plateaus in chemical shifts at 60-70 mM cholate in Figure 6 and so are weakly sensitive to the micelle surface as well.

Also, the ^{31}P nucleus may be safely assumed to be fully excluded from the hydrophobic binding pocket and to be proximate to the surface of the primary micelle, and the BNDHP ^{31}P chemical shift between 20-100 mM reflects continued changes in the local chemical environment. Secondary aggregation may therefore be described as a pseudo-cmc: that is, the onset of secondary aggregation is indicated in this work to occur ca. 50 mM and to consist of the

association of primary micelles, but does not show the highly discretized phase-separation behavior found at 7 mM and 14 mM cholate.

Secondary aggregation inhibits chirally selective guest-host interactions between BNDHP and cholate. In MEKC experiments, decreasing resolution of the R,S-BNDHP enantiomers was evident above 40 mM cholate (Figure 3). Furthermore, Figure 7 shows that $\delta(H_R)-\delta(H_S)$ values decline within the range 20-60 mM cholate for H5-H7, directly showing a loss of the chirally selective interactions between BNDHP and the cholate aggregates. As discussed above, secondary aggregates must have less solvent-exposed surface area. Steric effects from packing of primary micelles may therefore inhibit access of the naphthyl rings to the hydrophobic pockets or prevent access to other interactions that preferentially stabilize the binding of the S-isomer

The ^{31}P nucleus of BNDHP is an attractive NMR relaxation probe, since BNDHP is rigid, and the mechanisms for relaxation are very limited. In principle the ^{31}P relaxation data may be modeled to yield estimates of Stokes radii of macromolecular assemblies by treating the relaxation as a combination of contributions from the free and bound states. The ^{31}P CSA tensor was measured as a preliminary step towards such an analysis. To carry out this treatment, however, it is necessary to determine or model fractional populations in free and bound states and to account for the exchange contribution to T_2 relaxation. Further work in this area is needed and the use of diffusion NMR methods and relaxation in the rotating frame ($T_{1\rho}$) will be helpful in better understanding cholate aggregation in the presence of (R,S)-BNDHP. Nonetheless the NMR techniques used here have provided significant new insight into the complex behavior of bile salts and chiral recognition.

Conclusion

The work presented here provides a route to understanding bile salt micelle formation and chirally selective guest-host interactions in the presence of a model analyte. A strong correspondence between ^{31}P chemical shift resolution of the R,S-BNDHP enantiomers and the difference in mobilities in MEKC experiments was demonstrated. In addition, ^1H chemical shifts for H4-H8 (via $\delta(H_R)-\delta(H_S)$) of BNDHP also correlate with MEKC chiral resolution.

Interestingly, the NMR data indicate cmc-like aggregation processes occurring at cholate concentrations of 7 ± 1 mM and 14 ± 1 mM, even though MEKC chiral resolution is only obtained at cholate concentrations of about 14-15 mM or higher. We have attributed these observations to the formation of primitive structures consistent with dimers (7 mM cholate) and stable primary micelles (14 mM).

Our data are also consistent with further aggregation as cholate concentration increases which, according to Small's model, could be the assembly of primary micelles into higher order structures. Under the conditions studied here, secondary aggregation is observed to be an ill-defined process over the range 40-60 mM. The hydrophobic pocket sampled by H5-H7 protons of R,S-BNDHP is conserved in all aggregation states of cholate salts up to 100 mM. While the present results do not directly inform the design of current MEKC separations, the H5-H7 edge of R,S-BNDHP has been identified as a region of interest for better understanding structural mechanisms for chiral MEKC separations with bile salts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1, BNDHP **2, Cholate****Figure 1.**

Chemical structures of BNDHP (1) and Cholate (2). BNDHP exhibits chirality owing to restricted rotation about the ring-bridging bond marked by the asterisk (*).

Figure 2.

Comparison of MEKC data (column I) and ^{31}P NMR data (Columns II, III) for chiral discrimination of R,S-BNDHP in the presence of 80.0 mM cholate (a-f) and with 0 mM cholate (g-i) at pH 12.0. A 5 mM racemic mixture of BNDHP (2.5 mM each of R- and S-BNDHP) was used for (a) (d) and (g); 2.5 mM S-BNDHP was used for (b), (e) and (h); 2.5 mM R-BNDHP was used for (c), (f), (i). Detailed MEKC and NMR conditions are provided in the experimental section.

Figure 3. Plots of electrophoretic mobility (a) and ^{31}P chemical shift (b) of BNDHP as a function of cholate concentration.

Figure 4.

Spin-lattice (a) spin-spin (b) and T_1/T_2 ratio (c,d) relaxation data obtained via ^{31}P NMR of the R- (◆) and S- (■) isomers of BNDHP as a function of cholate concentration. Insets show only the concentration range above 30 mM cholate.

Figure 5. Representative ^1H NMR spectra for the R (solid lines) and S (dotted lines) isomers of BNDHP in the presence of increasing concentrations of cholate at pH 12 show cholate dependent perturbations of chemical shifts.

Figure 6. Complete chemical shift “titration” data showing the effect of increasing cholate concentration on the observed chemical shifts of BNDHP proton resonances.

Figure 7. Differences in ^1H chemical shifts for R and S BNDHP isomers with increasing cholate concentration, computed from the data in Figure 6.

Figure 8.

Log-log plots of ^1H chemical shift versus cholate concentrations for H3 and H4 of S-BNDHP are given (open circles), suggesting preliminary cholate aggregation at 7 mM and primary micelle formation at 14 mM cholate, respectively. The model of Eqn (1) is overlaid onto each (dashed line), where $\delta_{H4,\text{free}}^{S\text{-BNDHP}} = 8.1635$, $\delta_{H4,\text{bound}}^{S\text{-BNDHP}} = 8.045$, $\text{cmc} = 7\text{mM}$, and $\delta_{H3,\text{free}}^{S\text{-BNDHP}} = 7.5885$, $\delta_{H3,\text{bound}}^{S\text{-BNDHP}} = 7.645$, $\text{cmc} = 14\text{ mM}$. Models for all protons of R,S-BNDHP are given in the supplemental materials.