Spectrophotometric determination of the critical micellar concentration of bile salts using bilirubin monoglucuronide as a micellar probe

Utility of derivative spectroscopy

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We have developed a simple biologically non-invasive method for determining the critical micellar concentration (CMC) of bile salts using pure naturally occurring bilirubin IX α monoglucuronide (BMG), an important bile pigment present in virtually all mammalian biles. This methodology employs visible absorbance spectroscopy of BMG in bile salts over a range of bile salt concentrations that include the reported CMC. Using 100 μ M-BMG in 0.4 M-imidazole buffer at pH 7.8, we calculated that the CMC for sodium taurochenodeoxycholate is between 2.5 and 3.0 mM based on: (1) an abrupt change in λ_{max} in this concentration range, (2) a precipitous decrease in the amplitude of the absorbance shoulder at 450 nm, (3) a sudden decrease in the second derivative absorbance of BMG at 400 nm and an increase in absorbance at 470 nm, (4) a sharp change in the 4th derivative absorbance at 375 and 395 nm. In contrast, sodium taurocholate, a bile salt that reportedly does not have a CMC but continuously self-associates over a wide concentration range, exhibited none of these changes. The use of derivative spectroscopy enhances the ability to detect the CMC changes and also indicates the number of BMG species in solution and their relative energy states.

INTRODUCTION

Bile salts are amphiphilic molecules with the properties of self association in both aqueous and organic solvents. In aqueous solutions bile salts form large spherical or globoid aggregates with non-polar hydrophobic interiors and polar hydrophilic surfaces facing the aqueous environment (Carey, 1983). Over a narrow concentration range, referred to as the critical micellar concentration (CMC), some bile salts have a critical self-association, with a strong co-operative interaction of a number of bile salt monomers to form a micelle (Beher, 1977). The micelle formed at the CMC is thermodynamically more stable than higher concentrations of the monomer. Thus, increasing the concentration of the bile salt above the CMC will not increase the monomeric population of the bile salt that the micelle is in equilibrium with (Carey & Small, 1972).

Other bile salts such as sodium cholate (Beckerdite & Adams, 1985) and sodium taurocholate (NaTC) (Mazer *et al.*, 1979) appear to continuously self-aggregate over a range of concentrations in a stepwise or progressive fashion often beginning with dimers. These bile salts exhibit non-critical self-association. Since the change in aggregation occurs gradually and, therefore, is non-co-operative in these bile salts, the physical properties of these solutions should change gradually with increasing concentrations. Thus, when surface tension is plotted as

a logarithmic function of bile salt concentration, a sharp inflection point is seen at the point of critical micellization for bile salts that have a true CMC, whereas the change in slope is more subtle for bile salts that exhibit non-critical micellization (Carey, 1983).

Although for any set of physical-chemical conditions, only one value for the CMC exists for each individual bile salt, discrepant values have been reported by different investigators. Two major reasons exist for the varied results. First, the CMC of a given bile salt will vary depending on the physical-chemical environment. Thus, changes in pH, counter-ion concentration, solvent polarity and temperature have a marked effect on the CMC (Small, 1971). In addition, there are technical difficulties in measuring the CMC by any method that does not involve the addition of a 'reporter' molecule to the solution (Carey, 1983). Although the addition of a reporter molecule may simplify measurement of the CMC, the reporter molecule may itself, by the nature of its charge, shape or hydrophobicity, induce changes in the micelle which will affect the CMC. Thus, the reported value for the CMC of NaTCDC has ranged from 0.47 mM (Duane, 1977) to approx. 2.5 mM (Carey *et al.*, 1981). NaTC has an even wider range of reported CMC values, perhaps due to the fact that this bile salt has non-co-operative micellization (Carey, 1983).

Previous spectrophotometric determination of the CMC has involved the use of rhodamine 6G as a reporter

Abbreviations used: BMG, bilirubin IX α monoglucuronide; CMC, critical micellar concentration; UCB, unconjugated bilirubin IX α ; NaTC, sodium taurocholate; NaTCDC, sodium taurochenodeoxycholate; TC-BUF, taurocholate/imidazole/ascorbate buffer.

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molecule (Small, 1971). More recently Carey & Koretsky (1979) have determined the CMC of SDS using a solution containing unconjugated bilirubin IX α (UCB) at pH 10 as the reporter molecule. Since UCB is difficult to solubilize below pH 8.0 even in the presence of bile salts, it has not been used for determining the CMC of bile salts in the range of physiological pH until recently (Rodrigues & Spivak, 1987).

On the other hand, due to the presence of a highly polar glucuronic acid moiety which is ionized at physiological pH, BMG is soluble at physiological pH (Spivak & Yuey, 1985; Spivak *et al.*, 1987), especially in the presence of bile salts. Also, since BMG is a component of mammalian bile, its incorporation into the micelle as a reporter molecule simulates the natural biological interaction that occurs between bile salt and bile pigment and has the potential to shed some light on how this interaction occurs.

In this study, we demonstrate the utility of BMG as a natural reporter molecule for determining the true critical micellization of NaTCDC. We also demonstrate that NaTC does not exhibit a true critical micellization over a wide range of concentrations.

MATERIAL AND METHODS

Chemicals and buffers

BMG was prepared according to the method of Spivak & Carey (1985) and as later modified by Spivak & Yuey (1986). NaTC (> 98% pure by t.l.c.) and NaTCDC (> 99% pure by t.l.c.) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); NaTC was further purified by the method of Pope (1967). Imidazole and ascorbic acid were of the highest purity obtainable from Sigma. Water was doubly distilled and then de-ionized before use. All glassware was acid washed and rinsed thoroughly with distilled water before use. Taurocholate buffer solution (TC-BUF) contained 25 mM-NaTC, 5 mM-ascorbic acid and 0.4 M-imidazole, at pH 7.8.

Equipment

All spectrophotometric scans were performed on a Perkin-Elmer Model 553 UV-VIS spectrophotometer interfaced with a model 3600 micro-computer data station for digital scan recording (Perkin-Elmer, Elmwood Park, NJ, U.S.A.). The instrument was warmed up for at least 45 min before use and was electronically zeroed using the TC-BUF system in both the sample and background holder. The spectral slit width was 2 nm and the scan speed was 120 nm/min. Digital spectral recording to floppy disk was performed using IF552 software (Perkin-Elmer). Spectral analysis and plotting was performed after digital recording using Perkin-Elmer PECUV software and derivative analysis and carried out using the DERV2 OBEY file of PECUV (with a $\Delta\lambda$ of 10 nm over a data interval of 1 nm). For zero order spectra, the absorption at $\lambda_{\max}$ was digitally auto-expanded so that all spectra appear to have the same maximal absorption. This was performed so that relative absorbances could be easily compared and so that minor changes in BMG concentration (< 5%) that occur as a result of oxidation or hydrolysis (Spivak *et al.*, 1987) would not obscure relative absorption changes that occur with changing bile salt concentration. Since the

spectra are autoexpanded, we refer to relative absorbances and not absolute absorbance. Optically matched quartz cuvettes of 0.1 cm optical path length were used for all scans. Temperature of the scanned solutions was maintained at 26 °C using a thermostatically jacketed cuvette holder and Haake circulating temperature bath (Haake, Saddle Brook, NJ, U.S.A.).

Solutions

All scanned solutions contained 100 μ M-BMG, 5 mM-ascorbic acid (to prevent BMG oxidation) and 0.4 M-imidazole buffer at pH 7.80 ± 0.02 in addition to the appropriate bile salt concentration. Solutions in the reference cell contained the same solutes as those of the sample cell except for the presence of BMG. Solutions were prepared from BMG that was dried in a Speedvac evaporator (Savant Instruments, Hicksville, NY, U.S.A.) after h.p.l.c. preparation (Spivak & Yuey, 1986), and stored overnight at -10 °C. Buffer was added to the dry BMG solution to make a solution of approx. 500 μ M; since there are no gravimetric standards for BMG, the initial BMG concentration was determined by measuring the absorbance at 415 nm of a 20 μ l portion of concentrated BMG in 180 μ l of a TC-BUF solution and calculating the concentration from a spectrophotometric BMG standard curve of BMG in TC-BUF. The spectrophotometric standard curve of BMG was based on the h.p.l.c. determination of BMG as previously described (Spivak & Yuey, 1986). Once the concentration of BMG was accurately determined, the solution was then diluted with additional ascorbic acid buffer solution and bile salt to give the appropriate bile salt concentration and to give a final BMG concentration of 100 μ M. Since the buffer solution has a very high ionic strength, little change in the ionic strength occurs over the reported CMC ranges of these bile salts.

Purity and special handling of the solutions. All solutions were layered with argon and handled under very dim indirect light in order to minimize photo-oxidation and/or isomerization of BMG (Lightner, 1982). In addition, all solutions were kept on ice at 4 °C prior to scanning, and were scanned within 90 min of preparation in order to reduce the chance of hydrolysis of BMG (Spivak *et al.*, 1987). At the end of the experiment, the amount of BMG that was degraded by either oxidation or hydrolysis was determined by h.p.l.c. and was found to be less than 5% in every experiment.

RESULTS

Fig. 1 demonstrates the effect of changing bile salt concentration on the absorption spectra of BMG in NaTC and NaTCDC. As one can see from the absorption spectra and from Table 1, $\lambda_{\max}$ occurs in the 414–422 nm range and a shoulder occurs in the 439–467 nm range.

Although the spectra for the two different bile salts appear similar, on closer inspection, important differences are present. Whereas the relative absorbance in the area of the shoulder decreases nearly proportionally to the change in bile salt concentration for NaTC over the range of 1–12 mM [this concentration range encompasses virtually all of the reported CMC values for this bile salt (Carey, 1985)], a sudden change in the relative absorbance at the shoulder occurs for NaTCDC between 2.5 and 3.0 mM in the reported range of the CMC of this bile salt

Fig. 1. Zero-order spectra of 100 μM -BMG in imidazole/ascorbate buffer at pH 7.8 with varying concentrations of (a) NaTC and (b) NaTCDC

With increasing bile salt concentration, note the continuous decrease in the shoulder (439–467 nm) of NaTC, whereas NaTCDC exhibits a sudden decrease in the shoulder between 2.5 and 3.0 mM, which is where the CMC of this bile salt exists. In order to compare relative absorbances, the absorption at λ_{max} was digitally autoexpanded so that all spectra appear to have the same maximal absorption. The light-path length was 0.1 cm.

(Roda *et al.*, 1983). The gradual decrease in relative absorbance at the shoulder for NaTC is consistent with recent reports that NaTC does not have a true CMC, but continuously self-aggregates over a range of concentrations.

Along with the decrease in relative absorbance of the bathochromic shoulder as bile salt concentration increases, λ_{max} shifts hypsochromically, as one might expect (see Table 1). This pattern is a result of at least two different absorbing components in solution, one with a maximum near the area of the shoulder, and one with a maximum near the Soret band. With increasing bile salt concentration, the absorbing component at the shoulder becomes less prominent and the absorbing

component in the Soret band increases in intensity. Overlap of these two components produces a λ_{max} that exists between the shoulder absorbance and the true Soret band absorbance. From Table 1, it is apparent that there is no sudden change in λ_{max} over the concentration range of 1–12 mM for NaTC; however, NaTCDC exhibits a sudden shift in λ_{max} from 419 to 415 nm over the concentration range 2.5–3.0 mM. This abrupt change in λ_{max} is the result of a sudden increase in the relative intensity of the hypsochromic absorbing component that occurs at the CMC. This change in the two components can be seen more readily using second-derivative spectroscopy.

Fig. 2 demonstrates the utility of second-derivative

Table 1. Absorption maxima ($\lambda_{\max}$) of 100 μM -BMG in NaTC and 100 μM -BMG in NaTCDC

All solutions contained 0.4 M-imidazole/5 mM-ascorbate at pH 7.8. Note the sudden change in $\lambda_{\max}$ for NaTCDC between 2.5 and 3.0 mM; on the other hand, $\lambda_{\max}$ for NaTC changes gradually from 1 to 12 mM.

NaTC (mM)	$\lambda_{\max}$ BMG (nm)	NaTCDC (mM)	$\lambda_{\max}$ BMG (nm)
1	418	0.5	422
2	418	1.0	422
3	417	1.5	420
4	418	2.0	419
5	417	2.5	419~
6	417	3.0	415~
8	416	3.5	415
10	414	4.0	415
12	414	—	—

spectroscopy and reveals the presence of at least two separate components in solution. For concentrations of NaTCDC below 2.5 mM and NaTC below 5 mM, the minimum on the second derivative of the higher-energy component (i.e. the entity absorbing at lower wavelength) corresponds closely to the zero-order $\lambda_{\max}$ of BMG at 418 nm. Note that for concentrations of NaTCDC of 0.5–2.5 mM, and for concentrations of NaTC below 6 mM, there is a steady decrease in the relative absorbance at 418 nm of the second derivative of NaTCDC. However, at 3.0 mM-NaTCDC, there is a marked change in the absorbance with a minimum at 400 nm suddenly noticeable. This minimum is still present at 3.5 mM- and 4.0 mM-NaTCDC but is less prominent. Although this minimum at 400 nm is also seen for NaTC at concentrations above 6 mM, it develops gradually with increasing NaTC concentration. The paradoxical increase in the absorbance minimum at 400 nm for NaTCDC above 3.0 mM (implying a lower energy state), may be a result of the removal of BMG from a high-energy bile salt hydrophobic position as NaTCDC micelles continue to increase in size, or due to changes in the electronic configuration of the BMG molecule within the micelle without BMG changing its position.

Further spectral characteristics are revealed by fourth-derivative spectroscopy of NaTCDC (Fig. 3). The spectrum now has five minima and four maxima. Note that the sudden change in absorbances at ~ 375 nm and ~ 395 nm is clearly apparent as the CMC is approached. This pattern was not present for NaTC (spectrum not shown). Very few changes in the fourth-derivative spectra are seen for NaTCDC in the 470–495 nm area where interaction of monomeric BMG with bile salt monomers occurs.

DISCUSSION

We have shown that BMG can be used as a natural reporter molecule for the determination of the CMC of NaTCDC and also that NaTC does not have a true CMC but continuously self-aggregates over a range of reported CMC concentrations. These differences in the bile salt BMG interaction are manifested by sudden changes in the zero-order spectra of BMG in NaTCDC

in the area of the shoulder and in $\lambda_{\max}$, whereas the spectra of NaTC has a continuous change in both $\lambda_{\max}$ and the shoulder from 1–12 mM. The continuous decrease in the shoulder for NaTC relative to $\lambda_{\max}$ indicates that with continuous association of bile salts, electron transitions of BMG gradually develop a higher energy as the association with bile salt changes in a stepwise fashion with increasing bile salt concentration. On the other hand, the sudden decrease in relative absorbance at the shoulder for BMG in NaTCDC is consistent with a sudden change in association properties of NaTCDC that would be expected at the CMC. Because of sudden changes in association properties of BMG in NaTCDC at the CMC, electron transitions change suddenly to higher energy levels and a decrease in the shoulder relative to the Soret band is observed. Using derivative spectroscopy we have been able to show that the changes in the spectra can be sub-divided into several components and that micellization of NaTCDC is associated with sudden development of a minimum in the second derivative at 400 nm and sudden changes in the fourth derivative at a minima of ~ 375 and a maxima of ~ 395 nm. On the other hand, changes in the second derivative of NaTC appear gradually over a range of concentrations; the fourth-derivative spectra of BMG in NaTC did not show any distinctive change in absorbance with increasing NaTC concentration.

The second-derivative component at 400 nm most likely represents a more highly associated BMG state that is formed at the CMC of NaTCDC with the pyrrolic moiety of BMG associated with a hydrophobic position of the bile salt micelle while the polar glucuronic acid, and perhaps the lactam nitrogens and oxygens, are associated with the hydroxy groups of the bile salt. With increasing NaTC concentration, the n -number of this bile salt gradually increases and BMG gradually migrates from a more hydrophilic portion of the small micelle (low bile salt concentration) to a hydrophobic portion of the large micelle (high bile salt concentration). The second-derivative shoulder at 400 nm is probably a result of hydrogen bonding of BMG between the hydroxyl groups at the micellar bile salt perimeter and with BMG itself. Whereas binding of BMG to bile salt monomers may be associated with development of the high-energy 420 nm second-derivative negative absorbance seen in both NaTCDC and NaTC (in addition to the lower energy transitions seen in the fourth derivative at 470 and 495 nm), binding to the bile salt micelles may result in an even higher energy transition since such binding may involve hydrogen bonding of BMG across several bile salt molecule polar hydroxyl groups through lactam nitrogens and oxygens, through carboxylate ions of glucuronic and propionic acid, and through hydroxyl groups of the hexose sugar. These multiple hydrogen bonds, especially those involving the chromophore (Bell & Hall, 1981), would stabilize the ground-state of BMG so that photon transitions require higher energy. Consistent with this interpretation is the fact that 5 M-urea abolishes the 400 nm second-derivative negative absorbance (Spivak & Morrison, unpublished work) but not the 420 nm second-derivative negative absorbance. Presumably, 5 M-urea abolishes the 400 nm negative second-derivative absorbance because it interferes with complex intermolecular hydrogen bonding between bile salt micelles and BMG by substituting the smaller polar urea molecule. The 420 nm absorbance remains because it

Fig. 2. Second derivative spectra of (a) 2–12 mM-NaTC; (b) 0.5–4.0 mM-NaTDCD

The same BMG-buffer solution was used as in Fig. 1. For NaTC, note the continuous increase in the relative negative absorbance at 420 nm for concentrations at 2–8 mM. Also note the development of a 400 nm negative shoulder for NaTC beginning at 8 mM-NaTC and continuously increasing through 12 mM-NaTC, indicating continuous self-aggregation. On the other hand, note the sudden development of the 400 nm negative shoulder between 2.5 and 3.0 mM-NaTDCD, indicative of critical micellization. Spectra were derivatized from autoexpanded zero-order spectra.

represents the more simple, lower-energy hydrogen bonding between BMG and bile salt monomer or between BMG and urea. Further corroboration of the fact that bilirubin may act as a hydrogen-bonding cross-linking agent for micelles has come from our recent finding that bilirubin significantly increases the viscosity of micellar bile salt solutions but has only minor effects on non-micellar concentrations of bile salts (Rodrigues & Spivak, 1987).

Although dramatic changes are seen in the fourth-derivative spectrum in the 375–395 nm range with increasing NaTDCD concentration, very few changes in the fourth-derivative spectra are seen for NaTDCD in the 470–495 nm area where interaction of monomeric BMG with bile salt monomers occurs. This is undoubtedly a result of the fact that the concentration of BMG

associated with the monomeric form of the bile salt remains relatively constant with increasing concentration of bile salt. Most changes in the spectrum occur because of changes in the higher-energy portion of the spectrum between 380 and 430 nm. These changes may occur due to a shift of the bulk of BMG from aqueous self-association in a dimeric or multimeric configuration below the CMC to an intermicellar heterogeneous association (both with itself and with bile salt) above the CMC. Since micellar BMG would be less exposed to the aqueous environment through hydrocarbon shielding, such heterogeneous association would explain why non-enzymic hydrolysis of BMG is markedly retarded by micellar concentrations of bile salt (Spivak *et al.*, 1987). Incorporation of BMG into bile salt micelles may occur in a similar manner to the way lecithin is incorporated

Fig. 3. Fourth-derivative spectra of NaTCDC

The same BMG-buffer solution was used as in Fig. 1. A sudden increase in the negative absorbance at ~ 375 nm and an increase in the positive absorbance at ~ 395 nm occurs in the range of the CMC between 2.5 and 3.0 mM. Note that very little change occurs between 470 and 490 nm, implying that micellization is associated with significant changes in the higher-energy photon transition portions of the BMG spectrum and molecule.

into a micelle in the mixed-disc model of Mazer *et al.* (1980). The hypothesis of a similar configuration of lecithin and BMG within the micelle is born out by the fact that lecithin inhibits the non-enzymic hydrolysis of BMG (Spivak *et al.*, 1987) perhaps by competing for the same hydrophobic space as BMG within the micelle (Spivak *et al.*, 1987).

With falling-needle viscometry, a non-invasive technique for measuring the CMC, we have recently corroborated that the CMC of NaTCDC is between 2.5 and 3.0 mM in a buffer solution identical to the one present in these experiments, but in the absence of BMG (Rodrigues & Spivak, 1987). Viscometry also indicated that NaTC does not have a true CMC. Thus, BMG in low concentrations does not appear to perturb micelle formation.

Our reported values for the CMC of NaTCDC correlate well with values determined by solubilization (Hofmann, 1963) and surface tension techniques (Roda *et al.*, 1983). Values for the CMC of NaTC have varied considerably (Carey, 1983). This may be due not only to technique, but also the absence of a critical micellization phenomenon. Thus, some observers may have measured the CMC at the point of monomer to dimer transition, whereas others may have measured the CMC at higher aggregation levels.

The use of derivative spectroscopy to resolve and quantify mixtures of compounds in solutions with overlapping zero-order spectra has been well described (Botten *et al.*, 1977). This methodology requires that pure components of each sample have derivative spectra that do not significantly overlap in at least one of their minima or maxima. Until recently, such methodology had not been applied to the quantification of bilirubin. However, Merrick & Pardue (1986) have recently utilized

derivative spectroscopy for the simultaneous quantification of bilirubin and haemoglobin in solution. Although we have not established pure standards of bilirubin bound to bile salt monomer or bilirubin bound to bile salt micelle, the sudden changes that occur at micellization lend themselves to an estimate of the amount of bile pigment bound to a given bile salt species (e.g. monomer versus dimer versus bile salt micelle). Such an estimate is beyond the scope of this paper since it would require a multitude of scans in a variety of solvents similar to the analysis of Carey & Koretsky (1979) in an attempt to confirm the physical nature of each absorbing species.

Carey & Koretsky (1979) also found that NaTC has a significant effect on the spectrum of bilirubin; however, they used UCB at pH 10.0 as a model compound for bilirubin conjugates. At this pH, the carboxylic acids of UCB are fully ionized; the compound, therefore, has significant aqueous solubility. By using both zero-order and difference spectroscopy, they found two positive absorption peaks for UCB in taurocholate; one at 410–413 nm and one at 470–480 nm. Unlike our results, they found that the shoulder at the higher wavelength increases with increasing NaTC concentration. Although we find similar regions of absorption with BMG, the effect of NaTC on BMG is just the opposite, i.e. the shoulder decreases in intensity with increasing concentration of bile salt. Since this pattern holds true for NaTCDC also, it appears to indicate a fundamental difference in the properties of BMG at pH 7.8 (which is within the physiological range for bile pH) and UCB at pH 10.0 (which is not within the physiological range for bile). Solution pH and the presence of glucuronic acid obviously have a profound effect on the associative properties of bilirubin.

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