

Hemin Monomers in Micellar Sodium Lauryl Sulfate. A Spectral and Equilibrium Study with Cyanide[†]

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ABSTRACT: Spectral changes in the Soret region and equilibrium properties of hemin isolated from cow's blood have been investigated in the presence of aqueous solutions of sodium lauryl sulfate (SLS) and tetramethylammonium bromide (TMAB) at 25°. Micromolar concentrations of hemin in basic solution, normally dimeric, are observed to undergo large spectral changes in the presence of SLS and 0.1 M TMAB. A limiting spectrum occurs at about 1.5% SLS, notably above the critical micellar concentration. The spectral shift of peak position of hemin before and after the addition of the detergent from 386 to 400 m μ , respectively, as well as peak intensities and comparisons to the literature have been interpreted as resulting from dissociation of the hemin dimers into monomers with the subsequent intercalation of the monomers into the micelles of SLS. Spectra of the intercalated monomers are pH insensitive between 7.0 and 12.5.

Metalloporphyrins are known to be the active site of a variety of catalases, peroxidases, hemoglobins and myoglobins (Falk, 1963; Phillips, 1963). The biological environment imposed on metalloporphyrins is quite complex (Muirhead *et al.*, 1967) and no doubt plays an important if not a major role in determining the chemical reactivity of the metal center and its stability for a given oxidation state. A fruitful approach to understanding the hydrophobic and protic character of the protein as they influence heme reactivity has been to remove the prosthetic group and study its reactivity in aqueous and nonaqueous solvents of varying polarity and protic character (Caughey *et al.*, 1966). Such studies have often been complicated by the formation of heme dimers (Brown *et al.*, 1970) in equilibrium with monomers and axial coordination to the iron by solvents such as pyridine, resulting in a different specie than one would normally find in the protein environment (Corwin and Erdman, 1946).

Previous workers (Caughey *et al.*, 1966; Phillips, 1963; Porra and Jones, 1963) have shown that porphyrins are highly solubilized in the presence of detergents such as sodium lauryl sulfate (SLS),¹ cetyltrimethylammonium bromide, and Emasol 4130. This has been interpreted as arising from strong hydrophobic interactions between the detergent and the porphyrin overcoming porphyrin-porphyrin forces and ultimately solubilizing the porphyrin when the concentrations of the detergents reach their critical micellar concentration (cmc). Ferroprotoporphyrins (Phillips, 1963) have

The intercalated hemin solutions are observed to undergo a color change from green to red-orange in the presence of added potassium cyanide, indicating the formation of a dicyanohemin complex. The equilibrium constant associated with this system is evaluated to be 14.4 M⁻¹ at 25°. No monocyano-hemin complex is observed and the existence of an isosbestic point further suggests the presence of only two hemin species, namely a monoaquomonohydroxyhemin complex (MOH) and a dicyanohemin complex (M(CN)₂). The effects of an added counterion, TMAB, on the micellar hemin-cyanide equilibrium was investigated. The presence of the counterion was found to greatly enhance the stability of the dicyanohemin complex over the monoaquomonohydroxyhemin complex. A model, based upon electrostatics and the hydrophobic character of the micellar interior, is suggested to account for the above observations.

been added to an aqueous detergent solution and the accompanying spectral changes observed above 500 m μ have been ascribed to formation of monodisperse heme. Polet and Steinhardt (1969) have used flow techniques to study the reverse process, *i.e.*, the dimerization of hemin subsequent to acid denaturation of ferrihemoglobin leading to the release of hemin from the subunits.

The above suggested an initial study to examine the spectral and equilibrium characteristics of hemin solutions in the presence of micellar SLS and potassium cyanide. Such a system largely reduces several possible variables affecting hemin chemistry such as nearby polar groups and axial coordination of amino acid residues. At the same time hemin is still provided with a large macromolecular structure whose interactions are primarily hydrophobic, thus providing a simple model for hemoproteins.

Shick (1964) has found that SLS strongly interacts with TMAB (the counterion) in a well-defined fashion. Thus a further experimental parameter was to study the intercalated hemin with cyanide as a function of added counterion concentration to obtain information on how the outer electrostatic interactions at the micellar-water interface affect the iron center reactivity.

Materials and Methods

SLS was obtained from the Sigma Chemical Corp. and TMAB was obtained from Eastman Chemicals. All reagents were used without further purification and all solutions were made up by using distilled deionized water. Solid hemin was isolated by the method of Labbe and Nishida (1957) with the only minor change being that strontium bromide was used in place of the chloride salt. Hemin prepared from several different sources (dried cow's blood was obtained from Eastman Organic Chemicals and Matheson Coleman and Bell) gave

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¹ Abbreviations used are: SLS, sodium lauryl sulfate; TMAB, tetramethylammonium bromide; MOH·SLS and M(CN)₂·SLS refer to the monoaquomonohydroxyhemin and dicyanohemin complexes intercalated in the micellar SLS.

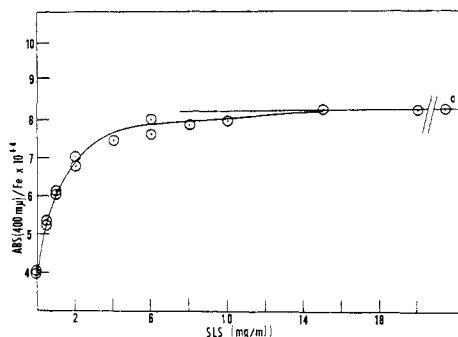


FIGURE 1: Graph of absorbance changes at 400 $m\mu$, divided by total iron, accompanying monomer formation. Hemin solutions range from 8.0 to 12.0 μM , $T = 25^\circ$, 0.1 M TMAB, and pH 10.00. Absorbance point at 2.0 mg/ml was pH independent from 7.0 to 12.5. Note that point "a" is at 3.0 mg/ml of SLS.

the same spectral and equilibrium characteristics and were used interchangeably. Two recrystallizations of the hemin were usually sufficient to remove any hemoprotein impurities as evidenced by no further change in the ultraviolet region of the spectrum. Stock solutions of potassium cyanide (about 0.5 M) were standardized by the method of Pierce and Haemisch (1958). The concentrated solutions were found to be stable, when well stoppered, over a period of about 2 weeks. The pH of all solutions was adjusted just prior to spectral analysis and determined with a Radiometer pH 26 meter with a GK2301B glass combination electrode. It was observed that the micellar solutions of SLS caused the electrode to slowly drift approximately 0.1 pH unit to the acidic side of where it had been initially standardized with prepared Beckman buffers. The pH drift, which occurred even in well-buffered solutions (*i.e.*, buffered with cyanide), was presumably due to a coating of the glass membrane with the micellar material. To overcome this difficulty the electrode was normally incubated with a 2% SLS solution about 10 min before standardization. Once standardized in this fashion the electrode no longer drifted and could be used to monitor the pH of hemin-SLS-cyanide solutions to ± 0.03 pH unit. The pH adjustments were made by the addition of either concentrated nitric acid or potassium hydroxide. High localized concentrations of base caused a temporary salting out of a white material (the SLS) but this eventually dissolved back into solution and all solutions used for spectra remained completely clear to the naked eye. Temperature control during pH and spectral measurements was $\pm 0.1^\circ$. Solutions were prepared by dissolving a small amount of solid hemin in a few milliliters of slightly basic solution and filtering off any particles of hemin not immediately solubilized. An aliquot of this solution was then added to a solution containing SLS and TMAB and brought to volume to give a 2% SLS and 0.1 M TMAB solution and hemin in the micromolar region. The total iron concentration of hemin solutions (essentially 100% dimerized at pH 10.5 in the absence of SLS) was measured by the use of the Perkin-Elmer 403 atomic absorption spectrophotometer. Standard iron solutions for comparison were made by using potassium ferricyanide as a primary standard. Such solutions contained iron in the 1–5 ppm range. The total iron concentration in the hemin solutions was then taken to represent the total concentration of hemin monomers. Combining this information with absorbance data (for a quartz cell 1.0-cm long) in the Soret region, permitted the valuation of extinction coefficients which were

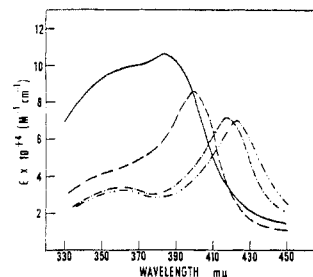
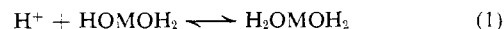


FIGURE 2: Extinction coefficient spectra in the Soret region for the various hemin species. (—) dimer at pH 10.5; (---) limiting spectra for monomer H_2OMOH in 2% SLS, 0.1 M TMAB, pH 10.00; (-·-·-) limiting spectra for dicyanohemin complex, 2% SLS, 0.1 M TMAB, pH 9.50, $CN^-_T \approx 0.1$ M; (····) limiting spectrum for dicyanohemin complex without SLS or TMAB, pH 9.50, $CN^-_T \approx 0.1$ M.

thereafter used to determine hemin concentrations of later experimental solutions. All spectra were obtained with a Cary 17 recording spectrophotometer. Free cyanide concentrations were obtained from knowing the total KCN added, the pH, and the K_a of HCN at 25° .

Results and Treatment of Data

Spectra. At 25° a series of spectra were obtained with hemin varying from 12 to 14.1 μM in aqueous solutions (0.1 M TMAB) as a function of varying amounts of SLS. Spectra were observed over the range from 350 to 450 $m\mu$ and a plot of absorbance (400 $m\mu$)/ Fe_{total} against SLS (mg/ml) was made (Figure 1). The leveling off point on the absorbance axis was used as a direct measure of the extinction coefficient of $0.82 \times 10^5 M^{-1} cm^{-1}$. The limiting spectrum of this new species is shown in Figure 2 and is the intercalated mono-aquomonohydroxyhemin complex ($MOH \cdot SLS$). At 2% SLS and 0.1 M TMAB the spectrum of the monomer is pH insensitive between about 7.0 and 12.5. It was observed, however, that below a pH of about 6.5 the greenish hemin monomer solution begins to undergo a color change to reddish pink, resulting in a peak shift from 400 to 390 $m\mu$, $\epsilon_{max} 0.85 \times 10^5 M^{-1} cm^{-1}$ (pH 4.0). This equilibrium is presumably due to the following (Phillips, 1960) (excluding charge balance)



No quantitative measurements of the K_a were made. All other spectral work was done at a pH above 9.0. Hence equilibrium 1 made no contribution to this study.

Dicyanohemin Complex [$M(CN)_2 \cdot SLS$] Spectra. The spectrum of the dicyanohemin complex (see Figure 2) was obtained as a limiting spectrum by maintaining a solution at a constant hemin monomer concentration of 9.10 μM 2% SLS, constant pH 9.50 and adding aliquots of KCN (0.5 M) until no further spectral changes occurred. The dicyanohemin complex is reddish orange in color with an absorption peak found at 422 $m\mu$. The dicyanohemin complex may be formed from hemin dimer solutions in the absence of SLS and TMAB (Phillips, 1960) and the spectrum of this material in the Soret region is shown in Figure 2 for comparison. The peak maximum for this is about 418 $m\mu$ a notable shift of 4 $m\mu$ for the same complex in 2% SLS. A spectrum of the hemin dimer at pH 10 in the absence of SLS and TMAB is also given for comparison purposes.

Equilibrium Studies. Figure 1 indicates that at about 1.5%

TABLE I: Equilibrium Parameters.

Fe _T (μM)	CN ⁻ _T (mM)	pOH	A ₄₂₂	CN ⁻ _{free} (mM)	K _{eq} (M ⁻¹)	Spectra
7.54	2.50	4.10	0.360	2.08	19.7	
7.54	2.50	3.80	0.302	2.27	16	
7.54	2.50	3.59	0.268	2.35	15	
11.3	6.00	4.25	0.732	4.66	24.4	
11.3	6.00	4.02	0.685	5.13	15.4	
11.3	6.00	3.75	0.603	5.50	10.7	
11.3	6.00	3.57	0.555	5.66	10.2	
13.0	8.00	9.96				1
13.0	8.00	3.90				2
13.0	8.00	3.76	0.810	7.39	18.1	3
13.0	8.00	3.50	0.720	7.61	12.2	4
13.0	8.00	3.17	0.620	7.81	11.8	5
13.0	8.00	2.88	0.510	7.90	10.1	6
13.0	8.00	2.75	0.460	7.93	8.7	7
K _{avg} = 14.4 M ⁻¹ ± 4.6						

SLS, hemin solutions exhibit a constant spectrum revealing the complete formation of monomer units. For all equilibrium studies a constant amount of SLS (2%) and 0.1 M TMAB was used. The data for a series of different solutions are presented in Table I and partially represented in Figure 3. At constant total cyanide and hemin monomer, varying the pH produces a series of spectra that exhibit an isosbestic point at 412 mμ. This suggests that only two species are present and is consistent with the following overall equilibrium (charges omitted)



$$K_{eq} = \frac{[\text{M}(\text{CN})_2 \cdot \text{SLS}][\text{OH}^-]}{[\text{H}_2\text{OMOH} \cdot \text{SLS}][\text{CN}^-]^2} \quad (3)$$

The concentrations of M(CN)₂·SLS and MOH·SLS were obtained from the following set of equations for a 1.0-cm cell path

$$A_{422} = \epsilon_{422}^{\text{MOH} \cdot \text{SLS}} [\text{MOH} \cdot \text{SLS}] + \epsilon_{422}^{\text{M}(\text{CN})_2 \cdot \text{SLS}} [\text{M}(\text{CN})_2 \cdot \text{SLS}] \quad (4a)$$

$$F_{\text{total}} = [\text{H}_2\text{OMOH} \cdot \text{SLS}] + [\text{M}(\text{CN})_2 \cdot \text{SLS}] \quad (4b)$$

·SLS refers to a specie solvated or incorporated into the SLS micelles. The K_{eq} is found to be 14.4 M⁻¹ at 25°.

Counterion Effect on Equilibrium. The addition of a counterion, TMAB, is observed to effect the equilibrium of the micellar hemin with cyanide. Figure 4 is a plot of the absorbance at both 400 mμ (the absorption peak of the MOH·SLS and 422 mμ [the absorption peak of M(CN)₂·SLS] as a function of added TMAB. At pH 10.10, constant total cyanide, constant hemin, and 2% SLS, it is observed that M(CN)₂·SLS complex is favored by the addition of the cationic counterion. A calculation shows that with no added TMAB the concentrations of MOH·SLS and M(CN)₂·SLS are 4.0 and 2.35 μM, respectively. In the presence of approximately 0.11

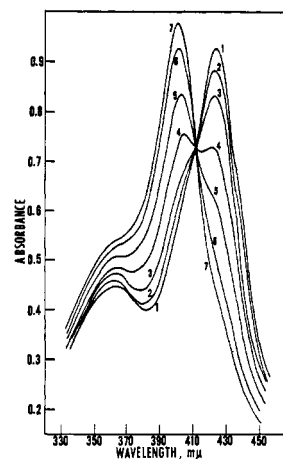


FIGURE 3: Equilibrium spectra taken in part from Table I. CN⁻_{total} = 8.0 × 10⁻³ M, 25°, 0.1 M TMAB; Fe_{total} = 13.0 μM. This shows how the spectra of the equilibrium (eq 2 and 3) are affected by pH. Curve 1 roughly corresponds to ~100% of M(CN)₂·SLS. As the pH increases a shift to the left in eq 2 results, ultimately leading to the formation of curve 7. This roughly corresponds to MOH·SLS. Intermediary curves reflect the position of equilibrium with changing pH.

M TMAB the concentrations are 1.3 and 5.0 μM, respectively, roughly doubling of the dicyanohemin complex.

Discussion

Spectral studies of the hemin monomer-dimer equilibrium in aqueous solution (Brown *et al.*, 1970) have found that the monomer protoferriheme (from limiting spectra) has an extinction coefficient of approximately 0.55 × 10⁵ M⁻¹ cm⁻¹ at 394 mμ. These spectral characteristics are distinctly different from that obtained with solutions containing the dimer exclusively (see Figure 2), but parallel quite closely the extinction coefficient and peak position of hemin in the presence of SLS micelles. Presumably the hydrophobic interaction between the micelle and hemin overcomes the hemin-hemin interactions leading to monomer formation. Such interactions have been previously noted for an iron(II) porphyrin (Phillips, 1963). The effects in the above case were observed by noting a sharpening of the spectral peaks above 500 mμ by the addition of detergent.

The Soret band for micrococcus catalase containing an iron(III) porphyrin (Brill and Sandberg, 1968) appears at 403 mμ with an extinction coefficient of 1.0 × 10⁵ M⁻¹ cm⁻¹. Thus, on the basis of spectral comparisons alone, the hemin

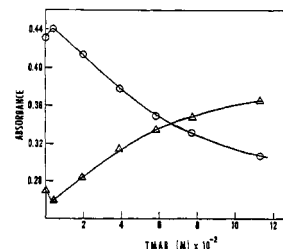


FIGURE 4: Shifting of hemin-cyanide equilibrium by the addition of TMAB. SLS at 2%, pH 10.10, T = 25°, CN⁻_{total} = 4.00 × 10⁻³ M, Fe_{total} = 6.35 μM. With no TMAB the dicyanohemin complex is 2.35 μM. At 0.12 M TMAB the concentration is 4.00 μM. Δ is absorbance at 422 mμ and ○ is absorbance at 400 mμ.

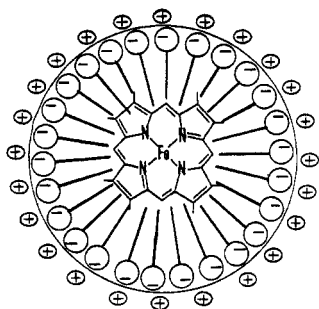


FIGURE 5: Possible arrangement of hemin monomer in spherically shaped micellar SLS. Negatively charged heads are the sulfate group of the SLS. Positively charged species are counterions of TMAB. Note that water and hydroxide species are not shown but would normally be placed above and below the plane of the metalloporphyrin. These are replaced by two cyanides in the formation of $M(CN)_2 \cdot SLS$.

that exists in SLS solutions is believed to be a monomer form. It is interesting to note that the spectral changes for hemin in SLS begin to occur when the concentration of SLS reaches the cmc. This region for SLS, in the presence of TMAB, can be readily calculated from the empirical equation derived by Shick (1964). For 0.1 M TMAB the cmc is 6.0×10^{-5} M or about 0.2% SLS. Denaturation of ferrihemoglobin by acid leads to the formation of free hemin (Polet and Steinhardt, 1969) with subsequent dimerization. The spectral changes in the above study are roughly the reverse of what changes occur as SLS is added to hemin dimers. This is an expected result since the two processes are essentially the reverse of one another.

Addition of cyanide to hemin-SLS solutions results in a change in color from green to red. This is reflected in a peak shift from 400 to 422 m μ . The extinction coefficient of the product $M(CN)_2 \cdot SLS$ is 0.69×10^5 M $^{-1}$ cm $^{-1}$. It is interesting to note that the addition of cyanide to micrococcus catalase (Brill and Sandberg, 1968) also gives rise to a red shift to about 425 m μ and extinction coefficient of 0.85×10^5 M $^{-1}$ cm $^{-1}$. The internal consistency of the derived equilibrium constant over a wide range of concentrations and pH values and existence of the isosbestic point supports the presence of only two species—a monomer and a dicyanohemin complex. No monocyanohemin complex is believed to exist based upon the data, and this is consistent with the equilibria investigated without detergents (Falk, 1963; Shack and Clark, 1947; Phillips, 1960).

A dicyanohemin complex may be produced in the absence of SLS, from a basic solution of hemin in which dimers predominate. Spectra of such solutions are indicated in Figure 2. It is to be noted that the peak position of the dicyanohemin complex in the absence of SLS is shifted approximately 4 m μ down toward the ultraviolet (uv) from that complex formed in the presence of SLS. This suggests that the respective environments of the two materials are different, presumably with the dicyanohemin complex intercalated in the micelle. The comparative sizes of the micelles (molecular weights lying between 7000 and 36,000 for SLS) (Oko and Venable, 1971; Schott, 1966) and the roughly spherical symmetry of SLS micelles (Reiss-Husson and Luzzati, 1964) would be expected to be large enough to incorporate the relatively smaller metalloporphyrins (Hoard, 1968). It would be difficult to imagine surface adsorption of the dicyanohemin complex on the micelle since both axial sites are occupied by

a negatively charged cyanide and would be expected to be repelled from the anionic head of the detergent.

Strong electrostatic interactions from counterions as well as hydrophobic ones were observed by previous workers (Stigter, 1964; Shick, 1964) in micelle formation. This is borne out in the present study (Figure 4) by the observation that the presence of a counterion, TMAB, will greatly increase the stability of the dicyanohemin complex over the MOH-SLS. This is readily understandable from electrostatic considerations if the counterion partially neutralizes the anionic sulfate heads, thereby reducing the total overall negative charge on the micelle. A reduction in the negative surface charge would favor the approach and penetration of CN^- through the membrane-like sheath imposed about the hemin at the micelle-water interface. A cross-sectional diagram of such a model is shown in Figure 5. This idea of electrostatic effects on the micelle surface is supported (J. Simplicio and K. S. Schwenzer, 1971, unpublished data) by the equilibrium constant measured for hemin-cyanide in the presence of micellar cetyltrimethylammonium bromide, containing a cationic polar head in which the equilibrium constant at 25° for eq 2 is approximately 5×10^4 M $^{-1}$.

The hemin-SLS system as found here may serve as a simple model for hemoproteins with the predominant environment being hydrophobic in nature. Furthermore, the effects of the counterion can be used to change, in a continuous fashion, the polarity and surface charge of the immediate environment of the surrounding macromolecular micelle. This serves as an added dimension of metalloporphyrin reactivity and sensitivity to an increasing polar environment. As a natural extension a study of the oxygen-sensitive Fe(II) in SLS is currently in progress.

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