Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 111, No. 4, August 1999, pp. 547–554

Surfactant-induced stabilization of four-coordinated hemes in reconstituted hemoglobins

B VENKATESH[1], S RAMASAMY[1], V SWARNALATHA[1], J SANTHANALAKSHMI[2], R ASOKAN[1], J M RIFKIND[3] and P T MANOHARAN[1]*

[1]Department of Chemistry and Regional Sophisticated Instrumentation Centre, Indian Institute of Technology, Chennai 600 036, India

[2]Department of Physical Chemistry, Madras University, Guindy Campus, Chennai 600 025, India

[3]Molecular Dynamics Section, Laboratory of Cellular and Molecular Biology, Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

e-mail: ptm@magnet.iitm.ernet.in

MS received 12 January 1999

Abstract. A transition from 5-coordination to 4-coordination metal centres of porphyrins in carbonmonoxy Fe(II), Cu(II) and Ni(II) hemoglobins is observed as a consequence of ionic surfactant interactions with metalloproteins through UV-Vis studies. SDS (anionic) and CTAB (cationic) surfactants are used. Values of heme partition coefficient (K) between globin and surfactant show that, in both CTAB and SDS, partitioning increases proportionately with surfactant concentration for a fixed concentration of hemoglobin. The interactions seem to be mainly hydrophobic in nature with metalloproteins, though coordination and coulombic interactions may also play a minor role in CTAB and SDS respectively. The present study provides new insight into reconstituted hemoglobin–surfactant interaction.

Keywords. Surface-induced stabilization; 4-coordinated hemes; reconstituted hemoglobins; hemoglobin–surfactant interaction.

1. Introduction

Biological functions of metalloproteins with metal porphyrins as prosthetic groups are sensitive towards changes in metal coordination, and coulombic, hydrophobic and hydrogen bonding interactions [1–3]. Some of these changes are achieved by the presence of ionic detergent micelles and surface active peptides [4–7]. It is known that interaction of surfactant with protein leads to the formation of a protein–amphiphile complex resulting in disruption of the tertiary structure of the proteins [8–11]. The resultant changes noticed during surfactant interaction with protein are (i) protein denaturation, (ii) sorption and (iii) changes in the α-helical content [8,11–15]. However, reports on the coordination changes at the active site during the denaturation of hemoglobin in the presence of ionic surfactants

*For correspondence

Abbreviations: Hb, hemoglobin; FeHbCO, carbonmonoxy hemoglobin; SDS, sodium dodecyl sulphate; CTAB, hexadecyltrimethyl ammonium bromide; CuPP, copper protoporphyrin IX; NiPP, nickel protoporphyrin IX; NiHb, nickel reconstituted hemoglobin; CuHb, copper reconstituted hemoglobin.

are seldom found in literature. Apart from this surfactant protein interaction, surfactant metalloporphyrin interactions are also known from the literature where the surfactants are capable of stabilizing the metalloporphyrins or heme through hydrophobic interactions. In this respect ionic micelles forming surfactants, such as sodium dodecyl sulphate (SDS, anionic) and hexadecyltrimethyl ammonium bromide (CTAB, cationic), are reported [16,17].

It is known that HbCO is in the R-state while CuHb and NiHb are found in the T-state, hence the structural information got from these modified proteins can be directly correlated to deoxyHb (T-state) [18,19]. In our present work, Ni(II) and Cu(II) reconstituted hemoglobins, along with the carbonmonoxide reacted normal human hemoglobin [Hb], have been subjected to interactions with SDS and CTAB, in order to find the extent of surfactant binding and the resultant changes at the active site in the two extreme states of Hb, namely the T and R states. The results form an interesting case study and are expected to yield an understanding of such interactions at the molecular level towards (i) cell surface–protein interactions, (ii) heme exposures, and (iii) denaturation of protein [12,20–22]. For the first time, we have observed the stabilization of 4-coordinated heme in metal ion reconstituted hemoglobins during their interaction with surfactants. Also partition coefficient (K) values for heme, between the globin and surfactant matrices, are estimated by using absorption spectral data.

2. Experimental

2.1 *Reagents*

Surfactants SDS and CTAB, protoporphyrin IX disodium salt, and DEAE-sephadex were obtained from the Sigma Chemical Co., USA. Sephadex G25 was obtained from Pharmacia Fine Chemicals, Sweden. and DE-23 and CM-23 cellulose were obtained from Whatman International, England. Tris buffer was obtained from American Bio-organics, USA. All remaining chemicals used were locally available analytical grade reagents.

2.2 *Equipment*

All the biological preparations were carried out in a cold room at 4°C. The protein was concentrated using a stirred cell from Amincon Division, USA. The changes in the metal coordination of heme were investigated by UV-Vis spectroscopic techniques. The absorbance measurements were made on a Varian-Cary-5-E UV-VIS-NIR spectrophotometer. Protein concentration was measured by metal estimation from ICP analysis.

2.3 *Experimental procedures*

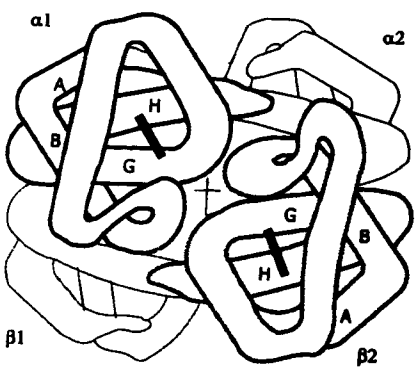
Human hemoglobin was first separated from RBC, and removal of minor components and purification were done as per the literature [23]. Reconstituted Ni(II) and Cu(II) hemoglobins were prepared by established methods [24]. In this study, all hemoglobins, CTAB and SDS solutions used were prepared in 100 mM sodium phosphate buffer at the biological pH of 7·2. To investigate the possible interactions between hemoglobin and surfactants using UV-Vis spectroscopic techniques, hemoglobin concentration was kept constant at 1×10^{-6} M while surfactant concentration was varied from 1×10^{-5} to 1×10^{-2} M adopting the titration method. Distilled and deionized water was used in all experiments. The partition coefficients (K) of heme between the globin and surfactant

matrices are calculated from $[OD]_k - [OD]_m = \Delta OD$, where $[OD]_k$ and $[OD]_m$ refer to the OD values in the absence and presence of surfactants and ΔOD is proportional to heme partitioning from the globin matrix. The extent of 5-coordinated heme lying unaffected is proportional to $\{[OD]_k - \Delta OD\}$. Thus the partition coefficient $K = \Delta OD/[OD]_k - \Delta OD_m$.

3. Results and discussion

It is well-known that NiHb and CuHb are in the T-state[18,19], and can be considered as good models for deoxyhemoglobins. These hemoglobins possess two different metal coordination sites such as 5-coordination and 4-coordination [6-coordination with weak axial interaction] at the active sites which are detectable in the Soret region of UV-visible spectrum. In the case of NiHb and CuHb, the 4-coordination site is identifiable by bands at 399 and 405 nm respectively and the 5-coordination site is represented by a peak at 418 nm in both cases[19]. In FeHbCO the absorption band occurs at 419 nm. The quaternary structure of hemoglobin and the structures of the surfactants are given in figure 1. Surfactant-induced modifications of metal coordinations can be monitored by observing the changes in the Soret band spectral region in the presence or absence of ionic surfactants.

Figure 2 shows the electronic spectra of NiHb, CuHb and FeHbCO, in the presence of varying amounts of SDS and CTAB at *p*H 7·2. In the case of CuHb with increasing SDS concentration, one of its split Soret bands, viz the 418 nm peak almost disappears at an SDS concentration of $2{\cdot}6 \times 10^{-4}$ M. The other peak at 405 nm is initially blue-shifted to 393 nm; with further increase in SDS concentration it gets red-shifted to 406 nm. The first effect, the reduction in the intensity of 418 nm peak, with the consequent formation of a



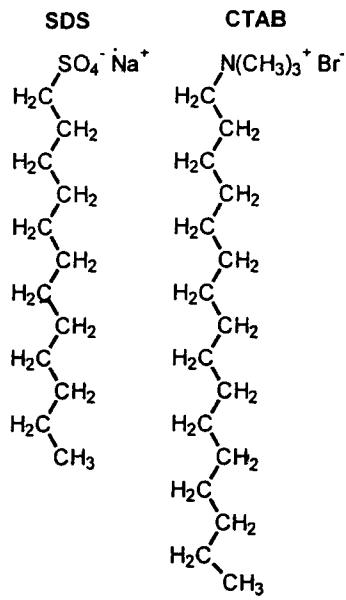


Figure 1. Schematic representation of the quarternary structure of hemoglobin and structure of the surfactants SDS and CTAB.

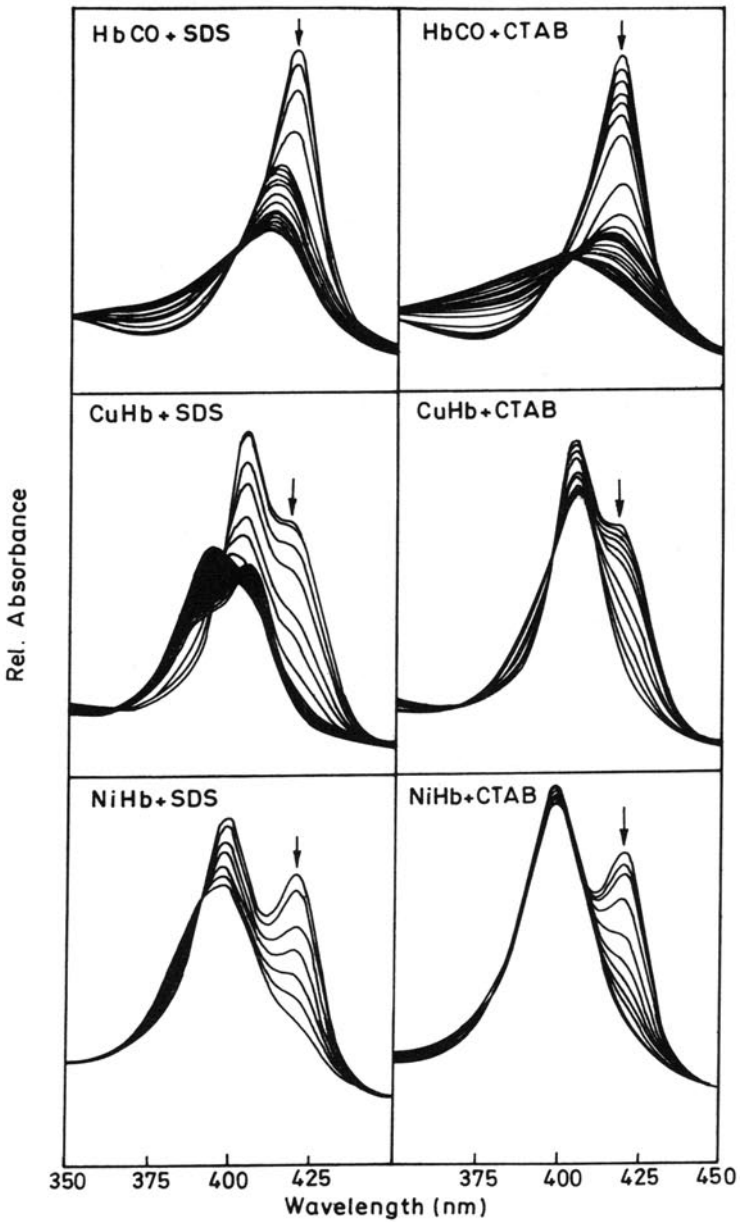


Figure 2. Electronic spectra of CuHb in 100 mM phosphate buffer, *p*H 7·2 (middle), NiHb in 100 mM phosphate buffer, *p*H 7·2 (bottom), FeHbCO in 100 mM phosphate buffer, *p*H 7·2 (top), with increasing addition of SDS and CTAB respectively, in the region 350–450 nm.

broad, blue-shifted band of 406 nm to 393 nm, can be attributed to the weakening of the axial bond in the 5-coordination site. The second effect, the slow formation of 406 nm peak with increased intensity due to increased SDS concentration, is attributed to the exposure of the 4-coordinated heme from the globin matrix. NiHb–SDS interaction is

similar to that of CuHb–SDS except that the peak position shifted to 398 nm is not substantially different from that of the 4-coordinated one viz 399 nm. The total disappearance of the 5-coordination site occurs in this case also at an SDS concentration of 2×10^{-4} M. Moreover, this 399 nm peak in NiHb initially broadens and blue-shifts to 398 nm which is subsequently red-shifted and sharpened at 401 nm with further increase in the SDS concentrations. These processes are under equilibrium control. The following scheme is proposed to explain the change under the influence of SDS:

$$\{(\text{VHb}) + (\text{IVHb})\} \xrightarrow{\text{SDS}} \{(\text{VIHb}) \rightleftharpoons (\text{IVHb})\}.$$

CuHb [λ_{max}] 418 nm	405 nm	393 nm	406 nm
NiHb [λ_{max}] 418 nm	399 nm	398 nm	401 nm

On the other hand, the CuHb–CTAB interaction interestingly shifts the 405 nm peak to 407 nm with simultaneous reduction in the intensity of the 5-coordination peak leading to its final disappearance at the CTAB concentration of $2{\cdot}4 \times 10^{-4}$ M. This again represents the exposure of the 4-coordinated heme. However, the intensity of the 5-coordinated peak in NiHb at 418 nm is proportionally reduced with the quantitative increasing amounts of CTAB finally disappearing at 3×10^{-4} M, with almost no reduction in the intensity of the 399 nm peak. With further increase in the concentration of CTAB, a minor shift of this 399 nm peak to 401 nm occurs. The subtle differences in the behaviour of NiHb and CuHb towards the surfactant is due to the differences in the T-conformation of these two as evidenced by –SH reactivity experiments reported earlier[25]. Furthermore, for a given Hb, CTAB brings out the sequence of reduction of 5-coordination and finally the exposure of the 4-coordinated heme more clearly than SDS does. In other words, the equilibrium process is slower with SDS than with CTAB. This phenomenon may be due to the higher hydrophobicity of CTAB compared to that of SDS which may be enough to shift the equilibrium such that the 4-coordinated heme from the globin matrix is exposed.

The nature of the SDS/CTAB interaction with FeHbCO [R-state] may be slightly different from that of Cu/Ni Hb [T-state] despite the evidence for metalloporphyrin exposure being detected in both cases. The reconstituted hemoglobins exhibit more extents of heme exposure than FeHbCO, which may be due to the more favorable interactions with the T-conformation. The Soret band at 419 nm in FeHbCO corresponding to strongly 5-coordinated [without considering CO bonding] iron undergoes almost a two-fold decrease in intensity with increased SDS concentration. This is further followed by a blue-shift to 410 nm which in turn indicates the tendency of the heme to move to 4-coordination at the SDS concentration of $\sim 4 \times 10^{-3}$ M. However, with CTAB the same 418 nm peak undergoes a greater blue-shift to 401 nm even at lower CTAB concentration $\sim 2 \times 10^{-3}$ M.

The possible reasons for the observed spectral changes in reconstituted hemoglobin on the addition of surfactant are as follows: (i) The 5-coordinated heme is converted to 4-coordinated heme but is still bound to the globin matrix, (ii) the 4-coordinated species in reconstituted hemoglobin arises from the dissociation of the tetramer into dimers or monomers, and (iii) total denaturation of the globin chain followed by heme ejection. The first process can be ruled out on the basis of the observation that in NiHb with increased addition of surfactant, we observe an increase in absorbance of 4-coordinated heme compared to that of 4-coordinated heme in NiHb (figure 3). Similar results are also obtained for CuHb. It may be noted that CuPP and NiPP exhibit bands at 403–408 nm

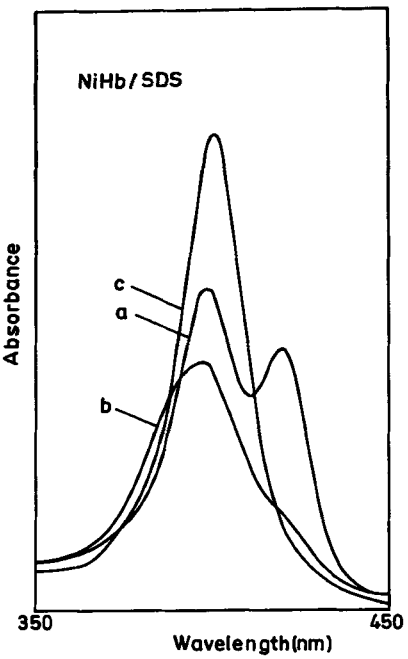


Figure 3. UV-visible spectra in 100 mm phosphate buffer, *p*H 7·2, for NiHb at different SDS concentration of $2{\cdot}5 \times 10^{-5}$ (a), 2×10^{-4} (b) and $3{\cdot}5 \times 10^{-3}$ M (c).

and 401 nm respectively in non-coordinating solvents, representing their 4-coordination character[26,27]. In the present results, the modifications in the metal coordination of metalloproteins in the presence of SDS and CTAB can be correlated to the CuPP and NiPP coordination changes in the respective spectral regions. The second possibility can also be ruled out based on previous CD experimental results[11,13], where it has been reported that the environmental change around the heme group alone involves destruction of the α-helical structure and change in the β-structure. This result even supports the third process. To further confirm this, deliberate additions of NaCl (aggregation favouring agent) to the surfactant–hemoglobin solutions are found to result in precipitation of surfactant components. The residue on redissolution in the buffer when subjected to UV-visible spectrophotometric studies showed the presence of metalloporphyrin in the surfactant media. Hence overall, the third process seems to be the most appropriate description for heme ejection on addition of surfactant.

The extent of heme exposure is proportional to partition coefficient (K) values and their dependence on surfactant concentrations for NiHb and CuHb are evident from figure 4. The higher K values indicate higher extent of heme exposure through stabilization of 4-coordinated heme. These values of partition coefficient show that there is difference in the extent of interaction between SDS and CTAB with NiHb and CuHb. The reactivity is reversed in NiHb compared to that of CuHb. This may be attributed to the different extent of T-state character based on PDS reactivity experiments[19].

Interpretations of these results on the following stepwise metalloprotein–CTAB interactions suggest: (i) The surfactant interactions weaken the proximal histidine [Fe–N_ε] coordinated to the metal centre; (ii) consequently there is a transition from 5- to

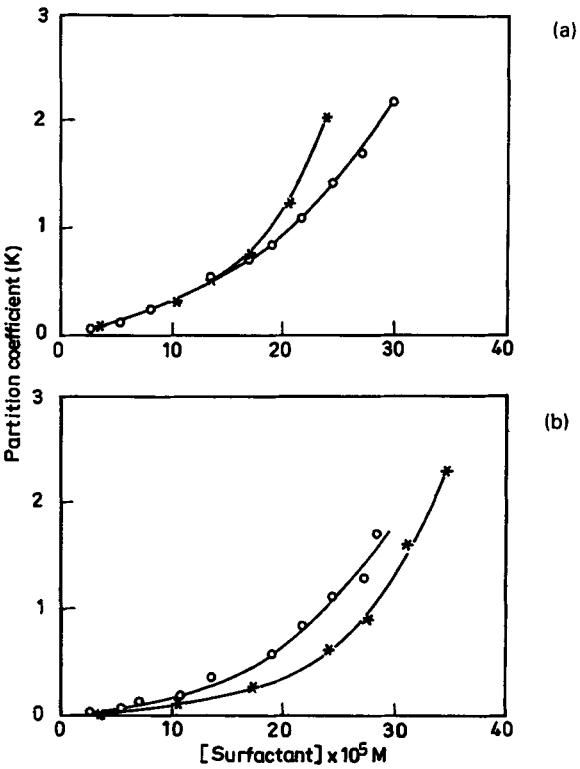


Figure 4. Plot of partition coefficient (K) as a function of SDS (*) and CTAB (O) concentration for (a) NiHb and (b) CuHb.

4-coordination of the porphyrin centre; (iii) the globin envelope of metalloporphyrin then hydrophobically interacts with the surfactant molecules, paving the way for chain unfolding followed by heme extraction into the hydrophobic micellar core. The hydrophobic interactions due to SDS and CTAB perhaps weaken the proximal histidine coordination and this penetrative additive effect may cause the ejection of heme.

4. Conclusions

According to the results of the present study, the interaction of the protein with surfactants and the resultant disappearance of the 5-coordination band are also observed at surfactant concentrations much lower than those for critical micelle formation values. With further increase in the concentration of surfactant, the ejection of heme from the globin matrix followed by stabilization of heme in the micelle media through hydrophobic interaction takes place. Despite these investigations, the specific interactions between surfactant–globin metalloporphyrin have to be rationalized with reference to protein conformational and micellar microstructural changes. Further UV-visible, Raman and EPR spectroscopic studies are under progress to verify the exact nature of such interactions and the consequent changes at different *p*H conditions.

Acknowledgements

This work was taken up as part of the project supported by the US-India Rupee Fund awarded to PTM and JMR. BV thanks the Council of Scientific and Industrial Research, New Delhi for a fellowship and PTM thanks them for an emeritus scientistship. JS thanks the Department of Science and Technology, New Delhi for a visiting fellowship.

References

1. Perutz M F 1970 *Nature (London)* **228** 226
2. Antonini E and Brunori M 1971 *Hemoglobins and myoglobins and their reactions with ligands* (Amsterdam: North Holland)
3. Venkatesh B, Manoharan P T and Rifkind J M 1998 *Progress in inorganic chemistry* (ed.) K D Karlin (New York: John Wiley and Sons) vol. 47, p. 563
4. Holt S L (ed.) 1982 *Inorganic reactions in organized media* ACS Symposium Series No. 177 (Washington, DC: Am. Chem. Soc.) chap. 9, p. 156
5. Fendler E J and Fendler J H 1975 *Catalysis in micellar and macromolecular systems* (New York: Academic Press)
6. Venkatesh B, Jayakumar R, Pandian R P and Manoharan P T 1996 *Biochem. Biophys. Res. Commun.* **223** 390
7. Jayakumar R, Mandal A B and Manoharan P T 1993 *J. Chem. Soc., Chem. Commun.* 853
8. Eremin A N, Metelitsa D I and Smettan G 1984 *Biokhimica* **49** 976
9. Jolivalt C, Minier M and Renon H 1993 *Biotechnol. Prog.* **9** 456
10. Hiramatsu K and Yang J T 1983 *Biochem. Biophys. Acta* **743** 106
11. Moriyama Y, Sasaoka H, Ichiyanagi T and Taketa K 1992 *J. Protein Chem.* **11** 583
12. Harrington J P, Newton P, Crumpton T and Keaton L 1993 *Int. J. Biochem.* 665
13. Hayashi T, Itagaki H, Fukuda T, Tamura U, Sato Y and Suzuki Y 1995 *Biol. Pharm. Pharmacol. Bull.* **18** 540
14. Das T K, Mazumdar S and Mitra S 1993 *J. Chem. Soc., Chem. Commun.* 1447
15. Birdi K S and Steinhardt J 1978 *Biochem. Biophys. Acta* **534** 219
16. Medhi O K, Mazumdar S and Mitra S 1989 *Inorg. Chem.* **28** 3243
17. Simplicio J and Schwenzer K 1973 *Biochemistry* **12** 1923
18. Manoharan P T, Alston K and Rifkind J M 1985 *Biol. Inorg. Copper Chem.* **1** 105
19. Manoharan P T, Alston K and Rifkind J M 1986 *J. Am. Chem. Soc.* **108** 7095
20. Szebeni J, Hauser H, Eskelson C D, Watson R R and Winterhalter K H 1988 *Biochemistry* **27** 6425
21. Hargrove M S, Whitaker T, Olson J S, Vali R J and Mathews A J 1997 *J. Biol. Chem.* **272** 17385
22. Rifkind J M, Abugo O, Levy A and Heim J 1994 *Methods Enzymol.* **231** 449
23. Williams R C and Tsay K-Y 1973 *Anal. Biochem.* **54** 137
24. Shibayama N, Morimoto H and Miyazaki G 1986 *J. Mol. Biol.* **192** 323
25. Manoharan P T, Alston K and Rifkind J M 1989 *Biochemistry* **28** 7148
26. McLees B D and Caughey W S 1968 *Biochemistry* **7** 642
27. Falk J E 1964 *Porphyrins and metalloporphyrins* (New York: Elsevier)