ton. Both these reactions can be expected to take place at such times, probably, shorter than 10^{-14} s. Which is the time required for proton transfer. The proton, formed as a result of de-electronation of hydrogen atom on Pt surface, remains without forming hydronium ion due to the reason mentioned above, close to the platinum surface and is readily available for electronation reaction. The electrons formed due to de-electronation of hydrogen atoms absorbed on the metal surface can become members of the conductance of Fermi electrons of the metal. The electronation of the proton (or hydrogen ion) and de-electronation of hydrogen atom is most probably occurring on the electrode surface and close to the inner Helmhotz plane of the electrical double layer populated by highly oriented water molecules. This exchange reaction is almost a solid state reaction and takes place at very high exchange rates that is necessary for a standard reference electrode. Thus the forward and reverse (or backward) reaction, viz. $H^+ + e \rightleftharpoons H$, can take place with zero free energy change i.e. $\Delta G^\circ = 0$ and consequently E° which is given by $-\Delta G^\circ/nF$ is also zero.

The finding provides a solid base for the electrochemical series built up on the intuitive assumption of W. Nernst that the standard potential of hydrogen can be assigned a value of zero.

1. Parthasarathy, S. and Srinivasan, K. R., *Curr. Sci.*, 1994, **66**, 56–59.
2. Sawyer, D. T. and Roberts Jr J. L., in *Experimental Electrochemistry for Chemists*, John Wiley, USA, 1974, p. 36.
3. Jeffery, G. H., Basset, J., Mendeham, J. and Denney, R. C., in *Vogel's Textbook of Quantitative Chemical Analysis*, English Language Book Society, UK, 1989, p. 61.
4. Samuel Glasstone, in *An Introduction to Electrochemistry*, Affiliated East West Press Pvt. Ltd., New Delhi, India, 1975, p. 139.
5. Davies, C. W., *J. Chem. Soc.*, 1938, 2093–2098.
6. Robinson, R. A. and Stokes, R. H., in *Electrolyte Solutions*, Academic Press, New York, 1985, p. 65.
7. Kortum, G., in *Treatise on Electrochemistry*, Elsevier, London 1965, p. 308.
8. Samuel Glasstone, in *An Introduction to Electrochemistry*, Affiliated East West Press Pvt. Ltd., New Delhi, India, 1975, p. 201.
9. Atkins, P. W., in *Physical Chemistry*, English Language Book Society, UK, 1985, p. 354.
10. Maier, C. G., *J. Am. Chem. Soc.*, 1930, **52**, 2159–2170.
11. Maier, C. G. and Raltson, O. C., *J. Am. Chem. Soc.*, 1926, **48**, 364–374.
12. National Bureau of Standards, USAA, 1952, Circular500, p. 1266.
13. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 1, p. 461.
14. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 1, p. 462.
15. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 1, p. 467.
16. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 1, p. 465.
17. Antropov, L. I, in *Theoretical Electrochemistry*, Mir Publishers, Moscow, 1972, p. 169.
18. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 2, p. 1238.
19. Bockris, J. O'M. and Srinivasan, S., in *Fuel Cells: Their Electrochemistry*, McGraw Hill Book Company, New York, 1969, p. 43.
20. Antropov, L. I., in *Theoretical Electrochemistry*, Mir Publishers, Moscow, 1972, p. 276.
21. Bockris, J. O'M. and Reddy, A. K. N., in *Modern Electrochemistry*, Plenum Press, New York, 1970, vol. 1, p. 487.

Received 8 September 1995, accepted 7 October 1995.

Selective denaturation of cytochrome c oxidase by ionic surfactants: Depletion of both the heme *a* residues from the enzyme

Tapan Kanti Das and Shyamalava Mazumdar

Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

UV-visible absorption and circular dichroism studies on interaction of ionic surfactants with bovine heart cytochrome c oxidase have been carried out in lauryl maltoside solution. Results showed that addition of the surfactant causes irreversible depletion of heme *a* from cytochrome *a* as well as cytochrome a_3 sites of this membrane protein at physiological pH. This selective denaturation by ionic surfactants is different from the effect of guanidine hydrochloride on this enzyme. Depletion of heme *a* causes complete inactivation of this ubiquitous respiratory enzyme.

IONIC surfactants, such as sodium dodecyl sulphate (SDS), are commonly used for denaturation of proteins[1–4]. There has, however, yet been no report on any systematic study on the nature of interaction of surfactants with hemeproteins. Moreover, cationic surfactants were considered to be less efficient in such denaturation of proteins, which has been disputed recently[5], lending it subject for further investigation. We have recently shown that surfactants, both cationic and anionic, can cause release of heme-NO species from nitric oxide complex of myoglobin[5]. Previous studies have shown that membrane proteins are generally more resistant to denaturation than other proteins. Cytochrome c oxidase (CcO, EC 1.9.3.1) is a mitochondrial membrane protein[6–11] with two heme *a* active sites, strongly ligated to one or two histidines of the protein. This enzyme is generally extracted in active form in neutral surfactants such as lauryl maltoside (LM), Triton X-100 (TX-100), etc. Structure and function of this multifunctional enzyme is a subject of frontier interest in recent years. The present report shows that the highly buried heme *a* groups of this enzyme can indeed be depleted by treatment with both cationic surfactant, cetyl trimethyl ammonium bromide (CTAB) and anionic surfactant, SDS, in lauryl maltoside solution. The biochemical effect of surfactants is a subject of growing concern and the present study demonstrates their fatal effect on this vital respiratory enzyme. Furthermore, this study provides an

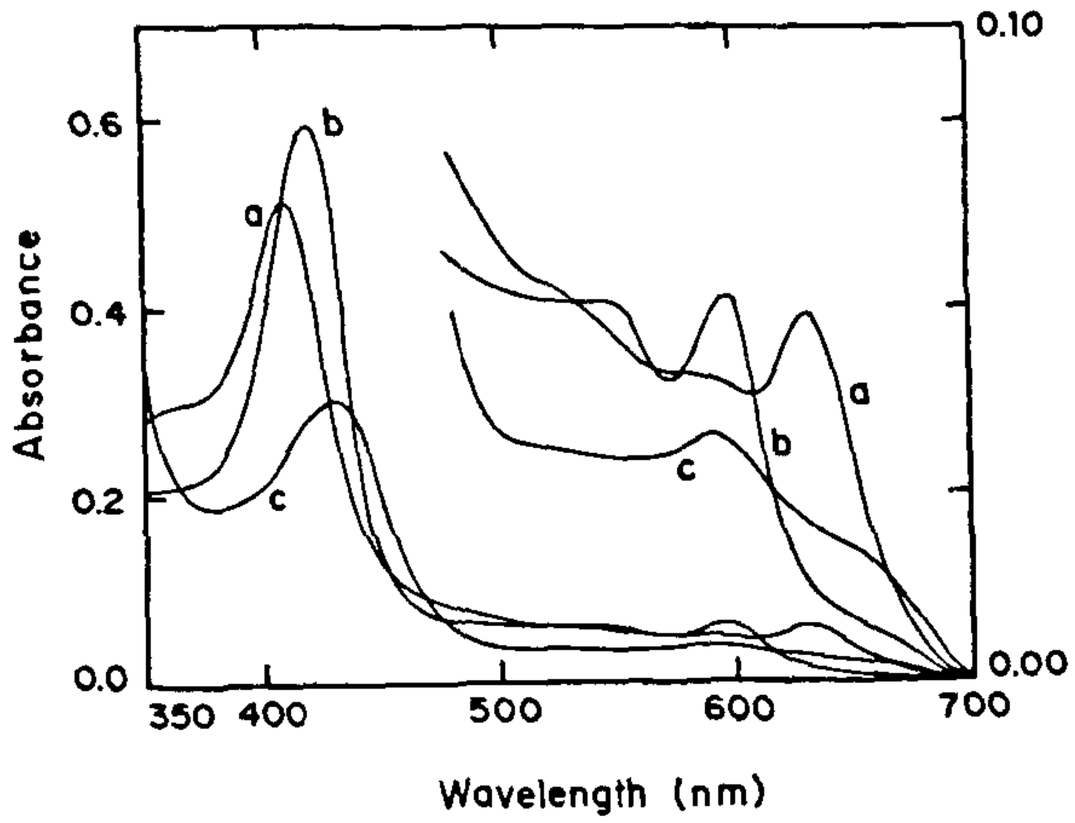

Figure 1. The optical absorption spectra of (a) CTAB-treated CcO. CcO (7 6 μM, 0 1% in lauryl maltoside, 50 mM sodium phosphate buffer, pH 7 4) was charged to a final CTAB concentration of 25 mM. (b) Native CcO spectrum (without CTAB) is given for comparison. (c) Dithionite-reduced CTAB – CcO. Minimal amount of solid dithionite was added to CTAB-treated CcO to effect reduction.

easy method of extracting heme *a* from the enzyme which would otherwise require more drastic conditions. The results have been interpreted in the light of electrostatic and hydrophobic interactions between the surfactant and the enzyme.

Bovine CcO was extracted from ox heart using reported methods[8,12]. The spectrum of bovine heart CcO in lauryl maltoside showed an irreversible change on addition of CTAB or SDS solution to the enzyme. Figure 1 shows the spectrum of CcO (7.6 μM) obtained after addition of 25 mM CTAB (trace *a*) in lauryl maltoside solution. The spectrum of the native enzyme in absence of surfactant is given in trace 'b' (Figure 1) for comparison. The spectrum of CTAB-treated CcO matched exactly with that of CTAB solution of heme *a* separately isolated from the native enzyme (data not shown, see ref. 13). Earlier report[13] of optical spectra of monomeric heme *a* encapsulated in surfactant solution also matched with the spectra shown in trace '*a*' of Figure 1. The Soret peak of CcO undergoes a large blue shift from 421 nm to 410 nm on addition of CTAB. This visible band of CcO at ~ 598 nm also disappears in presence of the surfactant to give a new charge transfer band at 631 nm characteristic of high-spin heme *a* species. Moreover, dithionite reduction of the solution of CTAB-treated CcO (trace 'c' of Figure 1) matched with the reduced heme *a* in CTAB obtained on addition of minimal amount of dithionite to heme *a* solution (data not shown). Similar observations were obtained when CcO was treated with the anionic surfactant, SDS. Unlike ionic surfactants, the neutral surfactants such as, Triton X-100, LM, etc., however, did not have any drastic effect on the spectra of the enzyme. Treatment of guanidine hydrochloride has recently[12] been shown to cause a red shift in the Soret band of CcO. This indicates that the environment around the heme *a* centre on treatment with ionic surfactant is different from that obtained on treatment with guanidine hydrochloride.

The coordination geometry of the two heme *a* centres (cytochrome *a* and cytochrome a_3 sites) in the intact native enzyme are different from each other, giving rise to a slight difference in their optical spectra (broadening of bands) in the native enzyme[9]. However, addition of the ionic surfactant results in complete disappearance of the spectral bands of CcO, indicating that both the cytochrome *a* and cytochrome a_3 sites are depleted from the enzyme to form heme *a* in micelles[14].

In order to further characterize the species obtained on addition of ionic surfactant to CcO, we have measured circular dichroism of the Soret region of CcO at different added concentrations of surfactants. Figure 2 shows that the ellipticity of the Soret region decreases steadily on addition of increasing amounts of CTAB. At surfactant concentrations above 30 mM (with 14 μM CcO) the CD spectra become almost constant with very small ellipticity in the Soret region. The presence of protein environment around the heme gives rise to large ellipticity of Soret band of hemes in hemeproteins[15]. Removal of the heme from the protein and its subsequent encapsulation inside micelles[14] result in an almost isotropic environment around the heme moiety, hence it has negligibly small ellipticity in the Soret region. This result further confirms that the species released on treatment of CcO with the surfactant is heme *a* which gets encapsulated inside the micelles formed by aggregation of the surfactants. The concentration of surfactant required to complete the reaction was found to be almost same for SDS and CTAB, indicating that both of these surfactants are almost equally effective in depletion of heme *a* from CcO. The concentration of guanidine hydrochloride required[12] to diminish the heme CD signal was (> 1 *M*) much more than the surfactant required in the present case, indicating that ionic surfactants are much more effective in releasing heme *a* from the enzyme. Control experiment with pure heme *a*

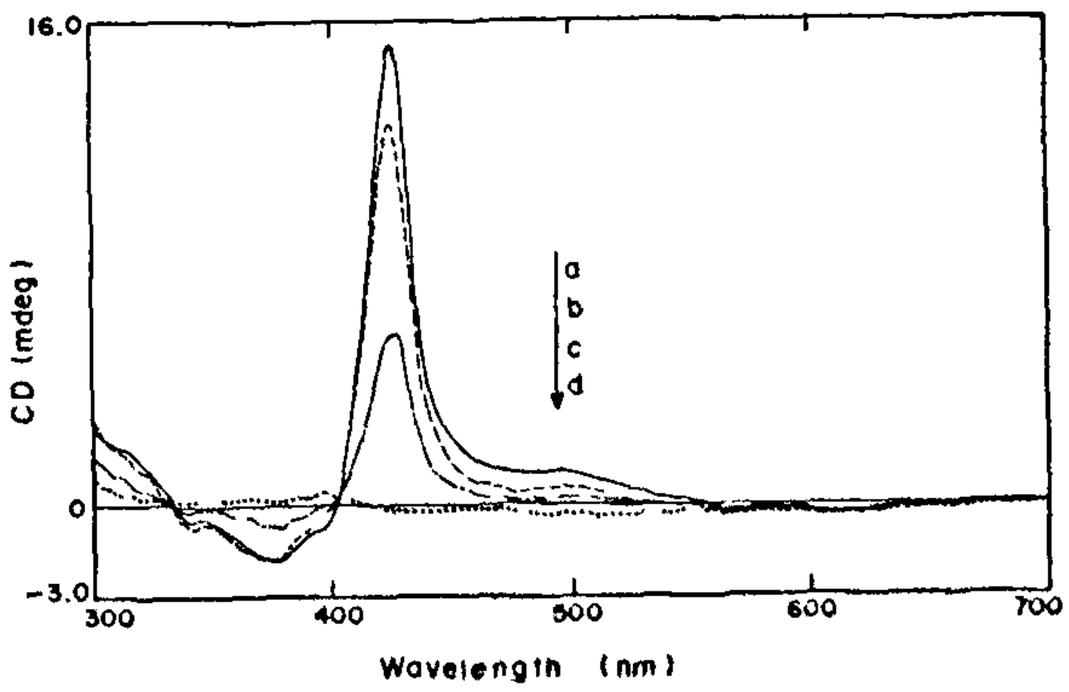

Figure 2. Changes in ellipticity in the visible region of CD spectrum of CcO in 0.1% lauryl maltoside, 50 mM sodium phosphate buffer, pH 7 4, on addition of CTAB aliquots. CcO concentration was 14 μM, cell path length was 10 mm Curves *a* to *d* represent CTAB concentrations of 0, 12.5, 22 8 and 39 mM respectively.

monodispersed in micelles (data not shown) did not show any significant CD in the near UV-visible region. In the UV region, CD bands around 220 nm were found to diminish up to 36% on addition of the surfactant (data not shown). Similar change in the CD in the 220 nm region has been found on guanidine hydrochloride[12] treatment of CcO. This indicated that the protein possibly gets partially unfolded on treatment with surfactant. However, because of the complexity of the structure of this multisubunit protein, the CD spectra does not provide any quantitative information regarding the unfolding of the structure.

CD and optical spectra of the cyanide complex of CcO (CcO – CN) have also been studied in presence and absence of ionic surfactants. The spectra of the cyanide complex were recorded within one hour after addition of buffered (pH 7.4) KCN solution to CcO when cyanide binds mainly to the cytochrome a_3 site[9]. The major Soret CD peak of CcO – CN was observed at 427 nm, which vanished on gradual addition of SDS or CTAB solution. The optical spectra of surfactant-treated CcO–CN matched quite well with the cyanide complex of heme *a* in CTAB. This result indicates that, addition of ionic surfactants causes breaking of iron–histidine bonds in cytochrome *a* as well as in cytochrome a_3 and results in formation of isolated heme *a* in micellar solution. Walter *et al.*[16] observed a partial spin state conversion of cyt *a* by incubating CcO in higher lauryl maltoside concentration at 40°C. A blue shift of Soret band and formation of a charge transfer band at 630 nm at the expense of the α-band (at 598 nm) was ascribed to a low to high spin transition of cyt *a*, probably because of axial Fe–N bond cleavage[16]. Our results show that this type of transition is complete by treating CcO with SDS or CTAB.

In order to characterize the heme *a* species obtained on treatment in micelles, NMR spectra of heme *a* in CTAB solution at pH~2 (Figure 3, upper spectrum) showed highly down field shifted broad signals due to the heme protons (possibly heme methyl protons). The paramagnetically shifted signals in CTAB solution of heme *a* are similar to those obtained from a $CDCl_3$ solution of heme *a* (lower spectrum in Figure 3), which is typical of high-spin Fe^{3+} heme species. The heme proton signals in micellar solution are generally very broad[14] as observed in the present case. Thus the heme *a* species released on treatment with CTAB on CcO is possibly a high-spin heme *a* complex with axial ligands water and hydroxo ion. The line width of the paramagnetically shifted heme proton signals broadens because of fast ligand exchange at ambient pH, hence they could not be detected at ambient pH using the 500 MHz NMR spectrometer.

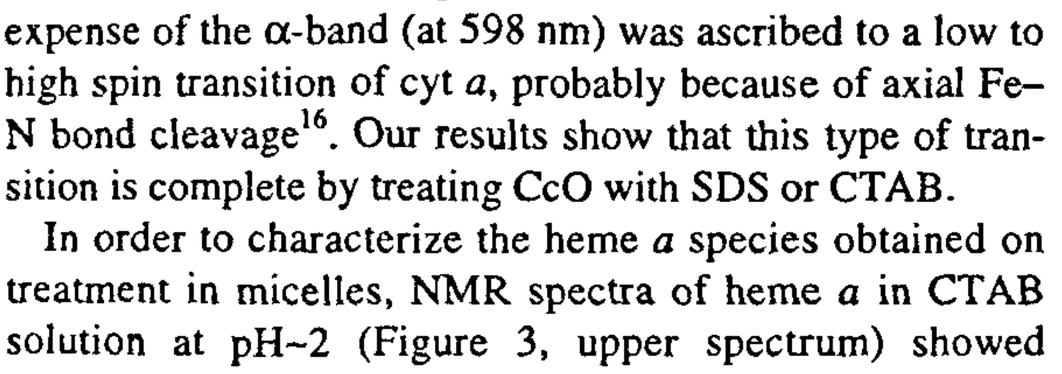

Figure 3. Proton NMR spectrum of heme *a* (~0.4 mM) in $CDCl_3$ (lower trace) and in 30 mM CTAB, 50 mM sodium phosphate buffer, pH~2 (upper trace) in D_2O. Temperature was maintained at 30°C. A multiple frequency irradiation pulse program was used to simultaneously decouple water and CTAB proton resonances

The present study, thus, demonstrates that ionic surfactants can cause irreversible release of heme *a* from cytochrome c oxidase. The deactivation of CcO and release of heme *a* by CTAB or SDS possibly involves electrostatic binding of the ionic surfactant to the enzyme. Both cationic and anionic surfactants possibly bind to the enzyme surface around its heme crevices to cause heme release. As the charged head groups of surfactant molecules anchor to the enzyme, their hydrophobic tails may interact with the hydrophobic amino acids and break the structure of the enzyme. The iron–histidine bonds possibly become severely weak because of the change in protein structure in the vicinity of heme centres, leading to release of heme *a* out of the enzyme. The heme *a* released from CcO is structurally different from the heme in other hemeproteins. The present method may help in designing heme reconstitution procedures with heme *a* and other hemes in CcO as well as in other hemeproteins.

1. Rao, K. S. and Prakash, V., *J. Biol. Chem.* 1993, **268**, 14769–14775.
2. Singer, S. J. and Nicholson, G. L. *Science*, 1972, **175**, 720–731
3. Tanford, C. and Reynolds, J. A., *Biochim. Biophys. Acta*, 1976, **457**, 133–170.
4. Helenius, A. and Simons, K., *Biochim. Biophys. Acta*, 1975, **415**, 29–79.
5. Das, T. K., Mazumdar, S. and Mitra, S., *J. Chem. Soc Chem. Commun.*, 1993, 1447–1448.
6. Saraste, M., *Q. Rev. Biophys.*, 1990, **23**, 331–366.
7. Copeland, R. A. and Chan, S. I., *Annu. Rev. Phys. Chem.*, 1989, **40**, 671–698.
8. Capaldi, R. A., *Annu. Rev. Biochem.*, 1990, **59**, 569–596
9. Brunori, M , Antonini, G., Malatesta, F., Sarti, P. and Wilson, M. T., in *Heme Proteins* (eds Eichhorn, G. L and Marzilli, L. G.) Elsevier, NY, 1988, vol. 7, pp 93–153
10. Gennis, R. B. and Ferguson-Miller, S., *Science*, 1995, **269**, 1063–1064, Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S., *Science*, 1995, **269**, 1069–1074.
11. Williams, R. J. P., *Nature*, 1995, **376**, 643; Iwata, S , Ostermeier, C., Ludwig, B. and Michel, H., *Nature*, 1995, **376**, 660–669.
12. Hill, B. C., Cook, K. and Robinson, N. C., *Biochemistry*, 1988, **27**, 4741–4747.
13. Yanagi, N., Sekuzu, I., Orii, Y. and Okunuki, K., *J. Biochem.*, 1972, **71**, 47–56.
14. Mazumdar, S. and Mitra, S., *Struct. Bonding*, 1993, **81**, 115–145.
15. Myer, Y. P., *Curr. Top. Bioenerg.*, 1985, **14**, 149–188.
16. Walter, S. A , Papadopoulos, P. G. and Baker, G. M., *J. Biol. Chem* , 1993, **268**, 4716–4719.

ACKNOWLEDGEMENTS. We thank Prof. Samaresh Mitra for his constant encouragement and support during the course of this work. NMR work was done at the National Facility of High Field NMR, TIFR, Bombay and we acknowledge help of Ms Uma Joshi and Mr Devidas Jadav in carrying out NMR experiments.

Received 6 July 1995, revised accepted 27 October 1995