Spectroscopic and Interfacial Properties of Myoglobin/Surfactant Complexes

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ABSTRACT The complexes of horse myoglobin (Mb) with the anionic surfactant sodium dodecyl sulfate (SDS), and with the cationic surfactants cetyltrimethylammonium chloride (CTAC) and decyltrimethylammonium bromide (DeTAB), have been studied by a combination of surface tension measurements and optical spectroscopy, including heme absorption and aromatic amino acid fluorescence. SDS interacts in a monomeric form with Mb, which suggests the existence of a specific binding site for SDS, and induces the formation of a hexacoordinated Mb heme, possibly involving the distal histidine. Fluorescence spectra display an increase of tryptophan emission. Both effects point to an increased protein flexibility. SDS micelles induce both the appearance of two more heme species, one of which has the features of free heme, and protein unfolding. Mb/CTAC complexes display a very different behavior. CTAC monomers have no effect on the absorption spectra, and only a slight effect on the fluorescence spectra, whereas the formation of CTAC aggregates on the protein strongly affects both absorption and fluorescence. Mb/DeTAB complexes behave in a very similar way as Mb/CTAC complexes. The surface activity of the different Mb/surfactant complexes, as well as the interactions between the surfactants and Mb, are discussed on the basis of their structural properties.

INTRODUCTION

The study of the interactions between surfactants and proteins is of significant scientific and technological importance. Surfactants are widely used in the purification and characterization of proteins, polyacrylamide gel electrophoresis in sodium dodecyl sulfate being a well-known example. Moreover, surfactants are attractive molecules to probe protein structure. Proteins and ionic surfactants share the property of having both charged groups and hydrophobic portions. This implies that the interactions between surfactants and proteins are intrinsically complex processes, involving different types of intermolecular forces. Therefore, small and well-characterized proteins are often chosen as targets for the formation of complexes with surfactants (Jones, 1992). Myoglobin is a favorable model protein, because it is suitable for a large number of spectroscopic techniques, including optical and magnetic resonance spectroscopies. Early studies (Kromphardt and Lübbers, 1958) showed that the addition of tetradecyl sulfate to ferric Mb lead to the appearance of a characteristic hemichrome absorption spectrum. The alterations of the electronic absorption spectrum of Mb due to sodium dodecyl sulfate were correlated with surfactant binding via numerous discrete steps depending on the concentration of SDS, which were below the critical micellar concentration (van den Oord and Wesdorp, 1969). Vibrational spectroscopy at the amide groups has shown that the addition of dodecylpyridinium bromide causes a decrease in α-helix conformation and that the magnitude of these changes is smaller for cationic than for anionic detergents (Wasylewsky et al., 1979). Dissociation of the prosthetic group was observed in Mb in the presence of cationic detergents by spectrophotometric and chromatographic experiments (Blauer et al., 1973). Volume effects that result from protein-detergent binding, protein conformational changes, and charge effects were studied by dilatometry (Katz et al., 1973).

Despite the large number of articles devoted to the characterization of Mb/surfactant complexes, a study that combines optical spectroscopy with surface tension measurements under identical experimental conditions is still missing. The aim of this work is to rationalize the effects of different types of surfactants on myoglobin. Both anionic (SDS) and cationic surfactants (cetyltrimethylammonium chloride and decyltrimethylammonium bromide) are considered. To accurately analyze protein/surfactant interactions, surface tension data are compared for the surfactants in water solution, in buffered solution, and in buffered solution in the presence of Mb. The dependence of Mb absorption and emission spectra on the surfactant concentration leads to interesting correlations with the surface tension measurements, which can be interpreted in terms of protein structural changes.

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Abbreviations used: Mb, myoglobin; CTAC, cetyltrimethylammonium (= hexadecyltrimethylammonium) chloride; SDS, sodium dodecyl sulfate; DeTAB, decyltrimethylammonium bromide; MOPS, 4-morpholinepropanesulfonic acid; 6cLS, six-coordinated, low-spin; 5cHS, five-coordinated, high-spin; 6cHS, six-coordinated, high-spin; *cmc*, critical micellar concentration; *cac*, critical aggregation concentration; CT1, charge-transfer band 1; Fe-PPIX, Fe-protoporphyrin IX; BSA, bovine serum albumin.

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EXPERIMENTAL SECTION

Materials

Horse myoglobin was purchased from Biozyme (Reinheitszahl = 4.8) (San Diego, CA) and used without further purification. The protein was dissolved in 20 mM 4-morpholinepropanesulfonic acid (Boehringer Mannheim, Ingelheim am Rhein, Germany) buffer, pH 7.0, and filtered with Millipore (Billerica, MA) Millex SV filters, before use. The concentration of the protein, determined spectrophotometrically using $\varepsilon = 157\ \text{cm}^{-1}\ \text{mM}^{-1}$ at 409 nm (Antonini and Brunori, 1971), was 8×10^{-6} M for both the surface tension and spectroscopic measurements. CTAC was purchased from Aldrich Chemicals (St. Louis, MO). SDS (99% purity) was purchased from Merck (Darmstadt, Germany). These surfactants were used without further purification. DeTAB was purchased from Fluka Chemie (Buchs, Switzerland) and twice recrystallized from acetone for the surface tension measurements. 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline was purchased from Eastman Kodak (Rochester, NY).

Spectroscopy

Electronic absorption spectra were recorded at room temperature (23 ± 1°C) with a double-beam Cary 5 spectrophotometer (Varian, Palo Alto, CA). The scan speed was 120 nm/min with 2 nm resolution. To obtain the relative abundance of the three species contributing to the spectra (see below), a simple data analysis was performed. In the case of Mb/SDS, we considered that the spectra at 0, 2×10^{-4}, and 10^{-1} M SDS were due to the spectra of the three pure species, i.e., native Mb, six-coordinated low-spin Mb, and five-coordinated high-spin Mb, respectively. The spectra of solutions at intermediate SDS concentrations were fitted by a weighted sum of the spectra of the three pure species with Microcal Origin software (Northampton, MA). The coefficients of the weighted sum could be related to the concentrations, because the relative absorption coefficients for the three species could be determined. The presence of a fourth species precludes this simple analysis for SDS concentrations $>3 \times 10^{-3}$ M. In the case of the complexes with CTAC, the spectra could not be fitted with the spectra of the three pure species, because the spectrum of the pure 6-cLS species cannot be observed alone. Therefore, the spectra were fitted with those of native Mb, of 5-c HS Mb at the highest CTAC concentration, and of the Mb/SDS 6-cLS complex. Fluorescence emission spectra were recorded at room temperature (23 ± 1°C) with either a Perkin Elmer LS50 (Wellesley, MA) or a Jasco FP-750 spectrofluorimeter (Easton, MD). The scan speed was 25 nm/min with 5-nm resolution.

Surface tension

Surface tension was determined with the duNouy ring method using a KSV Sigma 70 digital tensiometer (accuracy 0.1 mN/m) (Helsinki, Finland) equipped with a Metrohm 665 Dosimat (Buckingham, UK) allowing for an automatic surfactant concentration scan. The measurements were performed at 25.0 ± 0.5°C diluting a concentrated detergent solution contained in the thermostated vessel with water, MOPS pH 7.0, or Mb 8.0×10^{-6} M in MOPS pH 7.0. The solution was stirred for 1 min and allowed to rest for 5–10 min before each measurement. The force (W) necessary to detach a Pt/Ir ring from the solution is measured, and surface tension (γ) can be determined according to:

$$W = W_{\text{ring}} + 4\pi r\gamma. \tag{1}$$

RESULTS AND DISCUSSION

Myoglobin/sodium dodecyl sulfate

Fig. 1 shows the surface tension as a function of SDS concentration for water, MOPS buffer, and Mb in MOPS buffer solution. The curves in water and in MOPS buffer show the classical behavior of micelle-forming detergents. A minimum in the surface tension curve can be distinctly observed. This is the well-known effect of impurities with a surface activity higher than the surfactant itself (dodecyl alcohol in this case), that are preferentially adsorbed at the air/water interface, and desorb when micelles are formed due to their solubilization in the aggregates (Jönsson et al., 1998). In these cases, the concentration corresponding to the minimum surface tension (6.3×10^{-3} M) is considered as *cmc*. The *cmc* of purified SDS is 8.3×10^{-3} M (van Os et al., 1993). The effect of MOPS on the *cmc* of SDS is a shift to lower values (3.1×10^{-3} M), as expected for ionic surfactants in conditions of increased ionic strength. The similar slopes of the surface tension curves in the premicellar region for water and MOPS indicate that the surfactant adsorption at the interface is comparable in the two cases, as can be understood from the Gibbs equation:

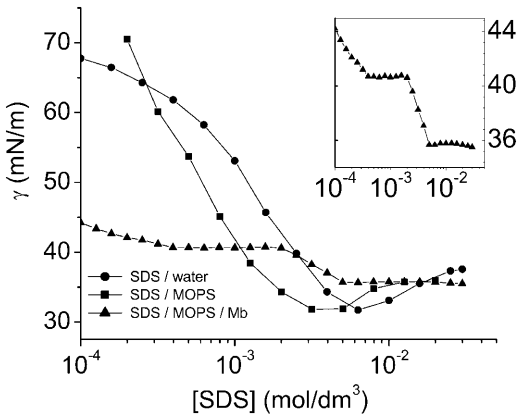

FIGURE 1 Surface tension versus SDS concentration for SDS in water solution (●), SDS in MOPS-buffered solution (■), and SDS in a MOPS-buffered solution containing 8×10^{-6} M Mb (▲). The inset shows the latter data points on an enlarged scale.

$$\Gamma_s = -\frac{1}{RT}\frac{d\gamma}{d\ln c_s}, \tag{2}$$

where Γ_s is the surfactant excess at the interface, γ is the surface tension, and c_s is the surfactant bulk concentration.

The interactions between surfactants and proteins can be interpreted in the framework of experimental and theoretical studies performed on surfactant/polymer interactions (Goddard, 1986). According to the pioneering work of Jones (1967, 1968), if the polymer concentration is kept constant, three characteristic surfactant concentrations can be defined. At low surfactant concentrations, the ternary system may exhibit a surface tension lowering with respect to the binary system, depending on the surface activity of the polymer. An increase in surfactant concentration results in a break in the surface tension curve, T_1. This point is often termed critical aggregation concentration, *cac,* and corresponds to the

surfactant concentration where the polymer-associated micellar aggregates become detectable. After T_1 a more or less constant surface tension region is generally observed until the polymer is saturated (T_2') with respect to surfactant adsorption. Beyond T_2', the activity of the monomeric surfactant starts to increase again and eventually an inflection, T_2, marks the appearance of ''free'' micelles. These observations hold even for more complex polymeric structures, such as proteins, but, given the heteropolymeric nature of peptides, specific binding sites can be present (Ananthapadmanabhan, 1993). The above-reported model has been confirmed for protein/surfactants by a number of different experimental techniques, such as binding isotherms (Jones, 1992), dynamic and static light scattering (Valstar et al., 2000), and steady-state and time-resolved fluorescence (Vasilescu et al., 1999).

The presence of Mb induces striking differences on the surface tension behavior in the dilute region. First of all, there is a consistent decrease of γ for a given surfactant concentration, indicating that the protein and/or the protein/surfactant complex have a considerable surface activity. Moreover, the slope of the surface tension curve is dramatically decreased with respect to the binary system indicating that SDS adsorption at the interface is poor, as follows from Gibbs equation. The inset reports an enlarged version of the curve for the protein-surfactant system. The *cac* can be clearly detected at 3.7×10^{-4} M SDS, so that the protein-driven micellar aggregation occurs at a concentration that is one order of magnitude lower than in the binary system; this finding indicates high interaction efficiency, because the excess free energy of interaction can be deduced from:

$$\Delta G = -RT \ln \frac{cac}{cmc}. \quad (3)$$

This quantity is related to the contribution of the polymer in driving the micellar aggregation and yields a value of 5.27 kJ mol^{-1} for Mb/SDS. The following concentration region is characterized by a flat slope, indicating that cooperative binding to the protein sites is triggered at the *cac* and continues until T_2'.

Another important parameter that quantifies protein-surfactant interaction is the binding ratio (Jean et al., 1999), defined as:

$$R = \frac{T_2' - cac}{C_p}, \quad (4)$$

where C_p is the protein concentration. In our case the saturation point occurs for $R = 200$, that is 200 SDS molecules are bound per myoglobin molecule.

The effects of SDS addition to Mb solutions have been monitored by electronic absorption spectroscopy. This technique provides, in fact, very useful information on the heme environment and coordination. Fig. 2 shows ultraviolet-visible spectra at increasing SDS concentrations. The spectrum of Mb (*solid line*), which has a sharp Soret band at 409 nm, Q bands at 504 and 535 nm, and a CT1 band at 634 nm, is characteristic of a six-coordinated high-spin heme with a histidine residue (His-93) and a water molecule bound at the fifth and the sixth coordination position of the iron atom, respectively (Eaton and Hochstrasser, 1968). SDS-treated Mb shows distinct changes compared to the native protein. Up to 2×10^{-4} M, the addition of SDS causes the formation of a new species at the expense of the 6-cHS heme, as is evident from the intensity decrease and red shift of the Soret band and the concomitant appearance of Q bands at 567 and 535 nm (*dashed line*). This spectrum, characteristic of a 6-cLS heme, is very similar to that of ferric cytochrome b_5 (Strittmatter and Velick, 1956), which has histidine residues as fifth and sixth ligands. Further changes in the spectrum are observed upon addition of higher amounts of the surfactant. A concentration of SDS higher than 3×10^{-3} M causes additional changes in the spectrum: the Soret band decreases further in intensity, broadens and blue shifts to 408 nm. Concomitantly, in the visible region, bands at 533, 568, and 635 nm are observed (*dotted line*). These changes are indicative of the formation of a new high-spin species, different from that of the native protein. The spectral features of this spectrum are unclear, because all bands are overlapped with those of both the 6-cLS and a fourth species, which dominates at still higher SDS concentration ($>7 \times 10^{-3}$ M). The spectrum of the latter species (*dashed-dotted line*), with maxima at 400, 491, 520, and 607 nm, closely resembles that of Fe-protoporphyrin IX in the presence of high SDS concentration at pH 8.3, which has been shown (Boffi et al., 1999) to be a 5-cHS species with hydroxyl as the fifth ligand. It appears, therefore, that the ligands of the Mb heme

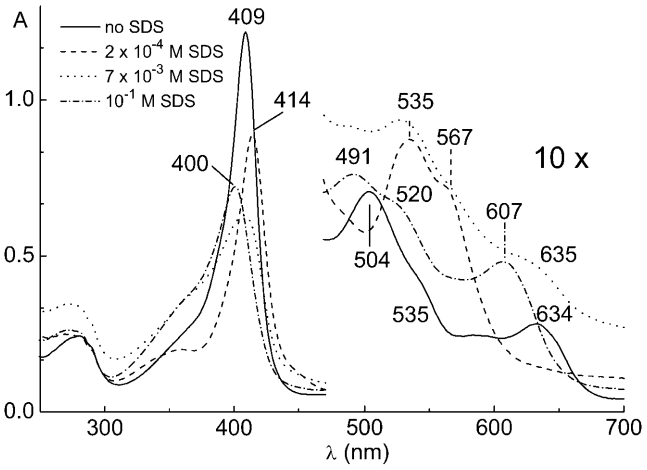

FIGURE 2 Electronic absorption spectra of 8×10^{-6} M Mb in MOPS-buffered solution at increasing SDS concentrations (see Fig. 2 key). The spectrum at 7×10^{-3} M SDS in the long-wavelength region has been shifted along the ordinate axis to allow better visualization.

chromophore (which is also Fe-PPIX) change in the presence of highly concentrated SDS: the Fe-His-93 bond is ruptured and the fifth ligand is likely hydroxyl.

Electronic absorption spectroscopy also allows us to assess that the structural modifications induced by SDS are reversible. In fact, the changes in the absorption spectra can be completely reversed by the addition of the cationic surfactant DeTAB, which apparently can displace SDS from the protein by means of its positively charged group. This effect is induced by an equimolar concentration of DeTAB and could be observed up to 10^{-2} M surfactant concentrations (data not shown). The displacement of anionic surfactant from Mb occurs with the simultaneous formation of mixed SDS/DeTAB aggregates. A detailed structural investigation on the nature of such aggregates is well beyond the aim of this work, although we noticed a considerable increase of turbidity of the protein solution that became opalescent. In analogy with some similar ternary systems (cationic + anionic surfactants in water), the formation of catanionic spontaneous vesicles seems probable (Tondre and Caillet, 2001).

The aromatic amino acid fluorescence of proteins is a sensitive probe for studying conformational transitions. The proximity of the two tryptophan residues (Trp-7 and Trp-14 on helix A) to the heme in native Mb results in a partial quenching of the tryptophan fluorescence. A blue shift of the maximum, compared to free tryptophan in aqueous solution, is a consequence of the shielding of the protein matrix. Fig. 3 shows representative fluorescence spectra of Mb at increasing SDS concentration. Up to 6×10^{-4} M SDS, the effect of adding SDS is a large increase of the fluorescence intensity, together with a gradual red shift of the maximum from 329 to 333 nm. This indicates that the tryptophan residues have moved away from the heme and have become more exposed to the solvent. No changes are observed in the spectra between 6×10^{-4} M and 10^{-3} M SDS. Between 10^{-3} M and 5×10^{-3} M SDS, the intensity slightly decreases, whereas the maximum shifts back to shorter wavelengths. The addition of a very high amount of SDS (between 5×10^{-3} M and 0.1 M) leads to a large intensity increase and further blue shifting (back to 329 nm), together with the appearance of a shoulder at ~305 nm.

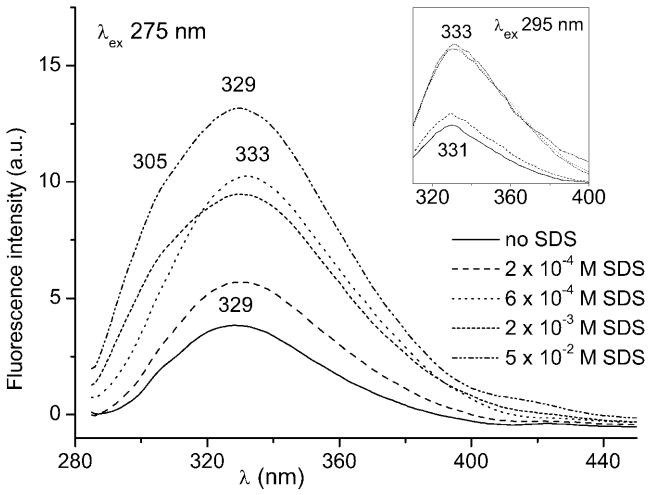

FIGURE 3 Fluorescence emission spectra of 8×10^{-6} M Mb in MOPS-buffered solution at increasing SDS concentrations (see Fig. 3 key). The excitation wavelength was 275 nm. The inset shows spectra with 295-nm excitation.

The spectral changes at high SDS concentrations can be attributed to an increased contribution of tyrosine emission. In fact, tyrosine fluorescence is blue shifted relative to tryptophan fluorescence. In native Mb, like in most proteins in their folded state, energy transfer from tyrosine residues to energy acceptors (tryptophan residues and heme) quenches the emission of tyrosine effectively. Tyrosine fluorescence was observed from apomyoglobin under denaturing conditions, i.e, in the presence of 6 M guanidine, but not from apomyoglobin at acid pH (Kirby and Steiner, 1970). The unfolding induced by high SDS concentrations can increase the separation between tyrosine fluorophores (Tyr-103 and Tyr-146) and energy acceptors, thus increasing tyrosine fluorescence. The fluorescence spectra obtained with 295-nm excitation (Fig. 3, *inset*), by which only tryptophan fluorescence is selectively excited, confirm this picture. These spectra display an intensity increase, but only a progressive red shift of the fluorescence maximum, at increasing SDS concentration.

SDS/protein complexes have been thoroughly investigated in view of their pertinence to the commonly used polyacrylamide gel electrophoresis in SDS. A considerable number of articles focus on the interactions between SDS and globular proteins, such as bovine serum albumin and lysozyme (Valstar et al., 1999). From a structural point of view, the so-called ''beads-necklace model'' has been proposed for the plateau region beyond the *cac*, based on a small angle neutron scattering study performed on the BSA/SDS system. Micelle-like clusters of surfactant are formed at precise sites of the protein with a smaller aggregation number than that of free micelles (Chen and Teixeira, 1986; Guo and Chen, 1990). A subsequent investigation with the aid of different molecular probe techniques (Turro et al., 1995) has confirmed this model and proved that the uncoiled protein wraps around the polar head region of the micelles, whereas no nucleation on its hydrophobic portions occurs. The same article gives also some insight on the pre-*cac* region, where binding to specific high-energy sites is detected; these sites are thought of as anchorages for the subsequent cooperative binding responsible for protein unfolding. A more recent work (Valstar et al., 2000) has extended this study to lysozyme/SDS, confirming the beads-necklace model, except that the more compact structure of lysozyme prevents a complete wrapping around micelles. A noteworthy observation for BSA/SDS is that even for a free SDS concentration as low as 10^{-5} M, approximately seven surfactant molecules are bound to one BSA molecule. The

interaction is modeled mainly as electrostatic (Ananthapadmanabhan, 1993), and it has analogies with the specific binding of three SDS molecules to cross-linked lysozyme, which has been observed by x-ray crystallography (Yonath et al., 1977).

These results on Mb can be interpreted in a very similar manner. Fig. 4 shows that the absorption data, the fluorescence data, and the surface tension are correlated when plotted as a function of surfactant concentration. The absorption spectra have been analyzed by a simple fitting procedure, to estimate the relative abundance of three out of the four different species observed: the native 6-cHS, the 6-cLS, and the 5-cHS. The presence of the fourth species makes the data analysis less straightforward at SDS concentrations higher than 3×10^{-3} M (Fig 4, *bottom panel, dashed line*), therefore the absorption data beyond this concentration have not been analyzed. Formation of the 6-cLS heme is observed at the lowest concentrations of SDS, i.e., in the pre-*cac* region. It was suggested that this species derives from the coordination of the distal His (His-64) N_ε atom, which is located on helix E, to the heme iron (van den Oord and Wesdorp, 1969). The crystal structure of both natural (Evans and Brayer, 1990) and recombinant (Maurus et al., 1997) wild-type horse Mb have been determined. In the former structure (1YMB Protein Data Bank code), His-64 N_ε is located at a distance of 4.81 Å from the Fe atom, whereas the distance is 4.41 Å in the latter structure (1WLA). Therefore, the observed His-64 coordination at low SDS concentration requires a slight movement of helix E. This can be a consequence of specific binding of the monomeric surfactant involving also ionic interactions with the protein. We have noticed that helix E contains five Lys residues: Lys-62 and -63, which are adjacent to the distal His-64, and Lys-77, -78, and -79. Lys-77 appears to be H-bonded with Glu-18 of helix A in both crystal structures. Lys-78 and Lys-79 are at H-bonding distances (from Glu-85 of helix F and Asp-4 of helix A, respectively) in the recombinant protein structure, although they are located beyond H-bonding distances in the native protein structure. On the basis of these observations, we propose that Lys salt bridge(s) can be a specific binding site for SDS in a monomeric form. Fig. 5 shows the arrangement of the heme, helix A, and helix E in Mb, based on the native protein structure. Surfactant binding at this site would break the interhelix salt bridge and allow more protein flexibility. The subsequent displacement of helix A would lead to a displacement of the Trp residues away from the heme, and other quenching residues, toward the solvent. This can explain the fluorescence intensity increase and the maximum red shift, respectively, which are observed at pre-*cac* SDS concentrations. A similar effect was

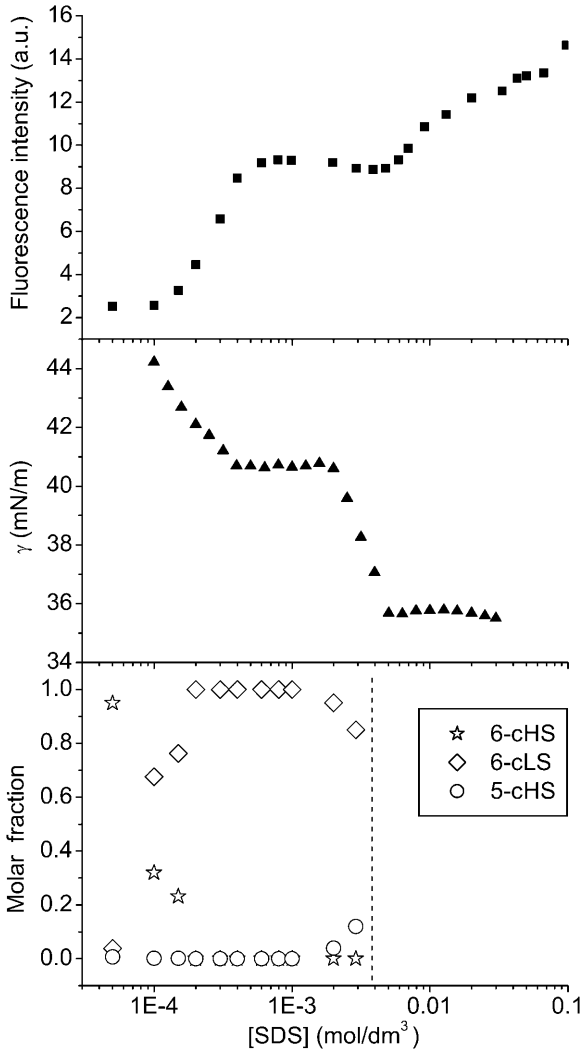

FIGURE 4 Plot of Mb fluorescence intensity versus SDS concentration (*top*). The surface tension of the solution is also reported for comparison (*middle*). Plot of the population of different Mb species, displayed as molar fraction, versus SDS concentration (*bottom*). The plot was obtained from the absorption spectra as described in Experimental Section. The dashed line indicates the region where a simple analysis of the spectra is prevented by the presence of a fourth species (see text).

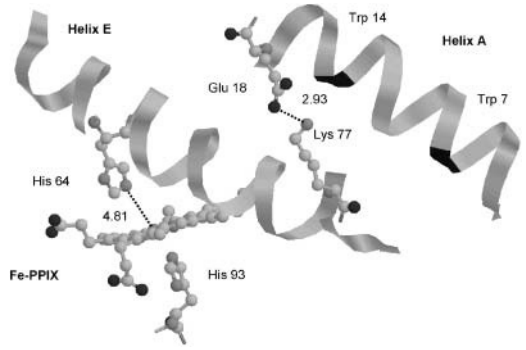

FIGURE 5 A schematic representation of helix E, helix A, Fe-PPIX, and His-93 (which is the fifth iron ligand) in Mb, according to the structural data (1YMB) (see text). The spatial arrangement of the residues His-64, Glu-18, and Lys-77 is shown, together with important interatomic distances (*dotted lines*, Å). The locations of Trp residues in Helix A are shown as black parts of the ribbon.

observed in the guanidine-induced unfolding of apomyoglobin, and it was ascribed to Trp-7 moving away from the quenching residue Lys-79 (Hargrove et al., 1994). The formation of surfactant aggregates does not further perturb the heme or tryptophan environments, because no changes in the absorption and fluorescence spectra are observed in the SDS concentration range from 6×10^{-4} to 10^{-3} M. Interestingly, saturation of the protein with the surfactant leads to a destabilization of helices G and H, to which tyrosine residues belong, as indicated by the enhanced tyrosine fluorescence.

Only surfactant concentrations higher than the *cmc* can induce a major protein unfolding at the heme pocket. The consequent opening of the heme pocket leads to a coordination change of the heme with the formation of the two different high-spin species. In one case the heme is possibly still bound to the protein whereas the other, $>9 \times 10^{-3}$ M SDS, corresponds to a complete detachment of the heme from the protein. This might be facilitated by the breakage of the salt bridges between the heme propionate groups and Lys-45 and His-97.

Myoglobin/cetyltrimethylammonium chloride

Fig. 6 reports the surface tension as a function of CTAC concentration for water, MOPS buffer, and Mb in MOPS buffer. Whereas the curve for CTAC in water shows the usual behavior, with a *cmc* of 1.1×10^{-3} M, the surfactant in MOPS buffer at pH 7.0 shows a markedly different activity. The *cmc* is shifted to 3.2×10^{-4} M and, for a given surfactant concentration, the surface tension is considerably lowered in the buffered system. The slope decrease in the premicellar region indicates, according to Eq. 2, a drop in surfactant adsorption at the interface. This unexpected behavior can only be attributed to the formation of an ionic pair between the surfactant and the anionic component of the buffer, i.e., 4-morpholinepropanesulfonate. This pair has a considerable surface activity, possibly due to electrostatic shielding of the polar headgroups operated by MOPS. The presence of Mb introduces some changes in the surface activity and aggregation of CTAC. The curve shown in the inset of Fig. 6 presents a similar pattern to that of Mb/SDS (Fig. 1, *inset*). However, some major differences can be found. The *cac* occurs at 7.0×10^{-5} M, and, if this value is compared to the *cmc* of the binary system, we obtain an interaction free energy of 3.7 kJ mol^{-1}, lower than that found for SDS. The end of the plateau beyond the *cac* marks the saturation of the protein. According to Eq. 4, this occurs when 15 CTAC molecules are bound to each protein molecule. These observations suggest a somewhat weaker interaction with respect to SDS, in agreement with the general, although poorly explained, observation (Anantha-padmanabhan, 1993) that cationic surfactants are expected to exert smaller perturbations on the protein structure.

Fig. 7 shows the electronic absorption spectra of Mb in MOPS-buffered solution in the presence of CTAC. The addition of small ($<3 \times 10^{-4}$ M) CTAC quantities causes a marked intensity decrease of the Soret band with a blue shift to 405 nm. The CT1 band disappears, whereas three new bands are observed in the Q-band region. The bands with maxima at 530 and 569 nm are typical of a 6cLS species. The band at 604 nm, together with a band at 572 nm, becomes evident upon further addition of CTAC ($\simeq 10^{-3}$ M). At this surfactant concentration the 6cLS form disappears and the Soret band has its maximum at 401 nm. No major changes are observed at higher concentrations of CTAC, except for a slight narrowing of all the bands. The latter spectrum closely resembles that of FePPIX in the presence of CTAC (data not shown), and has the features of the spectra of 5-cHS species. Therefore, this species is the counterpart of that observed in the presence of highly concentrated SDS. The heme group is not coordinated with a histidine residue from the protein, but it contains a weaker

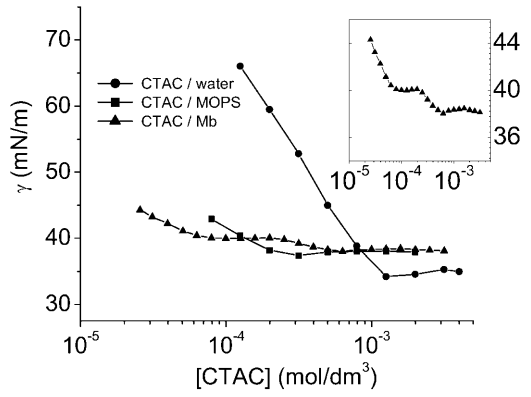

FIGURE 6 Surface tension versus CTAC concentration for CTAC in water solution (●), CTAC in MOPS-buffered solution (■), and CTAC in a MOPS-buffered solution containing 8×10^{-6} M Mb (▲). The inset shows the latter data points on an enlarged scale.

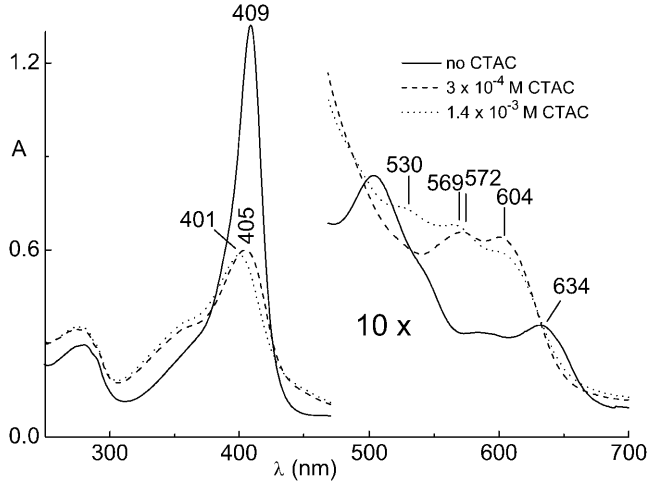

FIGURE 7 Electronic absorption spectra of 8×10^{-6} M Mb in MOPS-buffered solution at increasing CTAC concentrations (see Fig. 7 key).

ligand that might be a hydroxyl group, as in the case of Fe-PPIX in the presence of SDS. It is not clear if the heme chromophore loses all the interactions with the protein matrix and exits the hydrophobic pocket. However, it has been reported that in the presence of a similar cationic detergent, i.e., laurylpyridinium chloride, the prosthetic group can separate from Mb (Blauer et al., 1973; Yonath and Blauer, 1974).

As has been noted for the case of Mb/SDS, by use of a suitable compound the effects of CTAC on Mb can be completely reversed. Such a compound for the Mb/CTAC system was found to be the anionic compound 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline, which does not change the absorption spectra of Mb solutions when added alone, but can completely reverse the spectral changes induced by CTAC (data not shown). Also in this case, the formation of vesicles is suggested by the opalescence of the solution.

Fig. 8 shows the fluorescence spectra of Mb/CTAC solutions at increasing surfactant concentrations. The effect of CTAC addition is a progressive red shift of the fluorescence maximum to 335 nm accompanied by an intensity increase of the emission. Two marked differences are observed when the spectra are compared with those of Mb/SDS solutions. Firstly, the red shift is more pronounced for Mb/CTAC than for Mb/SDS, which indicates that the tryptophan residues are more exposed to the solvent when the protein is unfolded by CTAC. Secondly, the band shape does not change upon CTAC addition. This suggests that only the tryptophan fluorescence is enhanced, whereas the degree of quenching of the tyrosine fluorescence in the presence of CTAC remains as in the native protein.

As in the case of Mb/SDS, the spectral changes induced by CTAC and the corresponding changes in surface tension are correlated (Fig. 9). On the other hand, the overall picture

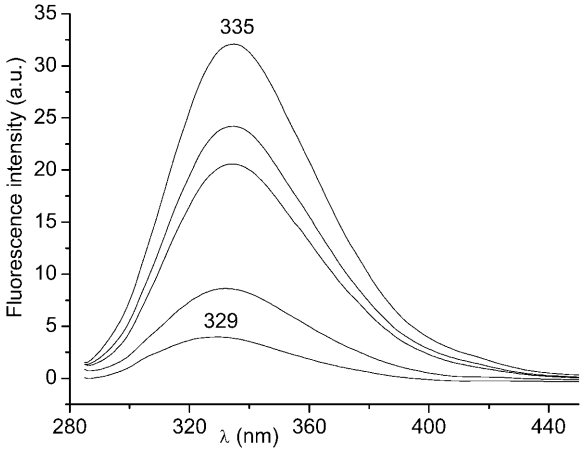

FIGURE 8 Fluorescence emission spectra of 8×10^{-6} M Mb in MOPS-buffered solution without CTAC (*lowest trace*) and at increasing CTAC concentrations: 1×10^{-4} M, 3×10^{-4} M, 9×10^{-4} M, 8×10^{-3} M, in the order of increasing intensity.

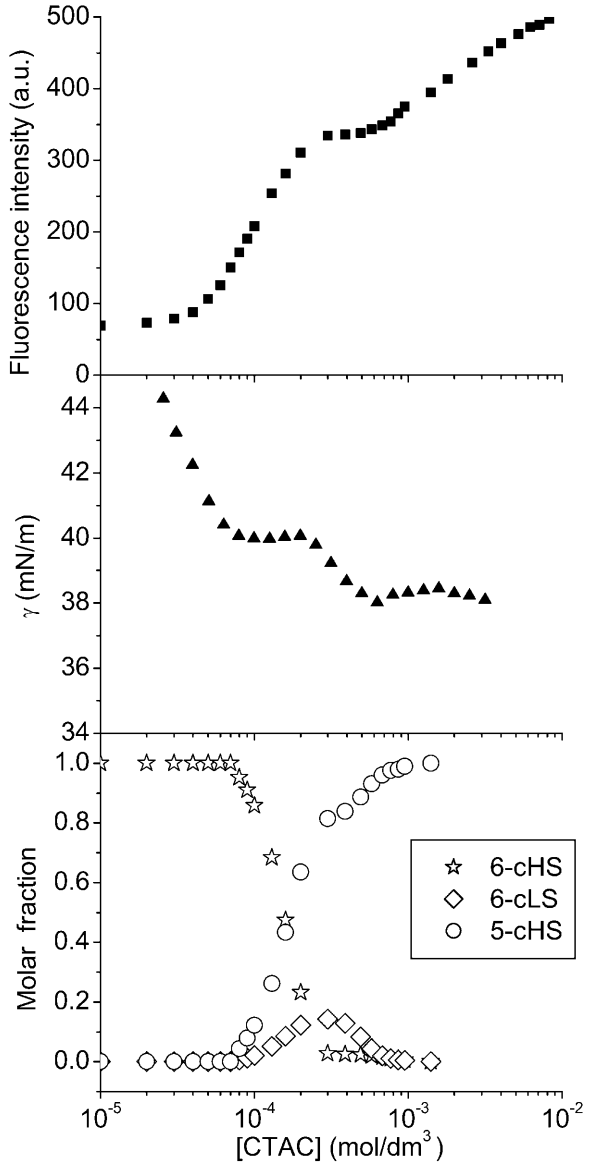

FIGURE 9 Plot of Mb tryptophan fluorescence intensity versus CTAC concentration (*top*). The surface tension of the solution is also reported for comparison (*middle*). Plot of the population of different Mb species, displayed as molar fraction, versus CTAC concentration (*bottom*) The plot was obtained from the absorption spectra as described in Experimental Section.

of the interaction between Mb and CTAC appears to be different when compared to the results obtained for Mb/SDS. The specific interaction, leading to the formation of a 6-cLS species in the complex of Mb with monomeric SDS, is missing. In fact, the lowest plot in Fig. 9 shows that only at concentrations much higher than the *cac* is a measurable amount of 6cLS formed. Although the heme sixth ligand is very likely to be the same as in the case of Mb/SDS, namely, His-64, the displacement of this residue appears to be triggered by the unfolding that accompanies the formation of protein-CTAC aggregates rather than by binding of monomeric surfactant molecules. The reason for this different

behavior could simply reside in the positive charge of CTAC. This might prevent the surfactant from approaching the protein site containing the salt bridges that are ruptured in the formation of the 6cLS (e.g., the Lys-77/Lys-78/Lys-79 triad discussed above) when the protein is in its native state. At higher concentrations of CTAC, the salt bridges in the unfolded protein possibly become accessible to the positively charged surfactant as in the case of SDS. It is also noted that the fluorescence intensity is not fully correlated with the formation of the 6-cLS species induced by CTAC, because the fluorescence slightly increases even below the *cac*. This may be due to a binding site for CTAC, different from that discussed above, which is effective in unshielding one or both of the tryptophan residues while leaving the heme coordination intact. CTAC aggregates appear more effective than CTAC monomers in unfolding the protein, as it is demonstrated by the steep fluorescence increase above the *cac*, and by the dramatic decrease in slope at the T_2' point. Micelle formation produces an apparent increase of the unfolding process, indicated by the large fluorescence increase above the *cmc*. A further difference between the Mb/SDS and Mb/CTAC complexes is that detachment of heme from the proximal histidine occurs at surfactant concentrations slightly above the *cac* of CTAC, whereas the effective SDS concentrations for this process are higher than the plateau following the *cac*. An electrostatic interaction between the ammonium group of CTAC and the negatively charged heme propionate groups may favor interaction between heme and CTAC, leading to heme dissociation from the protein at relatively low surfactant concentrations.

Myoglobin/decyltrimethylammonium bromide

Fig. 10 shows the surface tension plots for the system DeTAB in water, DeTAB in MOPS buffer, and DeTAB/Mb in MOPS buffer, as a function of surfactant concentration.

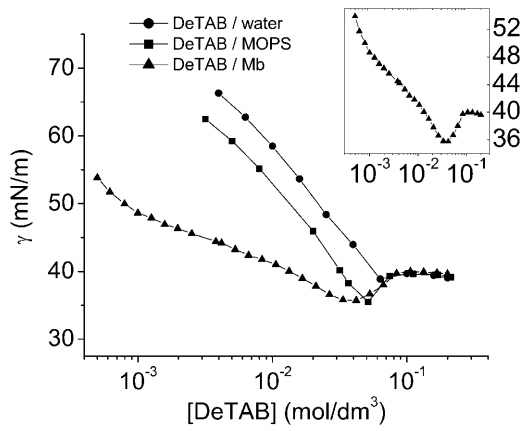

FIGURE 10 Surface tension versus DeTAB concentration for DeTAB in water solution (●), DeTAB in MOPS-buffered solution (■), and DeTAB in a MOPS-buffered solution containing 8×10^{-6} M Mb (▲). The inset shows the latter data points on an enlarged scale.

The *cmc* in water is found to be 60 mM, in good agreement with the literature value (Sams et al., 1972). The MOPS-buffered medium induces a slight shift of the *cmc* value to lower concentrations, together with the appearance of a minimum in the curve, which is also present in the curve of the protein-containing solution. The presence of a minimum is commonly attributed to surface-active impurities (see ''Myoglobin/sodium dodecyl sulfate'' section). In fact, surface tension curves of DeTAB in water before recrystallization from acetone also displayed a minimum (data not shown). In this case the source of these impurities is not easily recognizable. The comparison with surface tension results obtained for the other systems, in particular CTAC, rules out that amphiphilic impurities are due either to MOPS or to protein preparation. This peculiar behavior is therefore not ascribable to the effect of a single component (DeTAB, MOPS, or the protein) and should rather be interpreted in terms of a cooperative effect. However a detailed explanation would require further investigation that is beyond the aims of this article.

It is clear from a comparison of Figs. 6 and 10 that there are considerable differences between the behavior of CTAC and DeTAB solutions. The presence of MOPS has a dramatic effect on CTAC adsorption at the interface, suggesting a close interaction of the cationic surfactant with the MOPS anion, whereas for DeTAB the slope of the surface tension curve in the premicellar region is practically coincidental for water and MOPS-buffered solutions, indicating a scarce, if any, MOPS effect. Two factors might be contributing to such a different behavior: the shorter length of the alkyl chain (10 vs. 16 C atoms) and the different counterion (Br^- vs. Cl^-). A possible explanation for the poor affinity displayed by DeTAB for the MOPS anion is directly linked to the presence of a bromide counterion. In fact, the affinity of anions toward cationic micellar aggregates, that is the degree of counterion binding, has been found to follow the Hofmeister series (Leontidis, 2002). Several examples can be used to illustrate this feature: the *cmc* of CTAC in water is higher than the *cmc* of cetyltrimethylammonium bromide (1.4 mM and 0.82 mM, respectively; van Os et al., 1993), and the degree of counterion binding to micellar aggregates is considerably higher for bromide derivatives (Berr et al., 1992). This means that micelles formed by surfactants with bromide as a counterion have a lower interfacial charge. We can then suppose that the exchange of the bromide with MOPS anion is prevented by a strong competition of the bromide counterion. If we consider the surface tension curve in the presence of Mb, we do not observe the characteristic trend noted previously for SDS and CTAC (Figs. 1 and 6). Some slope change in the premicellar region can be detected by a closer inspection, but the presence of inflection points cannot be reliably assessed with surface tensiometry.

The electronic absorption spectra of Mb solutions at increasing DeTAB concentrations display an effect of the surfactant only at relatively high concentrations, i.e.,

$>2 \times 10^{-2}$ M (data not shown). Two species are formed with spectral features very similar to the 6-cLS and the high-spin species observed for Mb/CTAC. The 6-cLS species is more abundant than in the case of Mb/CTAC.

Fluorescence spectra of Mb/DeTAB (data not shown) also display features similar to those of the Mb/CTAC spectra. The emission maximum is shifted from 329 nm to 335 nm with an intensity increase upon DeTAB addition, indicating the exposure of the tryptophan residues to the solvent when the protein unfolds.

Because the surface tension curve lacks the characteristic features observed for SDS and CTAC, an interpretation of the nature of the interaction between DeTAB and Mb is not possible. It can be reasonably excluded, though, that monomeric DeTAB is active in inducing the formation of 6-cLS Mb, owing to the high surfactant concentrations required for this effect. The mechanism of action is possibly similar to that of CTAC, involving surfactant aggregation on the protein.

CONCLUSIONS

All examined Mb/surfactant complexes display multiple interaction modes, giving rise to conformational changes that can be interpreted in terms of different degrees of protein unfolding. The combination of spectroscopy and tensiometry helps in highlighting important aspects of Mb/surfactant interactions. Optical spectroscopy measures the response of the protein at important sites—the heme pocket, the tryptophan residues, the tyrosine residues—to increasing surfactant concentrations. Surface tension detects the adsorption of the surfactant at the air-water interface and surfactant binding to the protein.

The formation of a heme 6-cLS species through distal histidine (His-64) binding is common to the three surfactants. The mechanism for this coordination change, though, is not the same for SDS and for the cationic surfactants. SDS can act even in monomeric form through a specific mechanism—possibly breaking important salt bridges—which makes helix E more flexible. In the presence of CTAC, the distal histidine binds Fe only when the surfactant builds aggregates on the protein. DeTAB possibly acts in a similar way.

Fluorescence spectral changes accompany surface tension changes, indicating that the unfolding processes at the fluorophore sites are closely correlated with the surfactant activity. SDS and CTAC can both increase tryptophan fluorescence at monomeric concentrations, indicating a perturbation of helix A where both tryptophan residues are located. This interaction is also different for the two surfactants. CTAC can change the tryptophan environment leaving the heme coordination intact, whereas SDS affects both tryptophan and heme, which indicates that the perturbations induced by SDS on helix A and helix E are coupled. Moreover, SDS induces a strong change in the fluorescence band shape at concentrations above the *cac*, indicating a major rearrangement of the protein portion (helices G and H) where the tyrosine residues are located. This effect on tyrosine fluorescence does not occur in the presence of the cationic surfactants.

All the investigated Mb/surfactant complexes have in common the formation of pentacoordinated heme complexes, which are likely due to the detachment of heme from the protein and solvation into aggregates (in the CTAC case) or micelles (in the SDS case).

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