Anesthetic-Bacteriorhodopsin Interaction; Alcohol-Induced Biphasic Effects

Akira SHIBATA,*[,a] Shinobu KAWANAMI,[a] Kazumichi USHIO,[b] Satoru UENO,[a] and Takuya YAMASHITA[a]

Faculty of Pharmaceutical Sciences, The University of Tokushima,[a] Shomachi, Tokushima 770, and Itami Research Institute, Senju Pharmaceutical Co., Ltd.,[b] Itami 664, Japan. Received May 11, 1995; accepted July 24, 1995

The purple membrane is an appropriate model system for investigating the effects of anesthetics on the structures and functions of excitable cell membranes. The effects of anesthetically active alcohols (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol) on the structure of bacteriorhodopsin (bR) in the purple membrane were investigated by circular dichroism (CD) and absorbance measurements. These alcohols increased the 530-nm ellipticity of bR at low concentrations of the alcohols and decreased it at high concentrations. The addition of alcohol at a low concentration in the purple membrane aqueous suspension tended to favor the more folded-compact conformation of the bR by the enhancement of hydrophobic interaction. Alcohols at high concentrations caused the cooperative denaturation of bR due to the access of alcohol to the retinal chromophore. Our attention is mainly focused on the finding that alcohols at low concentrations induce a more folded conformation of bR.

Key words bacteriorhodopsin; anesthetic; 1-alcohol; hydrophobic interaction; circular dichroism

Anesthetics exert their influence in nerve cell membranes by interfering with nerve impulse conduction and transmission.[1,2] It has been a matter of controversy whether lipids or proteins constituting the nerve cell membrane are the primary action site of general anesthetics. The lipid solubility theory assumes that the membrane fluidizing and disordering effect by anesthetics leads to the constriction of the electrogenetic ion channels and then induces anesthesia.[3,4] Despite the abundance of reports on anesthetic-lipid interaction, those on the anesthetic-protein interaction are few. Protein theories of anesthesia have been divided among one implicating the presence of specific binding sites for uncharged anesthetics and another suggesting a nonspecific conformational change in the proteins.[5] It is advocated in the protein theory that anesthetics interact with the hydrophobic interior of the proteins and partially unfold them. Thus, a portion of the hydrophobic side chain in the protein interior would be exposed to water and undergo hydrophobic hydration by the formation of clathrates around the nonpolar side chains of amino acids in the proteins.

The 1-alcohols are the best-characterized samples of the homologous series which display cutoffs in anesthetic potency.[6,7] Studies on the effects of alcohols shorter than four or five carbon atoms on the gel-to-liquid crystalline phase transition of dipalmitoylphosphatidylcholine (DPPC) indicated that two main effects were observed[8,9]: that is, the main phase transition temperature of the lipid is depressed at low concentrations of alcohol but increased at high concentrations. It was subsequently shown by Simon and McIntosh[9] that this biphasic behavior induced by alcohols is a consequence of acyl chain interdigitation from opposing monolayers in the phospholipid bilayer structure.

The structural stability of globular proteins in aqueous solution in the presence of alcohols has also shown biphasic behavior.[10,11] The presence of alcohols in the low concentration range tends to stabilize and protect the protein structure against denaturation by denaturants, heat or low pH.[12] On the other hand, at a relatively high concentration of alcohols the protein is denatured, and its denaturation is characterized by an increase in the levorotation and in the viscosity of the protein solution.

We reported the effects of short chain alcohols (methanol, ethanol, propanol) on the secondary structure of poly(L-lysine), where these alcohols promoted the β-sheet conformation at low concentrations, but they supported the α-helix conformation when the concentrations exceeded the pharmacological level.[13]

We contend that anesthetics affect lipid membranes and proteins in nerve cell membranes indiscriminately, and all macromolecular structures are perturbed. In this study we have used bacteriorhodopsin (bR) in purple membrane fragments, with the excitable membrane proteins embedded in the lipid bilayer, isolated from Halobacterium halobium. An important characteristic of the biological function of the purple membrane is that it possesses a light-driven proton pump activity which is essential to the ATP synthesis process of this bacterium.[14] The purple membrane is made up of 25% of its dry weight by lipids and 75% by a single protein, bR, with a retinal chromophore bound in a 1 : 1 molar ratio with a Schiff-base.[15,16] The bR molecules are organized into a two-dimensional hexagonal lattice of trimers in the bilayer membranes.[17] Therefore, the purple membrane is an appropriate model system for investigating the effects of anesthetics on the structure and function of excitable cell membranes.

The objective of this study is to examine the effect of short-chain alcohols on the optical rotation and the absorbance of the purple membrane in the visible spectrum, and also to see how the ability of an alcohol to enhance hydrophobic interaction in the purple membrane suspension varies with alcohol chain length. In a recent study, it was suggested that anesthetic potency was linearly related to the ability to enhance a hydrophobic interaction in a protein solution.[18]

Materials and Methods

Purple membranes of Halobacterium halobium, strain S-9, were pre-

* To whom correspondence should be addressed.

pared according to the method of Oesterhelt and Stoeckenius.[15] After sucrose-gradient centrifugation, the membranes were washed three times with water and stored at 4 °C. Before use, the purple membrane fragments were suspended in 10 mM phosphate buffer (pH 7.4). The purple membrane preparations were incubated with a chosen alcohol for 20 h in the dark at 22 °C with stirring. The final bR concentration was 15.5 μM.

Circular dichroism (CD) was recorded with a JASCO J-600 spectrometer (Tokyo), equipped with a data processor, under a constant nitrogen flush. The instruments were calibrated with *d*-10-camphorsulfonic acid. Observed ellipticity (Θ_{obs}) was converted to molecular (or mean residue) ellipticity ($[\Theta]$) by the follow ing equation: $[\Theta]=100\times\Theta_{obs}/LC$, where C is the molarity of the bR and L is the cuvette light path length in centimeters. Two sets of measurements in the far-UV (200—250 nm) and the visible CD spectral regions (300—600 nm) were carried out by changing the wavelength and parameters of the apparatus.

Absorption spectra were recorded on a JASCO Ubest-30 spectrometer. A Teflon-stoppered cuvette with a light path length of 1.0 or 10 mm was used.

Results

The retinal chromophores bound covalently by means of a protonated Schiff base to the apoprotein in the purple membrane are dissymmetrically arranged with respect to each other.[19,20] The CD is very sensitive to subtle changes in the protein-induced screw sense of the chromophore arrangement and the local environments of the protein aromatic residues.

Figure 1 shows the effect of ethanol on the CD spectra of bR in the purple membrane in the 300 to 600 nm region at various concentrations of ethanol. Because of the gradual change in the ellipticity of bR by ethanol, an incubation period of 20 h was chosen. The control spectrum in the absence of ethanol exhibited bilobed bands with opposite signs together with a large negative CD band at 317 nm. The bilobed CD band, centered at *ca.* 568 nm, has been attributed to the sum of at least two components[21]: (1) a single positive Gaussian CD band resulting from asymmetric interactions between the retinal chromophores and the apoproteins. This CD band is often referred to as the monomer CD; and (2) an exciton band with a positive short wavelength lobe and an equally long wavelength lobe, which is due to the interaction between retinal chromophores on adjacent bR molecules in the purple membrane. The 317-nm negative CD band has been suggested to arise from π–π^* retinal transition.[19] The spectral feature implies that the CD spectrum of the retinal chromophore in the bR active site is very much controlled by its protein as well as by the lipid environment. The 530-nm ellipticity, as a measure of change in the protein conformation around the retinal chromophore, increases with the concentration of ethanol, reaching a maximum (C_{max}) (trace 3 in Fig. 1), then decreasing at higher concentrations, accompanied by a blue shift, and the band finally disappears. The biphasic nature of the ellipticity was also observed for the other short-chain alcohols used, methanol, 1-propanol, 1-butanol. 1-pentanol, and 1-hexanol. In the 595 and 317-nm negative CD bands, however, the presence of a lower concentration of alcohols caused no change in the ellipticity, and at higher concentrations their band intensities decreased monotonically.

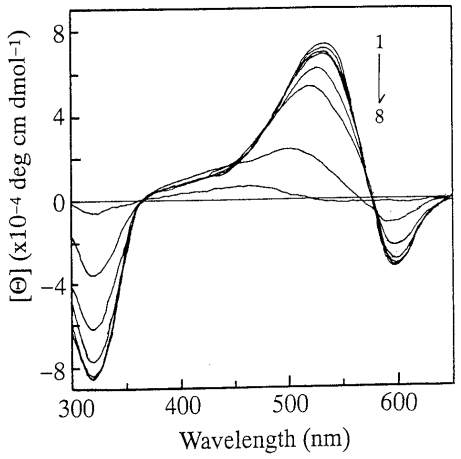

Fig. 1. Effect of Ethanol on the Visible CD Spectra of bR in the Purple Membrane in the 300—600 nm Region at Various Concentrations of Ethanol

The numbers 1—8 indicate the order of traces of CD spectra. 1, 0.45 M; 2, 0.28 M; 3, 0.12 M; 4, control; 5, 3.0 M; 6, 4.0 M; 7, 5.0 M; 8, 7.0 M.

At the alcohol concentrations which lead to the maximum 530-nm ellipticity, C_{max}, the effects of alcohols on an increase and a decrease in the protein ellipticity are equal. The values of C_{max} were plotted as $\ln C_{max}$ against the alcohol chain length in Fig. 2. The values of $\ln C_{max}$ decreased linearly with increasing chain length, indicative of the enhancing of the hydrophobic effect.

The bR CD band at 530 nm is analyzed by separating the data into those above and below the C_{max}. Figure 3 shows the 530-nm ellipticities of bR treated with a concentration below C_{max}, as a function of the concentrations for methanol, ethanol, propanol, 1-butanol, 1-pentanol, and 1-hexanol. These alcohols linearly increased the 530-nm ellipticity of bR, with an opposite direction in sign to the unfolding of bR, for all of the alcohols.

For the purple membrane suspension in the presence of alcohol at a low concentration, the initial rate of change in the optical rotation, $\ln(d[\Theta]/dC_{ad})_{c\to 0}$, in Fig. 3 is the appropriate term to represent enhancing of the hydrophobic interaction (see Discussion and Appendix for details). Figure 4 shows the results plotted as $\ln(d[\Theta]/dC_{ad})_{c\to 0}$ against the alcohol chain length. The relation between the hydrophobicity parameter and the alcohol carbon chain length was linear ($r^2=0.996$). The slope is comparable to the standard free energy change per CH_2 for the transfer of alcohols from an aqueous solution to a hydrophobic environment.

Figure 5 shows visible absorption (a) and CD (b) spectra

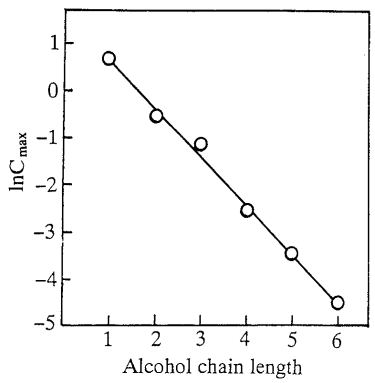

Fig. 2. Relationship between the Alcohol Concentrations Which Lead to the Maximum 530-nm Ellipticity of bR, C_{max}, and the Alkyl Chain Length of 1-Alcohols

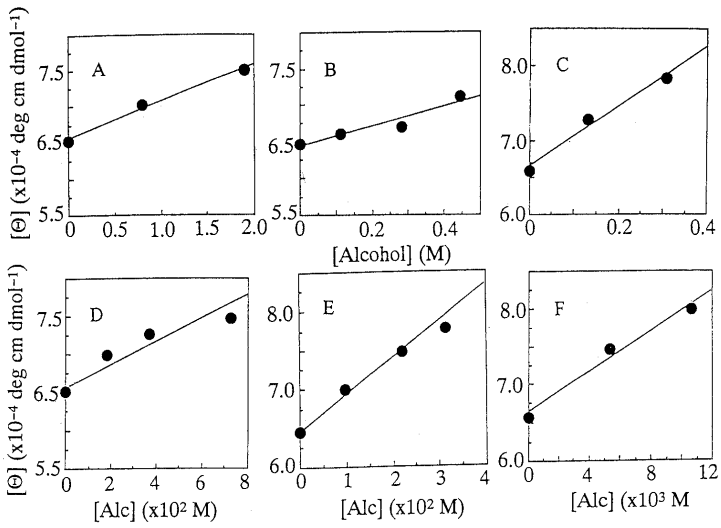

Fig. 3. The 530-nm Ellipticities of bR Treated with a Low Alcohol Concentration, Below C_{max}, as a Function of the Concentrations for Methanol (A), Ethanol (B), 1-Propanol (C), 1-Butanol (D), 1-Pentanol (E), and 1-Hexanol (F)

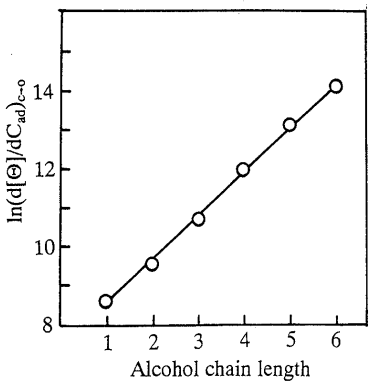

Fig. 4. Ability to Increase the 530-nm Ellipticity of bR as a Function of the Alkyl Chain Length of 1-Alcohols

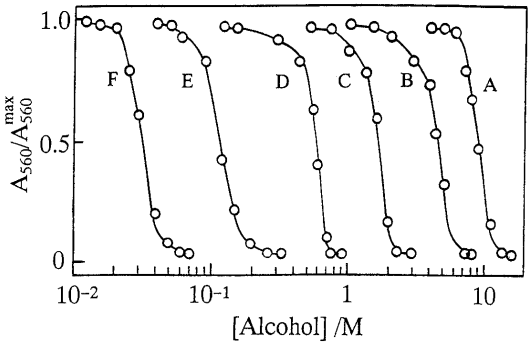

Fig. 6. Denaturation Curves for bR in the Purple Membrane Obtained with a Series of 1-Alcohols

The ordinate indicates the absorbance ratio of bR at 560 nm in the presence and absence of 1-alcohols. A, methanol; B, ethanol; C, 1-propanol; D, 1-butanol; E, 1-pentanol; F, 1-hexanol.

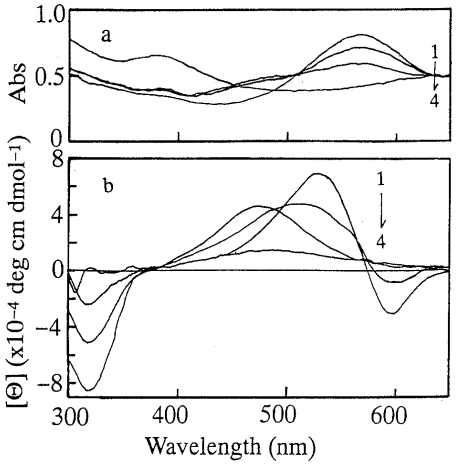

Fig. 5. Visible Absorption (a) and CD (b) Spectra of bR in the Presence of 1-Hexanol Concentrations above C_{max}

1, control; 2, 30 mM; 3, 40 mM; 4, 60 mM.

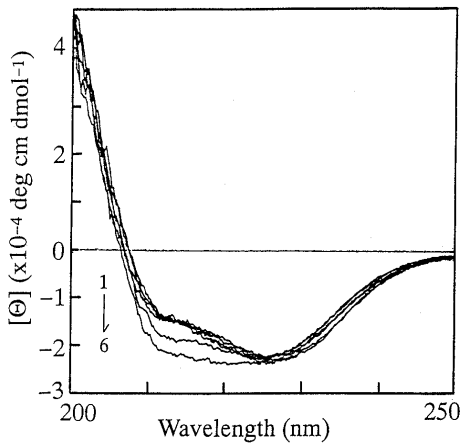

Fig. 7. UV CD Spectra of bR in the 200—250 nm Region at Various Concentrations of 1-Pentanol

1, control; 2, 30 mM; 3, 60 mM; 4, 120 mM; 5, 200 mM; 6, 250 mM.

of bR in the purple membrane in the presence of 1-hexanol above C_{max} (0, 30, 40, and 60 mM). The native bR gives rise to an absorption band at 560 nm due to strong π–π^* retinal transition. The addition of 1-hexanol shifted the absorption band from 560 to 380 through an intermediate band of 510 nm (isosbestic point) with the change in band intensity. 1-Hexanol above 30 mM resulted in blue shifts of the bilobed bands with opposite signs, whose zero amplitude coincided with the maximum of the absorption spectrum of the retinal chromophore in bR, accompanying a lowering of the band intensities, while the 317-nm bands showed only a lowering of their band intensities without

the band shift. The blue shift and the change in the band shapes of the 560-nm absorption and the 530-nm CD spectra are considered to be due to a change in retinal environment of bR involving denaturation of the purple membrane (PM).

Figure 6 shows denaturation curves for the PM obtained with alcohols. The absorbance at 560 nm is plotted against the concentration of the alcohols. The alcohol concentration required to denature the PM is readily estimated from the sigmoidal curve in the absorbance: 8.8 M for methanol, 4.5 M for ethanol, 1.7 M for 1-propanol, 6.1×10^{-1} M for 1-butanol, 1.2×10^{-1} M for 1-pentanol, and 3.3×10^{-2} M for 1-hexanol.

Figure 7 shows the UV CD spectra of bR in the region of 200—250 nm at various concentrations of 1-pentanol. The CD spectrum of bR in the absence of 1-pentanol is consistent with a model consisting of α-helices. Even the 1-pentanol concentration (120 mM) required to cause the bR denaturation did not change the band shape of the CD spectra. This implies that the denatured bR molecules also retain the α-helix structure in analogy with the native bR. However, the shapes of the CD bands of bR in the presence of ethanol above 200 mM differ noticeably from those for the native bR in aqueous solution.

Discussion

The effects of amphiphiles and nonpolar additives on proteins in an aqueous solution are often biphasic and vary between high and low concentration ranges. The effects of alcohols on the conformation of ribonuclease,[22] human serum albumin,[18] human transferrin[18] and lysozyme[18] are biphasic regarding the change in optical rotation of the proteins. Several nonplar gases (methane, ethane, propane, butane, and isobutane) shift the conformation of β-lactoglobulin and bovine serum albumin in aqueous solutions toward a more compact or folded form.[23] For proteins in aqueous solution in the presence of small amounts of an amphiphile, the shift of the optical rotation in the visible CD spectrum to an opposite direction relative to protein denaturation could be interpreted as due to the enhancement of the hydrophobic interaction in the protein solution.[11]

In this study, the effects of anesthetically active alcohols on the ellipticity of visible CD spectra of the purple membrane varied between high and low concentration ranges (Fig. 1). Thus far, the biphasic effects by short-chain alcohols have been found independently for the main phase transition of lipid bilayer membranes[8,9] and the conformation of globular proteins[10,11]; therefore, it is noteworthy that such a biphasic effect by short-chain alcohols was observed for bR in the purple membrane as a protein-lipid complex. Alcohols at a low concentation shifted the optical rotation of bR at 530 nm to an opposite direction relative to its denaturation, and the values of $\ln(d[\Theta]/dC_{ad})_{c\to 0}$ increased linearly with an increase in alcohol chain length (Fig. 4), indicative of the enhancement of the hydrophobic interaction in the bR protein. The seven α-helix rods of bR in the purple membrane are assembled by eight coiled connections.[24] These coils are composed of 70% hydrophilic residues and 30% hydrophobic residues. The enhancement of the hydrophobic effect involves the coaggregation of alcohol with the hydrophobic residues in the bR coiled portion.

The concept of conformation of the protein molecule is generally used, such as folded or unfolded conformation. When a protein is completely unfolded, all the nonpolar side chains of the protein are projecting out in the aqueous phase. It can be assumed that, for a native protein in a aqueous solution, there is an equilibrium between the nonpolar side chains, which are intramolecularly associated in the interior of the protein molecules, and those which are dissolved in the aqueous phase. Therefore, for the bR in aqueous solution in the presence of an alcohol at a low concentration, if the equilibrium is shifted toward the folded conformation and then a decrease occurs in the number of nonpolar side chains projecting in the water, enhancement of the hydrophobic interaction will be indicated as mentioned above. This process is thermodynamically unfavorable because of an increase in the chemical potential of bR. Because this effect is nonspecific and, as a first approximation, is distributed over the entire protein-solvent interface, any reduction in the protein-solvent interface would be thermodynamically favorable. As a result, the presence of an alcohol would tend to favor the more folded-compact conformation of the bR. Such a deformation of the bR conformation could result in a subtle change in the local environments of the retinal chromophore with the Schiff base linkage at Lys^{216} in bacterioopsin. Balasubramanian and Wetlaufer[25] showed, by following the changes in the optical rotation of a globular protein solution in the visible spectrum, that the anesthetic potency of various anesthetics (halothane, chloroform, diethyl ether, and propane), the reciprocal of the partial pressure of the anesthetic agent necessary to anesthetize 50% of the subjects, is linearly related to its ability to enhance the hydrophobic interaction of the protein solution. Assuming a similarity of the process of the binding of alcohols at the bR-water interface in the purple membrane and at the protein-water interface in the nerve cell membrane, the action of general anesthetics might be correlated more to the folding of protein nonpolar side chains rather than an unfolding.

When alcohols at high concentrations were added to the purple membrane suspension, the visible absorption and CD bands of bR showed a blue shift accompanying the lowering of their intensities, indicative of the cooperative denaturation of bR (Figs. 5 and 6).[26] These findings can be explained by assuming that the binding of alcohols increases the protein mobility in the membrane and leads to loss of the regularity of the arrangement of the chromophoric molecules in the membrane space lattice due to the excessive enhancement of hydrophobic interactions. Further addition of alcohol would lead to a penetration of alcohol into the bilayer membrane and finally to denaturation of bR due to access of the alcohol to the chromophore.

The CD spectrum between the 200 and 250 nm region provides information on the secondary structure of bR. The bR molecules denatured by alcohol retain the α-helix structure in analogy with the native bR. However, bR with alcohol at a much higher concentration appears to be in a conformation different from that of the native bR in

aqueous solution (Fig. 7). The addition of methanol to random-coil poly(L-lysine) at neutral pH transformed it into an α-helix when the methanol concentration reached 87—90 vol%.[27] When sodium octyl sulfate was added to random-coil poly(L-lysine), the α-helical conformation dominated at a surfactant concentration between 4 and 6 mM, but a β-sheet appeared above this surfactant concentration.[28] At higher alcohol concentrations, a change in the solvent property around the purple membrane can be expected. This would rearrange the interaction among water, alcohol, and bR molecules. These alcohol actions on the bR structure are essentially solvent effects.

Conclusion

The effects of anesthetically active alcohols (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol) on the structure of bR in the purple membrane were investigated by CD and absorbance measurements. We found biphasic effects of alcohols on the ellipticity of visible CD spectra of the purple membrane. These alcohols at low concentrations shifted the ellipticity of bR at 530 nm to an opposite direction relative to its denaturation, and the values of $\ln(\mathrm{d}[\Theta]/\mathrm{d}C_{\mathrm{add}})_{c\to 0}$ increased linearly with an increase in alcohol chain length, indicative of the enhancement of the hydrophobic interaction in the bR protein. The presence of alcohols at low concentrations, below $C_{\max}$ in the purple membrane aqueous suspension, would tend to favor the more folded-compact conformation of the bR by enhancement of the hydrophobic interaction. Such a deformation of the bR conformation could result in a subtle change in the local environments of the retinal chromophore with the Schiff base linkage at Lys^{216} in bacterioopsin. Decreases in the 530-nm ellipticities of bR in the presence of alcohols at high concentrations above $C_{\max}$ are due to the cooperative denaturation of bR.

The present results suggest that alcohol-induced anesthesia is correlated to the occurrence of a more folded conformation of membrane protein at low concentrations, below $C_{\max}$, rather than an unfolded one at high concentrations above $C_{\max}$.

Appendix

It is well established that the addition of small amounts of amphiphile (additive) to a micellar solution of surfactant causes the enhancement of the hydrophobic interaction.[29] The cmc lowering ability of an additive is explicitly linked to the distribution coefficient (K) between the micelles and the surrounding aqueous phase[30]:

$$[-\mathrm{d}(X)/\mathrm{d}C_{\mathrm{ad}}]_{c\to 0}\times 1/X_0 = aK \qquad (1)$$

where X and X_0 are the cmc (in mole fraction) in the presence and absence of the additive, C_{ad} is the mole fraction concentration of the additive, and a is a constant. Thus, $\ln(-\mathrm{d}(X)/\mathrm{d}C_{\mathrm{ad}})_{c\to 0}$ is proportional to $\ln K$ and hence to $-\Delta G_0$, the standard free energy of association of the additive with the surfactant. The term $\ln[-\mathrm{d}(X)/\mathrm{d}C_{\mathrm{ad}}]_{c\to 0}$ is appropriate to represent the enhancement of the hydrophobic interaction.

In an analogous manner, assuming that for a protein solution in the presence of an additive there is an equilibrium between the nonpolar side chains associated in the protein interior and those exposed to the aqueous phase, the relationship between the ability of the additive to increase optical rotation in the protein solution and the distribution constant (K') between the proteins and the aqueous phase is expressed as[18]:

$$[\mathrm{d}\Theta/\mathrm{d}C_{\mathrm{ad}}]_{c\to 0}\times 1/\Theta_0 = bK' \qquad (2)$$

where Θ and Θ_0 are the ellipticity of the protein in the presence and absence of an additive and b is a constant.

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