Eur. J. Biochem. *169*, 275–282 (1987)


# Spectroscopic properties of horse liver alcohol dehydrogenase in reversed micellar solutions

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(Received May 18/July 15, 1987) – EJB 87 0568

Catalytic and spectroscopic properties of alcohol dehydrogenase from horse liver, incorporated in reversed micellar media, have been studied. Two different reversed micellar systems have been used, one containing an anionic [sodium bis(2-ethylhexyl)sulfosuccinate, AOT], the other containing a cationic (cetyltrimethylammonium bromide, CTAB) surfactant.

With 1-hexanol as substrate the turnover number of the enzyme in AOT-reversed micelles is strongly dependent on the water content of the system. At low $w_o$ ($[H_2O]/[surfactant]$) ($w_o < 20$) no enzymatic activity can be detected, whereas at high $w_o$ ($w_o = 40$) the turnover is only slightly lower than in aqueous solution. In CTAB-reversed micelles the dependence of the turnover number on $w_o$ is much less. The enzymatic activity is in this case significantly lower than in aqueous solution and increases only slightly with an increasing water content of the reversed micelles.

Possible interactions of the protein with the surfactant interfaces in the reversed micellar media were studied via circular dichroism and fluorescence measurements. From the circular dichroism of the protein backbone it is observed that the protein secondary structure is not significantly affected upon incorporation in the reversed micelles since the far-ultraviolet spectrum is not altered.

Results from time-resolved fluorescence anisotropy experiments indicate that, especially in AOT-reversed micelles, interactions between the protein and the surfactant interface are largely electrostatic in nature, as evident from the dependence on the pH of the buffer used. In CTAB-reversed micellar solutions such interactions appear to be much less pronounced than in AOT.

Solubilization of biopolymers and especially of proteins in hydrocarbon media, via surfactant assemblies, called reversed micelles, has been extensively investigated during the last decade [1–7]. Structural information about these systems can be obtained via spectroscopic techniques, since reversed micelles form thermodynamically stable and optically transparent solutions. In two previous publications we have described some spectroscopic properties of fluorescent cytochrome *c* derivatives (from horse heart), incorporated in anionic- and cationic-reversed micelles [8, 9]. From these studies it became clear that electrostatic interactions between protein and surfactant interface are of considerable importance to the structure and dynamics of the encased cytochrome *c* molecules.

In this article, catalytic properties, fluorescence and circular dichroism results are presented of the well characterized protein alcohol dehydrogenase from horse liver (LADH) [10, 11], incorporated in reversed micelles, consisting of anionic sodium bis(2-ethylhexyl)sulfosuccinate (AOT) or cationic cetyltrimethylammonium bromide (CTAB) in isooctane. LADH consists of two identical subunits and has a relative molecular mass of 80000. Each subunit contains two tryptophan residues, which are very suitable intrinsic fluorescence probes for this study. From X-ray analysis [11] it is known that one tryptophan (Trp-15) is located near the surface of the protein, whereas the other (Trp-314) is buried inside the protein coil in a hydrophobic region near the subunit interface. The time-resolved fluorescence properties of the tryptophans in LADH have been described in a number of articles [12–16].

Incorporation of LADH in AOT-reversed micelles has been performed before by Meier and Luisi [17], and by Martinek et al. [18]. The group of Biellmann has recently reported on LADH catalysis in four-component reversed micellar systems with sodium dodecyl sulphate and cetyltrimethylammonium bromide as surfactants [19]. From the above-mentioned studies it was found that the enzymatic activity of the protein is preserved in the reversed micellar solutions and that in such systems both water-soluble and hydrocarbon-soluble substrates can be converted.

The aim of the present study is to obtain information about the structural and catalytic aspects of LADH incorporation in reversed micellar systems. In order to obtain information about charge effects, the spectroscopic properties of LADH incorporated in reversed micellar media have been studied with the protein dissolved in buffers of different pH (i.e. pH 8.8 and pH 7.0). The isoelectric point of the protein is about 8.7 [11], which means that at pH 8.8 the overall charge

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*Abbreviations.* AOT, Aerosol-OT [sodium bis(2-ethylhexyl)-sulfosuccinate]; CD, circular dichroism; CTAB, cetyltrimethyl ammonium bromide; LADH, horse liver alcohol dehydrogenase.

*Enzyme.* Horse liver alcohol dehydrogenase (EC 1.1.1.1).

of the protein is almost zero, whereas at pH 7.0 there is an overall positive charge. Of course, the effective pH of the water pools of the reversed micelles is not known, but it can be expected that at pH 8.8 the protein is less positively charged than at pH 7.0. If electrostatic interactions between protein and surfactant interface influence the spectroscopic properties of LADH, differences between these two systems arising from pH variation are expected to be observed.

Detailed information in this study has been obtained with the time-resolved fluorescence depolarization technique, which is a very sensitive method to observe changes in the environment of the fluorescent probes [20–22]. The rotational dynamics of the LADH molecule in reversed micellar systems could be related to effects of pH, $w_o$ and surfactant.

## MATERIALS AND METHODS

### *Chemicals*

LADH was obtained from Boehringer as a crystalline suspension in 20 mM potassium phosphate, 10% ethanol, pH 7.0. The crystals were centrifuged, dissolved in 20 mM sodium pyrophosphate/HCl, pH 8.8 or 20 mM potassium phosphate, pH 7.0 and dialyzed against the same buffer at 4°C for 24 h. The enzyme concentration was determined spectrophotometrically ($\varepsilon_{280\ nm} = 3.53 \times 10^4\ M^{-1}\ cm^{-1}$) [10]. The content of active sites was determined via a titration with NADH in the presence of 0.1 M isobutyramide [23] and amounted to about 95%. $NAD^+$ and NADH (both grade II) were purchased from Boehringer. AOT [sodium-bis(2-ethylhexyl)sulfosuccinate] was from Janssen Chimica and was purified according to the method of Menger and Yamada [24]. CTAB (cetyltrimethylammonium bromide; Serva) was used without further purification. Both surfactants were desiccated over $P_2O_5$. *p*-Terphenyl (scintillation grade) was obtained from BDH and was dissolved in ethanol (Merck, fluorescent grade). Isooctane (Uvasol, for fluorometry) and hexanol (P.A.) were from Merck.

### *Preparation of reversed micellar solutions*

Stock reversed micellar solutions were 0.24 M AOT in isooctane and 0.24 M CTAB in a 12% hexanol/isooctane (v/v) mixture. To these solutions were added: concentrated protein (80 μM), or $NAD^+$ (60 mM) solutions, buffer and isooctane until the desired water and surfactant concentrations were reached. An important parameter for reversed micellar solutions is the molar ratio of water to surfactant, which we will denote as $w_o$. The final surfactant concentration was always 0.2 M, the protein concentration was 40 nM for the activity determinations and 1.3 μM for the spectroscopic measurements. All concentrations are given with respect to the total volume.

### *Activity measurements*

Activity measurements were performed under saturating conditions for both substrate and cofactor. Oxidation of hexanol was performed with the enzyme dissolved in sodium pyrophosphate/HCl buffer, pH 8.8. The $NAD^+$ concentration was 0.5 mM ($K_{m,NAD^+}$ = 10.0 μM in aqueous solution and ≈ 50 μM in AOT-reversed micelles [18]). The substrate concentration was 2 mM in aqueous solution and in AOT-reversed micelles ($K_{m,hexanol}$ = 0.4 mM in water and ≈ 0.1 mM in AOT-reversed micelles [18]). Both for aqueous and AOT solutions 2.9 ml of sample, containing protein and cofactor, was prepared. The reaction was started by addition of 100 μl of a 60 mM solution of hexanol in buffer or in isooctane. In the CTAB system the substrate is also cosurfactant. In the case of CTAB two reversed micellar solutions were prepared, one containing protein, the other one $NAD^+$. The reaction was started by mixing equal amounts of these solutions. Initial reaction rates were determined by monitoring the absorbance changes at 340 nm. An absorption coefficient of 6.22 $mM^{-1}\ cm^{-1}$ was used to determine NADH concentration in both aqueous and hydrocarbon solutions. All experiments were carried out at 25°C.

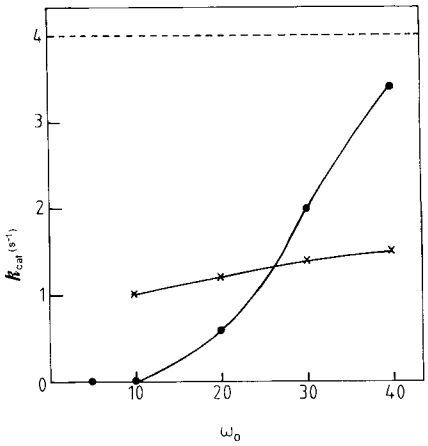


Fig. 1. *Turnover number of horse liver alcohol dehydrogenase with hexanol as substrate in AOT (–·–·–) and CTAB (–×–×–) reversed micellar solutions as a function of* $w_o$ *at 25°C.* Buffer used is sodium pyrophosphate/HCl pH 8.8. Also shown is the activity of the dehydrogenase in aqueous pH 8.8 solution with hexanol as substrate (– – – –)

### *Spectroscopic measurements*

Fluorescence emission spectra were recorded on an Aminco SPF-500 spectrofluorimeter. Slit widths were 4 nm.

Circular dichroism was measured with a Jobin Yvon Mark V Autodichrograph. Data were collected with a Silex microcomputer. Cuvets with 0.5 mm path length were used.

The experimental set up for the time-resolved fluorescence measurements has been described in previous publications [16, 25]. The excitation wavelength was 300 nm and the emission was selected with a 337-nm (10-nm bandpass) interference filter. Polarized fluorescence decay data were collected in two subgroups (1024 channels each) of the multichannel analyzer and transferred afterwards to a MicroVax II computer.

Analysis was performed with the reference convolution method, published before [16]. The reference compound was *p*-terphenyl dissolved in ethanol. For this compound fluorescence lifetimes of 1.064 ± 0.005 ns at 20°C and 1.057 ± 0.005 ns at 30°C have been reported [16]. In the analysis of the data presented here, a reference lifetimes, $\tau_r$, of 1.06 ns was fixed. All experiments were carried out at 25°C and were duplicated.

## RESULTS AND DISCUSSION

### *Enzymatic activity*

The enzymatic activity of LADH, which was measured under saturating conditions for both hexanol and $NAD^+$, will

Table 1. *Fluorescence and anisotropy decay parameters of LADH at 25°C*

| pH | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\bar{\tau}^a$ | $\beta$ | $\phi$ |
|---|---|---|---|---|---|---|---|
| | | ns | | ns | | | ns |
| 8.8 | 0.69 ± 0.01 | 3.33 ± 0.05 | 0.31 ± 0.02 | 6.30 ± 0.03 | 4.25 | 0.25 ± 0.01 | 33.7 ± 0.8 |
| 7.0 | 0.70 ± 0.01 | 3.53 ± 0.04 | 0.30 ± 0.01 | 6.26 ± 0.05 | 4.36 | 0.24 ± 0.01 | 34.2 ± 0.8 |

[a] $\bar{\tau} = \sum_i \alpha_i \tau_i$ .

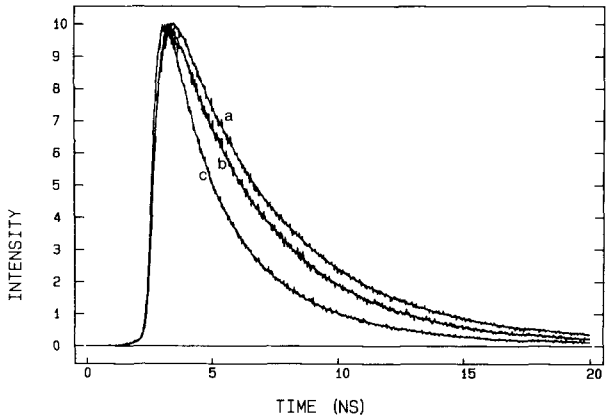


Fig. 2. *Fluorescence decay curves of horse liver alcohol dehydrogenase at 25°C in (a) aqueous solution, pH 8.8; (b) AOT-reversed micellar solution.* $w_o = 20$, *pH 8.8 and (c) AOT-reversed micellar solution* $w_o = 20$, *pH 7.0.* Excitation and emission wavelengths were 300 nm and 337 nm respectively. Time equivalence per channel was 0.030813 ns. The curve obtained for LADH in aqueous solution pH 7.0 (not shown) is almost identical to curve a. The fluorescence decay parameters are listed in Table 1 (curve a) and in Table 2 (curves b and c). Intensity is in arbitrary units

be presented here in terms of the turnover number; $k_{cat}$, which is the number of moles of $NAD^+$ reduced (mole enzyme)$^{-1}$ (second)$^{-1}$. For LADH in pH-8.8 buffer we have found $k_{cat} = 4.0\ s^{-1}$. This value is substantially lower than the one reported by Martinek et al. [18] who found $k_{cat} = 10\ s^{-1}$ with hexanol as substrate. However, Boehringer reports a specific activity of LADH with ethanol of 2.7 U/mg, which corresponds to $k_{cat} = 3.6\ s^{-1}$. In their article Martinek et al. show that there is almost no dependence of $k_{cat}$ on an increasing chain length of the substrate when normal aliphatic alcohols are used from ethanol up to octanol, so we may conclude that the value we found is close to the Boehringer specifications.

The results, obtained in AOT and in CTAB-reversed micellar systems are shown in Fig. 1. In AOT micelles at $w_o = 5$ and at $w_o = 10$ no catalytic activity can be detected. At higher water contents there is activity, which increases monotonically with increasing $w_o$ and at $w_o = 40$, $k_{cat}$ is only slightly lower than in aqueous buffer solution. These results are in very good aggreement with the ones published by Martinek et al. [18]. The decreased activity at low $w_o$ can be ascribed to changes in the microenvironment of the enzyme, like pH, ionic strength and H-bonding changes. In CTAB micelles there is enzymatic activity at the lowest water content studied ($w_o = 10$). This activity is strongly reduced in comparison to the aqueous LADH solution. With increasing $w_o$ the activity slightly increases, but even at the highest water content ($w_o = 40$) there is still a large difference with the aqueous $k_{cat}$ value. In a recent publication by the group of Biellmann [19], activity measurements of LADH in SDS and in CTAB-reversed micellar systems are presented. The CTAB system used in the latter work is quite different from ours. The buffer was 50 mM Tes pH 7.5, the organic solvent was cyclohexane and most results were obtained with 1-butanol as cosurfactant/substrate. However, the trend in the reported $V_{max}$ values as a function of $w_o$ is the same as found by us. $V_{max}$ is considerably lower than in water and it is only slightly dependent on the water content of the system. The authors argue that the decreased activity is caused by a distorted binding of the coenzyme affecting its dissociation constant.

*Fluorescence properties of LADH in aqueous solutions*

The steady-state tryptophan fluorescence upon 300-nm excitation in aqueous solution is the same at pH 8.8 and at pH 7.0. The emission spectrum has a single peak with its maximum at 330 nm. Time-resolved fluorescence properties of LADH in aqueous solution, measured at 344 nm upon 300-nm laser excitation, have been presented in a previous publication [16]. The fluorescence decay can be described by a sum of two exponentials $[\alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$, with $\alpha_i$ the preexponential factors and $\tau_i$ the fluorescence lifetimes). In this work the emission wavelength was 337 nm (337 nm was used instead of 344 nm because this is closer to the emission maximum of the protein fluorescence and thus more fluorescence intensity is obtained) and therefore the decay parameters differ slightly from the ones reported in [16]. The results from the analysis are presented in Table 1. The results obtained at pH 8.8 and at pH 7.0 differ only very little. The two lifetimes are believed to be independent, where the short one is due to Trp-314 and the long one to Trp-15 emission [12, 14]. In a recent paper by Demmer et al. [15] this simple model is opposed. Based on quenching experiments with potassium iodide the authors state that the fluorescence decay must be described by a sum of at least three exponentials. In the latter model a single lifetime of about 4 ns is assigned to Trp-314 and the two other ones ($\approx 1.7$ ns and $\approx 6.5$ ns) to Trp-15. The double exponential decay of Trp-15 is explained by conformational heterogeneity of this solvent-exposed residue. Fitting of our data with a sum of three exponentials is also possible and yields results which are quite comparable to the ones of Demmer et al. [15] but the quality of the fits as judged from the reduced $\chi^2$ value, the Durbin Watson parameter, the plot of the weighted residuals and the autocorrelation function of the weighted residuals [26] does not improve upon introduction of the third exponential and therefore we will restrict ourselves to the double-exponential model.

The anisotropy decay of LADH has been reported to be dependent on the excitation and the emission wavelengths because of the non-spherical shape of the protein and the fluorescence originating from two different electronic tran-

Table 2. *Fluorescence decay parameters of LADH in AOT-reversed micelles at 25°C*

| pH | $w_o$ | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\alpha_3$ | $\tau_3$ | $\bar{\tau}$[a] |
|---|---|---|---|---|---|---|---|---|
| | | | ns | | ns | | ns | |
| 8.8 | 5 | 0.41 ± 0.01 | 3.2 ± 0.3 | 0.19 ± 0.03 | 5.7 ± 0.2 | 0.40 ± 0.03 | 0.11 ± 0.02 | 2.4 |
| | 10 | 0.39 ± 0.01 | 3.2 ± 0.2 | 0.16 ± 0.02 | 5.7 ± 0.2 | 0.45 ± 0.02 | 0.12 ± 0.01 | 2.2 |
| | 20 | 0.38 ± 0.01 | 3.1 ± 0.2 | 0.15 ± 0.02 | 5.7 ± 0.2 | 0.47 ± 0.03 | 0.07 ± 0.02 | 2.1 |
| | 30 | 0.38 ± 0.01 | 3.1 ± 0.3 | 0.14 ± 0.03 | 5.7 ± 0.3 | 0.48 ± 0.04 | 0.04 ± 0.01 | 2.0 |
| | 40 | 0.38 ± 0.01 | 3.1 ± 0.2 | 0.14 ± 0.03 | 5.7 ± 0.2 | 0.48 ± 0.04 | 0.04 ± 0.02 | 2.0 |
| 7.0 | 5 | 0.41 ± 0.01 | 2.2 ± 0.1 | 0.29 ± 0.02 | 4.9 ± 0.2 | 0.30 ± 0.01 | 0.21 ± 0.03 | 2.4 |
| | 10 | 0.45 ± 0.01 | 2.1 ± 0.1 | 0.26 ± 0.01 | 4.7 ± 0.1 | 0.29 ± 0.01 | 0.35 ± 0.03 | 2.3 |
| | 20 | 0.47 ± 0.01 | 2.1 ± 0.1 | 0.20 ± 0.03 | 4.7 ± 0.1 | 0.33 ± 0.02 | 0.40 ± 0.03 | 2.0 |
| | 30 | 0.47 ± 0.01 | 2.1 ± 0.1 | 0.20 ± 0.03 | 4.7 ± 0.1 | 0.33 ± 0.02 | 0.41 ± 0.03 | 2.0 |
| | 40 | 0.47 ± 0.02 | 2.1 ± 0.1 | 0.20 ± 0.02 | 4.7 ± 0.2 | 0.33 ± 0.01 | 0.42 ± 0.05 | 2.0 |

[a] See Table 1 for definition.

sitions ($^1L_a$ and $^1L_b$) [16]. An influence of the non-spherical protein shape on the rotational dynamics of LADH was also observed by Beechem et al. [13] via multiple dye fluorescence anisotropy experiments. The observed anisotropy decays can be fitted with a single-exponential function [$\beta \exp(-t/\phi)$, with $\beta$ the preexponential factor and $\phi$ the rotational correlation time]. Parameters are listed in Table 1. Again the results at the two different pH values are almost identical.

### *Spectroscopic properties of LADH in reversed micellar solutions*

The far-ultraviolet (190–250 nm) circular dichroic spectrum of LADH does not change significantly upon changing the pH of the aqueous solution from 8.8 to 7.0, indicating, that the overall protein structure is nearly identical at both pH values. This agrees well with our observation that the time-resolved fluorescence properties hardly change at varying pH. CD spectra obtained in reversed micellar solutions are, within the experimental error (which is about 10% for these solutions), identical to the ones in the aqueous solutions. Therefore we conclude that no significant changes of the overall protein conformation occur upon incorporation of LADH into the reversed micelles. A similar observation was made by Meier and Luisi [17], who have measured circular dichroism of LADH in pH 8.8 buffer and in AOT-reversed micelles.

Analysis of the fluorescence decays of LADH in AOT-reversed micelles reveals complex decay patterns. Three fluorescence lifetimes are needed to describe the observed decays satisfactorily. Typical examples are shown in Fig. 2. The parameters for the unconstrained fits of the data are presented in Table 2.

First the results, obtained at pH 8.8 will be discussed. At this pH the values of $\tau_1$ and $\tau_2$ are quite close to each other. It has been noted by O'Connor et al. [27] that recovery of decay parameters is not always straightfoward when the decay times do not differ by more than a factor of 2. Acceptable fits are also obtained when $\tau_1$ is fixed at a value of 3.33 ns, which is also found in the aqueous solution. It is tempting to conclude that the fluorescence from the buried tryptophan residue (Trp-314) does not change upon incorporation in the reversed micelles but, if this were so, the preexponential factor $\alpha_1$ should also be of the same order of magnitude as in the aqueous solution, which is not observed. Therefore we believe that the best explanation of the observations is that both

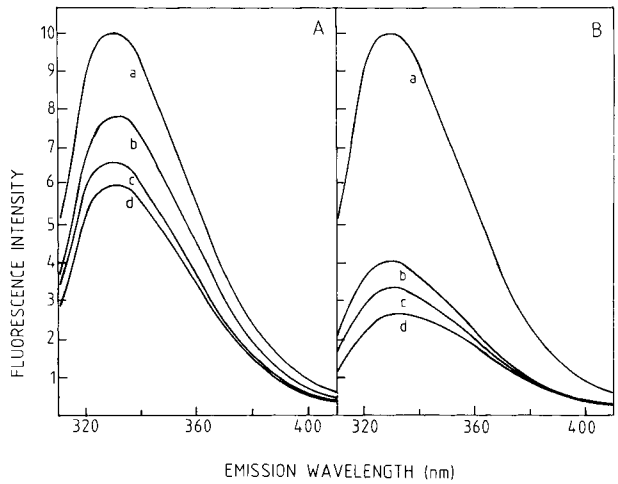


Fig. 3. *Uncorrected fluorescence emission spectra of horse liver alcohol dehydrogenase upon 300-nm excitation at pH 8.8 (A) and at pH 7.0 (B) (25°C).* Curve a is the spectrum obtained of LADH in aqueous solution; curves b, c and d are obtained in AOT-reversed micellar water pools at $w_o = 5$, $w_o = 10$ and $w_o = 40$ respectively

tryptophan residues have a contribution to the third lifetime. For Trp-15 this can be due to a solvent effect because this residue is exposed but for Trp-314 it is more likely that a change in the conformation of the protein in its neighbourhood causes the alterations. This conformational change can only be a subtle one because it was not observed via the circular dichroism of the protein. Circular dichroism of the aromatic residues of the protein might yield more information about the changes in the neighbourhood of the tryptophans but even at the highest possible protein concentration in the reversed micellar systems we were not able to detect any signal in this spectral region. The fluorescence lifetimes are only very slightly dependent on the water content. So the interactions between the protein and the micelles, which cause the change of the fluorescence lifetimes, are not very much dependent on the water pool size.

The same conclusion can be reached when the emission spectra are considered (Fig. 3A). Upon incorporation into AOT micelles the emission is quenched and with increasing $w_o$ this effect gets stronger. We do not find a shift of the emission maximum in the spectra. These observations contradict with the ones reported by Meier and Luisi [17] who found

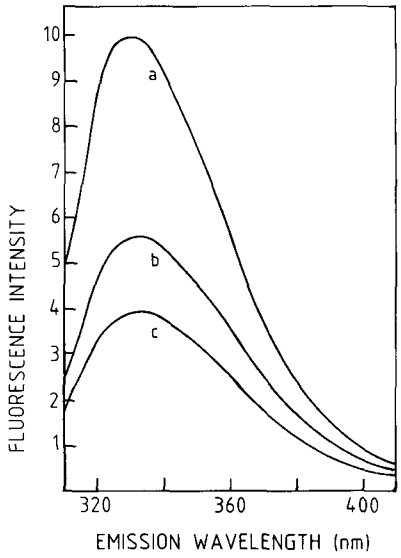


Fig. 4. *Uncorrected fluorescence emission spectra of horse liver alcohol dehydrogenase in aqueous AOT solutions (pH 7.0) at 25°C.* Excitation wavelength was 300 nm. Curve a corresponds to the spectrum obtained at [AOT] = 0 and 0.1 mM. Curve b is measured at 0.5 mM AOT and curve c is the spectrum at 1.0 mM, 2.0 mM and 5.0 mM AOT

a blue shift of the emission maximum and a quenching which is stronger at $w_o = 19$ than at $w_o = 42$. The reason for this discrepancy is not clear.

When the protein is dissolved in pH-7.0 buffer the overall charge will be more positive than at pH 8.8 and thus stronger interactions are expected with negatively charged AOT interfaces. At pH 7.0 it is not possible to obtain good fits of the observed fluorescence decays with $\tau_1$ fixed at the value found in aqueous solution; therefore it is obvious that both tryptophan residues are affected. The differences between the fluorescence properties in aqueous solution and in reversed micelles are indeed more pronounced at pH 7.0 than at pH 8.8. This is also observed via the emission spectra, which are shown in Fig. 3B. In addition to the quenching of the fluorescence a red shift of the emission maximum, which varies from 2 nm at $w_o = 5$ to 8 nm at $w_o = 40$, is found at pH 7.0.

A good explanation for the unexpected increasing changes of the fluorescence of LADH in reversed-AOT micelles with increasing $w_o$ cannot be given at this moment. The changes of the fluorescence properties are apparently not correlated to the enzymatic activity, because $k_{cat}$ is most close to the value in aqueous solution at $w_o = 40$.

Emission spectra were also measured of LADH in aqueous surfactant solutions at different surfactant concentrations. The spectra obtained in AOT solutions at pH 7.0 are shown in Fig. 4. At an AOT concentration of 0.1 mM the spectrum is identical to the one in normal aqueous solution.At 0.5 mM AOT the emission is quenched and the maximum is shifted about 4 nm to the red. The spectra obtained at 1.0, 2.0 and 5.0 mM AOT are identical. The red shift is the same as at 0.5 mM but the quenching is stronger. This stepwise change of the emission spectrum is an indication that also in aqueous AOT solutions LADH interacts with surfactant aggregates. The changes take place when the surfactant concentration is raised above the critical micellar concentration. At pH 8.8 similar observations were made.

In CTAB-reversed micelles the results of the fluorescence decay experiments (Table 3) are easier to interpret. Three exponentials are also needed to fit the data but the values of $\alpha_1$ and $\tau_1$ are almost identical throughout the whole $w_o$ range to the ones in aqueous solution. This is found both at pH 8.8 and at pH 7.0. Examples of decay curves are shown in Fig. 5. The tabulated values are obtained from unconstrained fits of the data. It can be concluded that the fluorescence of Trp-314 is not affected in this case and that the third fluorescence lifetime is due to Trp-15 emission only (probably caused by conformational heterogeneity). The fact that there are almost no differences in the properties of the protein at the two different pH values is an indication that electrostatic interactions between LADH and the CTAB interface are much less pronounced than with AOT.

Both at pH 8.8 and at pH 7.0 the steady-state fluorescence of the protein is quenched upon incorporation in the CTAB-reversed micelles. At low $w_o$ the quenching is stronger than at high $w_o$. The maximum of the fluorescence is at the same wavelength as in the aqueous solution. In aqueous CTAB solution no effect on the LADH fluorescence can be observed up to surfactant concentrations of 5 mM. This is different from the case of AOT where comparable effects are found in aqueous and in hydrocarbon micellar solutions, indicating that the changes in the fluorescence properties in the CTAB-reversed micellar system are not due to direct interactions between the protein and the sufactant. Some properties of the water pools like the high local bromide concentration may cause the quenching of the fluorescence in the reversed micelles.

*Rotational dynamics of LADH in reversed micellar solutions*

The rotational dynamics of LADH, as measured via the fluorescence anisotropy decay, change markedly upon incorporation of the protein in reversed micellar media, especially when AOT is the surfactant. The anisotropy decay parameters, obtained in AOT micelles at pH 8.8 and at pH 7.0 are given in Table 4. In most cases, the observed decays must be described by a sum of two exponentials. A typical example of a double exponential anisotropy decay is shown in Fig. 6. The same phenomenon was observed with fluorescent cytochrome *c* derivatives, incorporated in AOT or in CTAB-reversed micelles [8]. With LADH, the appearance of the second (fast) component in the decay can be explained by a rapid internal motion of a tryptophan residue, superimposed on the overall rotation of the protein. When this component is due to motion of only one of the tryptophans, a model must be used to fit the data, in which the fluorescence of the other tryptophan residue is not coupled to this fast component [16]. With the application of such a model it should in principle be possible to determine which tryptophan is involved in this fast motion. However, it was not possible to distinguish between the different possibilities of coupling fluorescent lifetimes with rotational correlation times. The reason for this is that the relative contribution of the fast component to the total anisotropy decay is only very small.

At pH 8.8 the fast component is only observed at $w_o < 30$. From $w_o = 5$ up to $w_o = 20$ the preexponential factor, $\beta_1$, associated with the fast component, decreases and the concomitant rotational correlation time gets shorter. At pH 7.0 a double exponential decay is observed up to the highest $w_o$ value studied ($w_o = 40$). The value of the rotational correlation time at pH 7.0 also decreases with increasing $w_o$ but the preexponential factor, $\beta_1$, is less dependent on $w_o$ than

Table 3. *Fluorescence decay parameters of LADH in CTAB-reversed micelles at 25°C*

| pH | $w_o$ | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\alpha_3$ | $\tau_3$ | $\bar{\tau}$[a] |
|---|---|---|---|---|---|---|---|---|
| | | | ns | | ns | | ns | |
| 8.8 | 10 | 0.75 ± 0.06 | 3.5 ± 0.2 | 0.14 ± 0.08 | 5.2 ± 0.5 | 0.11 ± 0.04 | 0.07 ± 0.04 | 3.3 |
| | 20 | 0.71 ± 0.03 | 3.6 ± 0.3 | 0.19 ± 0.06 | 5.0 ± 0.4 | 0.10 ± 0.04 | 0.05 ± 0.03 | 3.5 |
| | 30 | 0.70 ± 0.05 | 3.6 ± 0.2 | 0.21 ± 0.05 | 5.2 ± 0.5 | 0.09 ± 0.04 | 0.05 ± 0.02 | 3.6 |
| | 40 | 0.69 ± 0.05 | 3.7 ± 0.4 | 0.20 ± 0.05 | 5.4 ± 0.5 | 0.11 ± 0.03 | 0.06 ± 0.03 | 3.6 |
| 7.0 | 10 | 0.72 ± 0.07 | 3.5 ± 0.1 | 0.13 ± 0.07 | 5.3 ± 0.4 | 0.15 ± 0.04 | 0.09 ± 0.03 | 3.3 |
| | 20 | 0.74 ± 0.07 | 3.5 ± 0.1 | 0.16 ± 0.07 | 5.2 ± 0.4 | 0.10 ± 0.03 | 0.09 ± 0.03 | 3.5 |
| | 30 | 0.73 ± 0.08 | 3.5 ± 0.1 | 0.17 ± 0.08 | 5.2 ± 0.4 | 0.10 ± 0.03 | 0.09 ± 0.04 | 3.5 |
| | 40 | 0.74 ± 0.08 | 3.6 ± 0.1 | 0.17 ± 0.08 | 5.3 ± 0.4 | 0.09 ± 0.03 | 0.09 ± 0.04 | 3.6 |

[a] See Table 1 for definition.

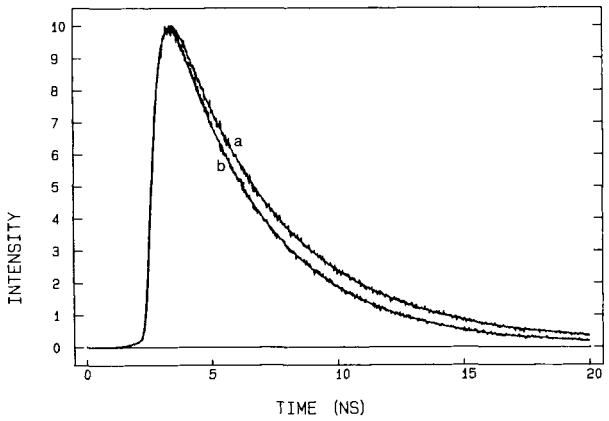


Fig. 5. *Fluorescence decay curves of horse liver alcohol dehydrogenase at 25°C in (a) aqueous solution pH 8.8 and (b) CTAB-reversed micellar solution* $w_o$ = *20, pH 8.8.* Experimental conditions were identical to the ones described in the legend of Fig. 2. The curves, obtained at pH 7.0 are not shown because they are almost identical to the ones shown here. Decay parameters are given in Table 1 (curve a) and in Table 3 (curve b)

Table 4. *Fluorescence anisotropy decay parameters of LADH in AOT-reversed micelles at 25°C*

| pH | $w_o$ | $\beta_1$ | $\phi_1$ | $\beta_2$ | $\phi_2$ |
|---|---|---|---|---|---|
| | | | ns | | ns |
| 8.8 | 5 | 0.05 ± 0.01 | 1.3 ± 0.5 | 0.200 ± 0.003 | 190 ± 47 |
| | 10 | 0.04 ± 0.01 | 0.9 ± 0.2 | 0.204 ± 0.002 | 132 ± 33 |
| | 20 | 0.02 ± 0.01 | 0.6 ± 0.2 | 0.220 ± 0.003 | 90 ± 21 |
| | 30 | – | – | 0.239 ± 0.001 | 58 ± 4 |
| | 40 | – | – | 0.241 ± 0.001 | 35 ± 2 |
| 7.0 | 5 | 0.05 ± 0.01 | 1.7 ± 0.6 | 0.222 ± 0.002 | 203 ± 64 |
| | 10 | 0.04 ± 0.01 | 1.1 ± 0.3 | 0.216 ± 0.003 | 201 ± 44 |
| | 20 | 0.04 ± 0.01 | 1.2 ± 0.4 | 0.207 ± 0.004 | 115 ± 19 |
| | 30 | 0.04 ± 0.01 | 0.9 ± 0.5 | 0.205 ± 0.003 | 71 ± 12 |
| | 40 | 0.04 ± 0.01 | 0.9 ± 0.3 | 0.204 ± 0.003 | 62 ± 10 |

at pH 8.8. The shortening of the rotational correlation time with increasing water pool size can be due to a decreasing viscosity in the neighbourhood of the protein, which is expected when the micelles grow.

The slow decaying component of the anisotropy represents the overall rotation of the protein, which is determined by the motion of the protein within the micelle and the motion of

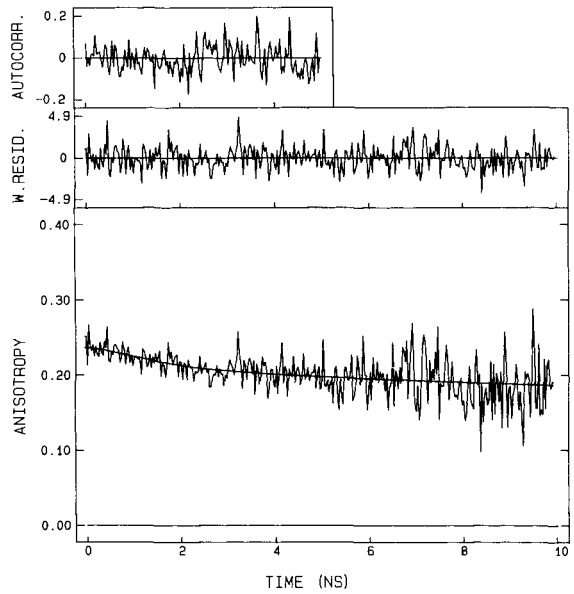


Fig. 6. *Fluorescence anisotropy decay of horse liver alcohol dehydrogenase in AOT-reversed micellar water pools,* $w_o$ = *20, pH 7.0 at 25°C.* Shown are the first 10 ns of the decay. Experimental conditions were the same as in Fig. 2. The decay is fitted with a double-exponential function. The quality of the fit is indicated by the weighted residuals and the autocorrelation of these residuals, shown in the upper pannels of the plot. The anisotropy parameters are listed in Table 4

the micelle in the organic solvent. Assuming that these motions are independent, the observed rotational correlation time, $\phi_2$, is composed of $\phi_{int}$ (describing internal motion within the micelle) and $\phi_{mic}$ (describing overall micellar rotation) as follows (cf. [8, 28]):

$$\frac{1}{\phi_2} = \frac{1}{\phi_{int}} + \frac{1}{\phi_{mic}}. \quad (1)$$

Especially at low $w_o$ values, where the observed rotational correlation times are long, the error in the reported $\phi_2$ values is very large. The reason for this is that, on the time scale of the fluorescence experiment ($\approx$ 20 ns), there is almost no decay of the anisotropy (Fig. 6). When the rotational correlation times get shorter the accuracy of the parameters improves.

At both pH values in AOT-reversed micelles the trend is that $\phi_2$ decreases monotonically when the size of the water pools increases. Because it is not likely that $\phi_{mic}$ decreases with increasing $w_o$, the latter observation must be related to

Table 5. *Core radii ($r_c$) and rotational correlation times ($\phi$) of AOT and CTAB-reversed micelles at 25°C*

| $w_o$ | AOT | | CTAB | |
|---|---|---|---|---|
| | $r_c$ | $\phi$ | $r_c$ | $\phi$ |
| | nm | ns | nm | ns |
| 5 | 1.2 | 7 | – | – |
| 10 | 1.7 | 12 | 1.9 | 41 |
| 20 | 3.4 | 47 | 3.1 | 85 |
| 30 | 4.3 | 80 | 4.1 | 140 |
| 40 | 5.5 | 145 | 5.2 | 223 |

Table 6. *Fluorescence anisotropy decay parameters of LADH in CTAB-reversed micelles at 25°C*

| pH | $w_o$ | $\beta$ | $\phi$ |
|---|---|---|---|
| | | | ns |
| 8.8 | 10 | 0.230 ± 0.002 | 135 ± 21 |
| | 20 | 0.231 ± 0.002 | 111 ± 21 |
| | 30 | 0.233 ± 0.002 | 105 ± 15 |
| | 40 | 0.229 ± 0.001 | 73 ± 8 |
| 7.0 | 10 | 0.228 ± 0.002 | 123 ± 19 |
| | 20 | 0.229 ± 0.002 | 86 ± 9 |
| | 30 | 0.229 ± 0.001 | 77 ± 7 |
| | 40 | 0.227 ± 0.001 | 67 ± 6 |

an increased mobility of the protein within the micelles. At pH 8.8 the observed rotational correlation times are shorter than at pH 7.0. Sizes and rotational correlation times of empty AOT- and CTAB-reversed micelles as earlier determined with time-resolved luminescence quenching [8] are presented in Table 5. Since the LADH molecule can be considered as a prolate ellipsoid with long and short semiaxes of 11 nm and 6 nm respectively, we may conclude that only at $w_o = 40$ is the water pool of an empty AOT micelle large enough to accomodate the protein. This means that up to $w_o = 30$ the micelles have to grow to be able to solubilize a protein molecule. When the protein is totally immobilized in the micelle ($1/\phi_{int} = 0$) the size of the micelle can be calculated from $\phi_2$ [8]. The diameter of the water pool at $w_o = 5$, pH = 8.8 is then about 12 nm, which is close to the largest dimension of the LADH molecule (11 nm). The decreasing rotational correlation times at higher $w_o$ can then only be explained if the micelles are larger than expected and if there is some internal mobility of the protein in the water pool. At $w_o = 40$ a rotational correlation time of 145 ns is calculated for the empty AOT micelles. Substituting this value for $\phi_{mic}$ in Eqn (1), values of 46 ns and 108 ns can be calculated for $\phi_{int}$ at pH 8.8 and at pH 7.0, respectively. These numbers are only valid when the size of the micelle does not increase upon incorporation of a protein molecule but, even if there is a considerable expansion of the micelle, the internal mobility of the protein is markedly higher at pH 8.8 than at pH 7.0. Such a result strongly suggests that at pH 7.0 the interactions between the protein and the AOT interface are much more important than at pH 8.8, providing evidence for an electrostatic nature of these interactions. It is noteworthy that the activity of the enzyme follows the same tendency as observed for the rotational dynamics in AOT-reversed micelles.

In CTAB-reversed micelles, single-exponential anisotropy decays are found at both pH values (decay parameters listed in Table 6). As with AOT as surfactant, the rotational correlation times, which represent the overall rotational dynamics of the protein, decrease monotonically with increasing $w_o$. The observed rotational correlation times in the CTAB-reversed micelles are somewhat longer at pH 8.8 than at pH 7.0 (especially at $w_o = 20$ and at $w_o = 30$). So it seems that in the cationic micelles also (local) electrostatic interactions play a role and that at pH 8.8 the protein is more associated with the interface than at pH 7.0. This effect was not observed with the fluorescence lifetime measurements.

The rotational correlation times in the CTAB-reversed micelles at $w_o = 10$ of 135 ns and 123 ns at pH 8.8 and at pH 7.0, respectively, can not be explained by the existence of a spherical reversed micelle with a core diameter of 11 nm (being the largest dimension of the LADH molecule) and the protein totally immobilized in it because in that case a rotational correlation time, $\phi$, of about 250 ns would be expected [8]. Internal mobility of the protein within the micelle or a non-spherical micelle shape could explain the observed rotational correlation times. The present experiments do not allow us to distinguish between these two possibilities. For empty CTAB-reversed micelles at $w_o = 40$ we have calculated a rotational correlation time of 223 ns (Table 5). When this value is substituted in Eqn (1) for $\phi_{mic}$ we can calculate $\phi_{int}$ for the protein in CTAB-reversed micelles. At pH 8.8 a value of 109 ns is found and at pH 7.0 $\phi_{int} = 96$ ns. These values, which are valid when the micelles do not expand upon protein incorporation, are similar, in contrast to those determined in AOT micelles. So at least at $w_o = 40$ the electrostatic interactions are less important in CTAB than in AOT. The observed effects in the reversed micellar media agree well with what could be expected, based on charge effects of the protein and the surfactant interface.

## CONCLUDING REMARKS

The interactions between LADH in reversed micellar systems and surfactant interfaces are largely electrostatic in nature. This can be concluded from the fact that a change of pH from 8.8 to 7.0 causes differences in the structural and dynamic properties of the protein molecule especially in AOT-reversed micellar water pools.

The overall structure of LADH is only very little affected upon incorporation in the reversed micellar systems. The circular dichroism of the protein backbone hardly changes in the reversed micelles. On the other hand, the time-resolved fluorescence properties are markedly different in aqueous solution and in reversed micellar solutions. This means that the fluorescent lifetimes especially are mainly dependent on local changes in the environment of the tryptophan residues in the protein and do not report on the overall properties of the protein molecule.

With increasing $w_o$, both the activity and the rotational mobility of LADH in reversed micellar water pools increase. In AOT solutions the differences between low and high $w_o$ are larger than in CTAB solutions.

Part of this work was supported by the Netherlands Foundation of Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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