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MICELLAR SOLUBILIZATION OF BIOPOLYMERS IN HYDROCARBON SOLVENTS. II. THE CASE OF HORSE LIVER ALCOHOL DEHYDROGENASE

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The chemical characterization of horse liver alcohol dehydrogenase solubilized in isooctane via reverse micelles formed by the anionic surfactant di (2-ethyl-hexyl) sodium sulfosuccinate (AOT) and water (0.6 to 4% v/v) is presented. The enzyme's catalytic activity toward acetaldehyde reduction is markedly dependent upon $w_0 = [H_2O]/[AOT]$, and upon the pH of the stock aqueous solution (pH_{st}), from which the hydrocarbon enzyme solution is prepared. Kinetically, the micellar solution appears to follow a normal Michaelis-Menten behavior, with a turnover number which, under the optimal conditions ($w_0 = 42$, $pH_{st} = 8.8$), appears to be higher than in bulk water. The affinity between enzyme and NADH, as judged from direct binding studies (quenching of the protein fluorescence), is much reduced with respect to water if concentrations refer to the water pool of the micelles, and comparable to water if concentrations refer to the overall volume (hydrocarbon plus water pool). Also, the K_m values are much higher if concentrations refer to the water pool. Ultraviolet absorption studies show that the aromatic chromophores are not significantly perturbed on going from a water solution to the micellar solution. The essentially aqueous environment of the protein in the reverse micelles is confirmed by fluoresence studies. Circular dichroism studies show that the enzyme's conformation in the micelles is similar to that in water; however, under certain conditions, small but significant changes of the main chain folding seem to occur, which do not impair enzymatic activity. The spectroscopic properties of NADH in the hydrocarbon phase (fluorescence and circular dichroism) are also investigated. The potential of the LADH-NADH system for technical applications (oxidoreduction of lipophylic substrates) is discussed.

INTRODUCTION

In this paper, we present data pertaining to horse liver alcohol dehydrogenase (LADH) solubilized in isooctane with the help of the anionic surfactant di (2-ethyl-hexyl) sodium sulfosuccinate (AOT). As is well known, LADH is composed of two chemically equivalent subunits of 40,000 Daltons each, and it requires NAD(H) to catalyze the following reaction (1):

$$RCHO + NADH + H^+ \rightleftharpoons RCH_2OH + NAD^+ \quad (1)$$

where R is an alkyl or aryl group. The enzyme is well characterized both kinetically (2) and spectroscopically (3, 4), and its x-ray structure is known in detail (1).

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We will utilize circular dichroism (CD), fluorescence, and activity data in order to test the structural model outlined in the preceding paper (18). Also, the behavior of LADH in the micellar hydrocarbon solution should be representative for other coenzyme-dependent oligomeric enzymes. Furthermore, LADH is interesting technologically in view of its lack of substrate specificity; because of this fact, it has been often utilized as a catalyst for several organic reactions (5).

MATERIALS AND METHODS

LADH was purchased from Böehringer in the form of an ethanol suspension, and was purified as described previously (3). The kinetic assay in water was carried out according to the method of Theorell and Yonetani (6). NADH of the highest quality was also obtained from Boehringer. The same extinction coefficients were used to calculate concentrations in water and in the hydrocarbon solutions, i.e., $3.53 \times 10^4\ M^{-1}\ cm^{-1}$ for LADH and $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ for NADH, respectively. The micellar hydrocarbon solutions were prepared as described in the preceding paper (18). Ultraviolet absorption measurements were carried out with an Acta MVI (Beckmann), fluorescence spectra with an Aminco 1000 (a corrected instrument), and circular dichroic measurements with a Jasco J40.

All experiments in the micellar enzyme solutions were carried out at 30°C. The activity studies presented in this work were carried out by adding acetaldehyde (diluted with the hydrocarbon) to the micellar enzyme-NADH hydrocarbon solution. In the binding studies, a micellar solution with NADH was added to a miceller solution of the enzyme at the same w_0 value. The enzyme concentration (overall) used for determining dissociation constants was 1 μM or lower. It was 2.5 μM or higher for titration of enzyme active sites. As buffer, we have used glycine-NaOH-0.1 M (pH 8.8–10), or phosphate 0.1 M (pH 6–7.5).

RESULTS AND DISCUSSION

Boundary Considerations

In all the studies presented in this paper, a low AOT concentration was used (50 mM), to enable us to carry out reliable spectroscopic investigations in the far UV region. We had difficulties solubilizing LADH at room temperature, but at 30°C (to which all data reported in this paper refer), the enzyme could be readily solubilized in the hydrocarbon phase with as little as 0.6% water ($w_0 = 6.6$). This influence of temperature on solubilization is in

keeping with the results presented by Zulauf and Eicke (7), according to which the size of the micelles (and therefore the space available for a possible host molecule) increases with increasing temperature and/or w_0. Before presenting data pertaining to LADH solubilized in the hydrocarbon phase, a few considerations are needed in order to better define the micellar system. Consider, for example, that under our conditions, only a small fraction of the micelles (0.5–2%) are filled by enzyme molecules, the rest being available for NADH and substrate (and, of course, water). In keeping with the view advanced in the previous paper, the protein-containing micelles, the NADH-containing micelles, the micelles containing the LADH-NADH complex, and the unfilled micelles should be considered to be in rapid equilibrium, i.e., exchanging rapidly the guest molecules. Though the enzyme and coenzyme are only soluble in the water pool, substrates such as acetaldehyde will be divided between the water and the hydrocarbon phase.

It is also useful to consider the dimensions of the micellar aggregates, as calculated with the rough model presented in the preceding paper (18). At $w_0 = 20$ (where the enzyme is still active), the water pool has a radius of ca. 52 Å, and a layer of water of ca. 12 Å depth separates the enzyme surface from the surfactant layer. The aggregation number S_n is 670, and the number of water molecules per micelle is around 13,300.

An elaboration is also necessary in regard to the "local pH" of the reverse micelles: when we prepare an enzyme micellar solution by adding enzyme from an aqueous stock solution with a given pH (defined as pH_{st}), we cannot simply assume that the pH inside the water pool of the micelle (pH_{wp}) will be the same. Also, it is possible that the pK of certain ionizable groups is different in the micelles than in water (8). In the case of protein solubilized in hydrocarbon reverse micelles, this point has been raised already in the case of ribonuclease (9) and α-chymotrypsin (10). At the present stage, it is difficult to define the local pH or pK as a function of the various micellar parameters [detailed studies are in progress in our group on the basis of ^{31}P-NMR measurements (11)]. For the purpose of this paper, one should recognize that the change in pH and/or pK of ionizable groups can affect the enzymatic activity, and, particularly, the comparison between activity in water and in the micelle should be viewed in this perspective.

Finally, one should keep in mind the duality in expressing the concentration in a reverse micellar system, depending on whether one operates with water pool (wp) concentrations or with overall (ov) concentrations. Correspondingly, there will be two K_m values to consider, $(K_m)_{ov}$ and $(K_m)_{wp}$, which are numerically related by the factor f [see the preceding paper (18)]:

$$(K_m)_{ov} = (K_m)_{wp} \cdot f \tag{2}$$

with

$$f = F_w + P(1 - F_w) \tag{3}$$

where F_w is the water volume fraction, and P represents the previously (18) defined partition coefficient of the substrate (the enzyme is assumed to be soluble only in the water pools). Notice that when $P = 1$, $(K_m)_{wp} = (K_m)_{ov}$ (the system is homogeneous with respect to S concentration). For substrates that are preferentially soluble in the water pool, i.e., $P < 1$, $(K_m)_{wp} > (K_m)_{ov}$. For example, when $P = 0.1$ and $F_w = 0.01$, $(K_m)_{wp}$ will be ca. 10 times larger, and when $P = 0.01$ and $F_w = 0.01$, it will be 50 times larger than $(K_m)_{ov}$.

Spectroscopic Characterization of the LADH-NADH System in the Micellar Phase

Fluorescence and circular dichroism properties of NADH have been extensively investigated for water solutions (3), and it is widely accepted that they are markedly affected by the environment and the conformation of the coenzyme molecule. Thus NADH can be considered as a useful probe to test the micellar environment. In particular, if NADH were exposed to the hydrocarbon solvent, or if it directly interacted with the surfactant molecules, one would expect large alterations of the fluoresence and/or CD spectra with respect to those for water solutions.

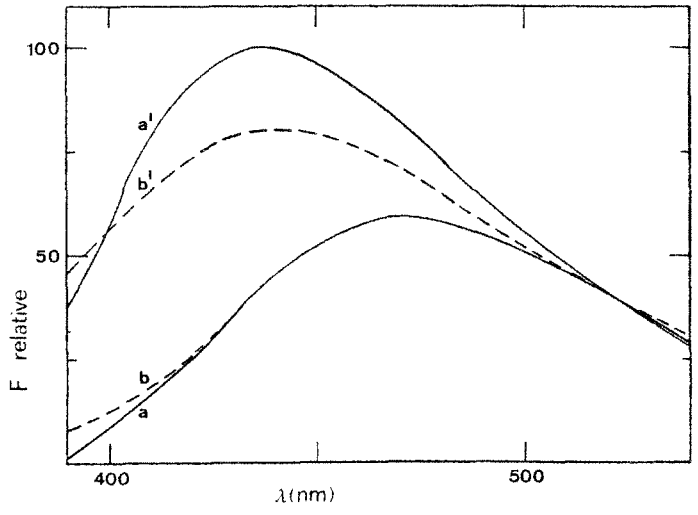

FIG. 1. Emission spectra of NADH in water, pH 6.2, and in isooctane-AOT-H_2O (- - -) $w_0 = 42$, pH_{si} 8.8. Curves (a) and (b) are the free forms; (a') and (b') represent NADH (11.6 μM) in a solution containing 2.8 μM LADH.

Figure 1 shows that the emission spectra of free NADH in water and in the micellar environment at $w_0 = 42$ are practically the same. Also note from Fig. 1 that λ_{max} of the bound form is the same in water and in the micellar system.

Figure 2 shows the CD spectra of NADH in water and in the hydrocarbon system in different micellar conditions. The form of the spectrum and the intensity in the 340 nm region does not change (or changes only slightly) on going from one system to another. There seem to be some decrease of the ellipticity in the far UV region at lower w_0 values, but again λ_{max}, as well as the form of the Cotton effect, does not change appreciably. In conclusion, these spectroscopic data suggest that the NADH is in an environment very similar to that of bulk water.

Let us now consider the spectroscopic properties of the protein. Figure 3 shows the absorption spectra of LADH under different conditions. In water around neutral pH, the ratio of the absorbance at 280 and 260 nm is

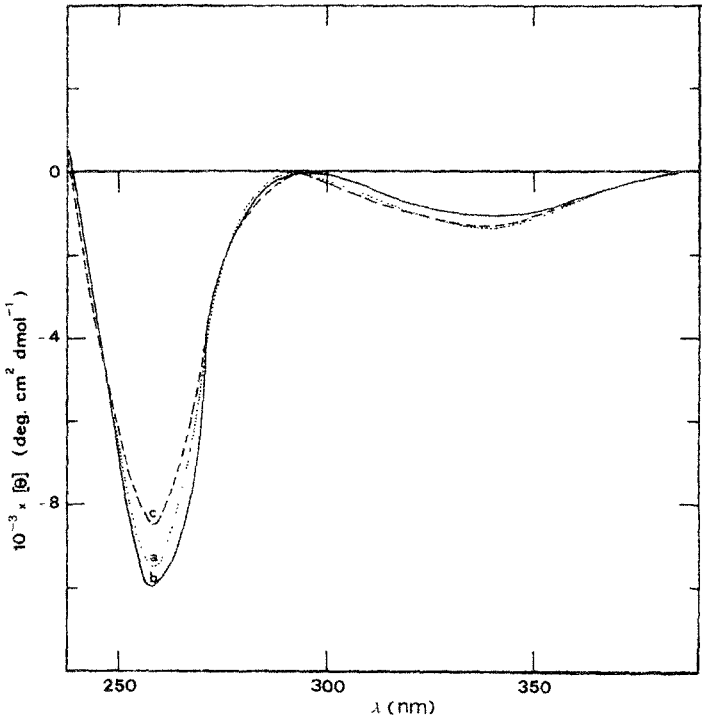

FIG. 2. Circular dichroism spectra of NADH in water, pH 6.2, curve (a), and pH 8.8, curve (b). Also shown are the spectra in the micellar solution, $w_0 = 42$, pH_{st} 8.8, curve (a), $w_0 = 19$, pH_{st} 8.8, curve (c), and pH_{st} 9.2, $w_0 = 19$, curve (c).

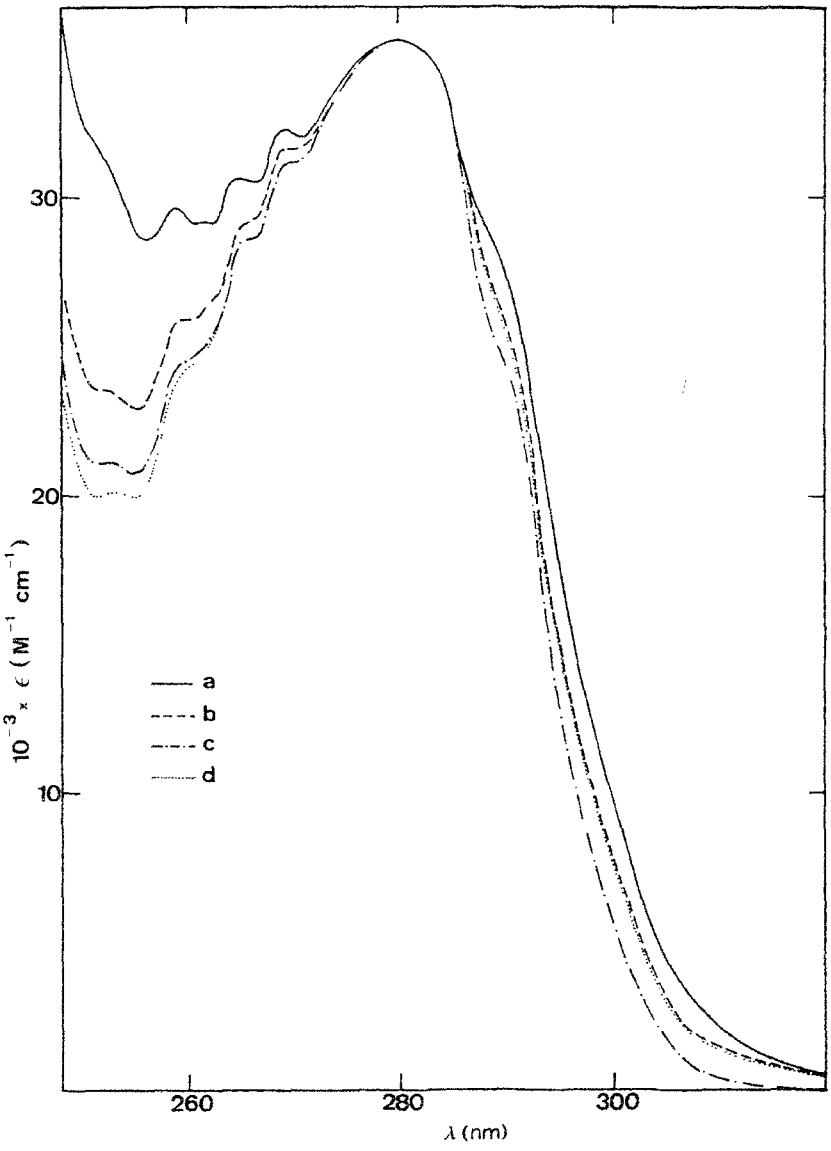

FIG. 3. UV-absorption properties of LADH in water, pH 9.6, curve (a), pH 8.8, curve (b), pH 7.0, curve (d), and in the micellar solution, $w_0 = 42$, pH_{st} 8.8, curve (c).

1.44. By increasing pH, due to the ionization of tyrosine, this ratio decreases (Fig. 3a), and there is a concomitant increase of the absorption in the 300 nm region. When we inject an enzyme solution at pH 8.8 (Fig. 3b) into the micellar phase with a final $w_0 = 42$, we obtain the spectrum of Fig. 3c, namely, a spectrum which is very close to that of a neutral aqueous solution (Fig. 3d). Thus it appears as if the pK of the enzyme's tyrosine(s) experiences a jump toward higher values on going from an aqueous alkaline solution into the micellar solution. Other than that, the environment of the protein is not significantly altered.

Figure 4 reports circular dichroism properties of LADH in the far UV spectrum in water [which are in agreement with previous results (3)], and in the micellar phase. For proteins, this spectral region is dominated by the amide chromophore, and any perturbations should mostly reflect changes in the peptide backbone conformation. The intensity in the region 215–230 nm and the general character of the spectra are similar for water and for the hydrocarbon phase. Thus these data indicate that, although the peptide

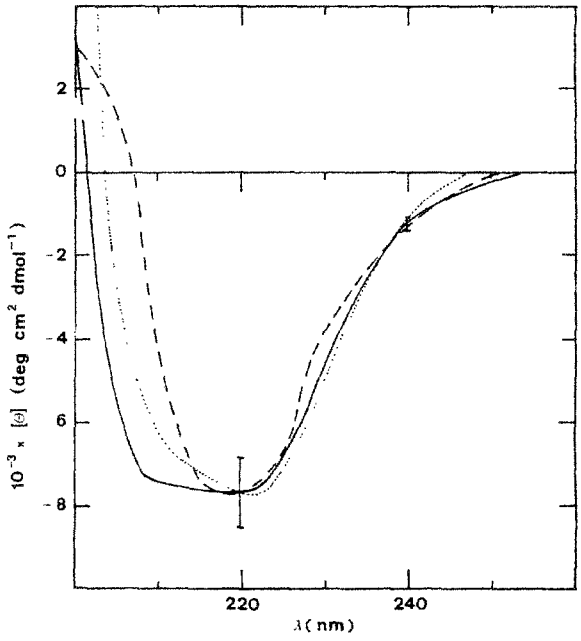

FIG. 4. Circular dichroism of LADH in water, pH 7.0(· · ·), and in the hydrocarbon micellar solution, $w_0 = 19$, pH_{st} 8.8. (– – –), and $w_0 = 42$, pH_{st} 8.8 (———). Vertical bars represent the instrumental noise level.

backbone probably undergoes some small rearrangement, the main conformational features remain similar for the two systems. This is in agreement with previous data obtained for ribonuclease in AOT-isooctane micelles where, under the investigated conditions, the CD spectra for water and for the micellar phase were very close to each other (9).

Let us consider now fluorescence spectra, as reported in Fig. 5. Here, differences between the water and the hydrocarbon phase are more marked. The general trend in the micellar solution is a decrease of the quantum yield, and a shift of the emission maximum toward the blue. In order to interpret these data, one should remember that LADH has two tryptophan residues per subunit, which have been spatially located by x-ray analysis (1). Trp-15 is close to the surface and is probably exposed to the solvent, while trp-314 is

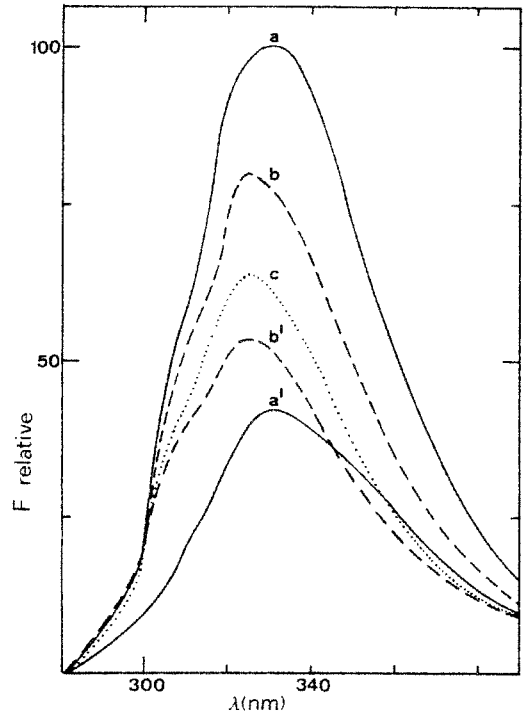

FIG. 5. Flourescence spectra of LADH in water and in hydrocarbon micellar solution. Curve (a) is free LADH in water, pH 6.2, curve (b) in micelle at $w_0 = 42$, pH_{st} 8.8, curve (c) in micelle at $w_0 = 19$, pH_{st} 9.2, curve (b') is as in (b) but in the presence of 11.6 μM NADH, curve (a') is as in (a), but in the presence of 11.6 μM NADH. The enzyme concentration is 2.8 μM.

buried inside the protein coil, and close to the interaction domain of the two subunits. It has also been shown that quenching of the LADH fluorescence in water solution by iodide involves mostly trp-15, so that the emission spectrum after quenching can be attributed almost exclusively to trp-314 (4). Fluorescence data in the micelles can be interpreted in these terms, i.e., by assuming that in the micellar phase, the exposed tryptophan is preferentially quenched by solvent water molecules. This also implies that the blue shift of λ_{max} observed in the micelles is an apparent one, i.e., it due to the decrease of the intensity of the red side component.

Upon NADH binding, the protein emission is quenched, and this provides a simple metnod to determine the coenzyme dissociation constant (2, 13). As shown in Table 1, $(K_d)_{ov}$ is very close to K_d found in water solution, whereas $(K_d)_{wp}$ is much larger. The quenching of the protein fluorescence by NADH also permits us to titrate the enzyme active site concentration (by using solutions with a greater enzyme concentration than those used for binding studies). Figure 6 shows one such titration experiment. The binding capability of our micellar enzyme solutions was generally 10–20% lower than that found in water solution (which was typically 95% of the theoretical value). It is possible that this small inactivation of the enzyme in the hydrocarbon-AOT solution is not due to a property of the micelles as such, but it comes about when the aqueous enzyme stock solution is abruptly injected into the hydrocarbon phase (a kind of "solubilization-shock' effect).

As already mentioned, these binding experiments are carried out by mixing the micellar solution of the enzyme with a micellar solution of the

TABLE 1. **Kinetic and Binding Properties of Horse Liver Alcohol Dehydrogenase (LADH) in the Hydrocarbon Micellar Solutions and in Water**[a]

System	K_m^{NADH}, (μM)	K_m^S (μM)	v/e (s^{-1})	K_d^{NADH} (μM)
LADH in water, pH 7.1	10[b]; 11.3[c]	375[b]; 110[c]	114[d]	0.4[e]; 0.3[f]
LADH in water, pH 9.0	3	400	5	0.65; 0.99
LADH in micelle,[g] $w_0 = 42$, pH_{st} 8.8	23 (610)	300 (1900)	80	0.35 (9.25)
LADH in micelle,[g] $w_0 = 19$, pH_{st} 9.2	20 (1175)	n.d.	30	n.d.

[a] Data in water are at 23.5°C, data in the micellar system at 30°C. The uncertainty interval of the micellar parameters is ca. 20%.
[b] From Ref. (14).
[c] From Ref. (2).
[d] From Ref. (15).
[e] From Ref. (16).
[f] From Ref. (17).
[g] The water pool value is given in parentheses [see Eq. (2)].

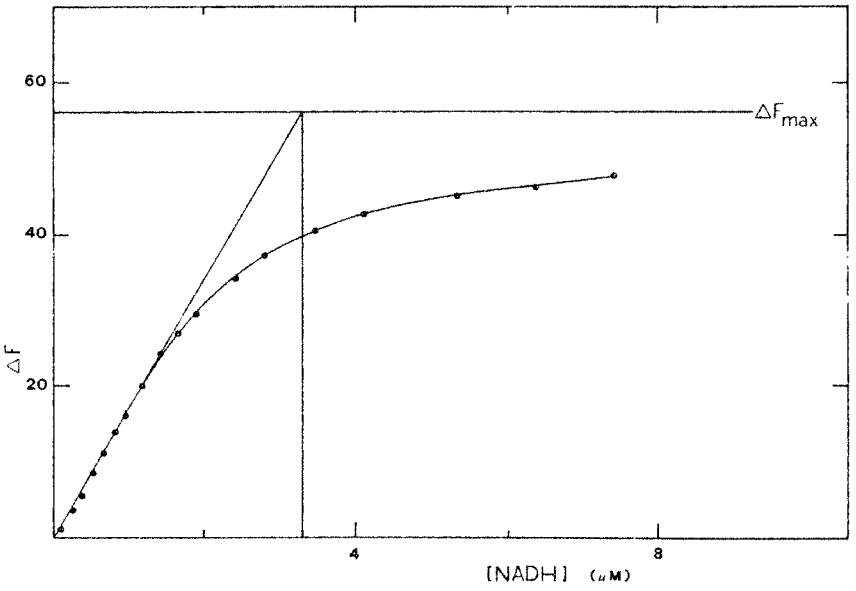

FIG. 6. Quenching of LADH fluorescence by NADH and titration of the enzyme's active sites in the micellar solution, $w_0 = 42$ and pH_{st} 8.8. The enzyme concentration (overall) was 2.35 μM, and the titration point is at 3.3 μM NADH, which corresponds to 70% of the theoretical binding sites. ΔF_{max} is the maximal fluorescence quenching obtained by extrapolating at infinite ligand concentration (13).

coenzyme at the same w_0. We observed that the fluorescence had already reached the equilibrium value after the mixing time (ca. 15 s). This indicates that, at least in the concentration range used in these binding experiments, the time necessary for the exchange of material among the micelles is shorter than this mixing time.

Enzymatic Activity of LADH in Reverse Micelles

The first question to consider is whether in the micellar system there are new parameters that may influence the activity other than the well-known ones (concentrations, temperature, pH, etc.), which are important in water. One of such 'new" parameters is w_0, the molar ratio of water to the surfactant. As we have seen in the preceding paper (18), w_0 can be directly correlated to the size of the water pool, and therefore it is indirectly connected with the environmental changes caused the enzyme's confinement in the micelle. Thus it will be important to study the dependence of the enzymatic reaction velocity upon w_0.

Figure 7 reports the reaction velocity as a function of w_0 and pH_{st}. For each pH_{st} value, a bell-shaped curve is obtained, each having a distinct maximum by a w_0 value, which we will indicate as $(w_0)_{max}$. The position of this maximum shifts toward the right [larger $(w_0)_{max}$ values] by decreasing pH_{st}. This behavior is only in a very minor way due to the changes of water concentration itself. In fact, when the water content is changed at constant w_0 (this can be done by simultaneously changing the water and the AOT content), the reaction velocity does not change markedly (see insert Fig. 7).

It is important to clarify to what extent the behavior illustrated in Fig. 7 is due to enzyme inactivation in certain ranges of w_0 and pH_{st}. In order to answer this question, we have carried out titration experiments of the enzyme binding sites with NADH, at $w_0 = 42$, pH_{st} 8.8, and $w_0 = 30$, pH_{st} 9.2. Under these two sets of conditions, the enzyme's kinetic activity differs by a factor of 6 (see Fig. 7); however, the binding capability of the enzyme is in both cases in the range 70–80% of the theoretical value. It appears then that the decrease of the reaction velocity in certain w_0 and pH_{st} regions is not simply due to enzyme inactivation.

Another point of interest is the comparison between the kinetic behavior in water and in the micellar phase, as evaluated by classical

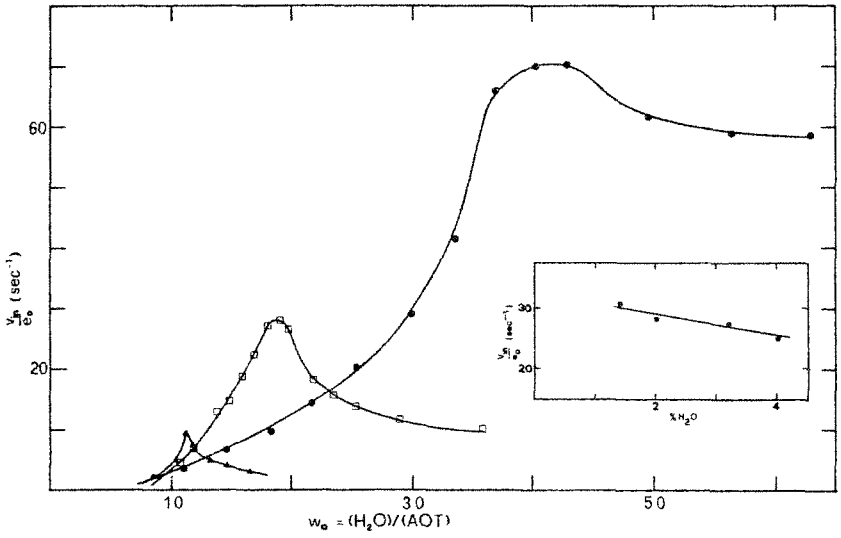

FIG. 7. Turnover number (v_{in}/e_0) of LADH in the micellar solution as a function of w_0 and $pH_{st} = 9.6$ (▲), 9.2 (□), 8.8 (●), with glycine-NaOH 0.1 M buffer. The enzyme concentration (overall) was in the range 1.7×10^{-8} M, $(CH_3CHO) = 1.9$ mM, $(NADH) = 80$ μM, conditions under which the enzyme is practically saturated (see Table 1). In the insert, the dependence of the turnover number on the water concentration at constant $w_0 = 19$, $pH_{st} = 9.2$, is given.

steady-state experiments. Figure 8 reports Eadie plots for the reduction of acetaldehyde, at $w_0 = 19$, pH_{st} 9.2, and $w_0 = 42$, pH_{st} 8.8. Data are collected in Table 1. The maximal turnover number at $w_0 = 42$ and pH_{st} 8.8 is 80 s^{-1}. The turnover number of the enzyme at pH 8.8 in water is around 5 s^{-1}, and it would then appear that the enzyme in the micelle is much more active than in water solution. However, as we have already mentioned, the local pH (pH_{wp}) is lower than pH_{st}, and it is not possible as yet to precisely assess its value. This makes a direct comparison between the micellar and the water solution very difficult.

As far as the evaluation of K_m is concerned, we have to deal again with the problem of choice of the concentration coordinates. In the case of acetaldehyde, one is confronted with the additional problem of the partition of the substrate between the water phase and the hydrocarbon phase (a first approximation value of $P = 0.12$ has been obtained). As summarized in Table 1, values of $(K_m)_{wp}$ are much larger than in water, and $(K_m)_{ov}$ is about the same as in water.

As mentioned in the experimental part, acetaldehyde can be added to micellar phase as a hydrocarbon solution. It was interesting to see whether other hydrocarbonlike substrates would be catalytically transformed by the LADH-NADH micellar system, in particular, those which, because of their poor solubility in water, have not been considered so far in the wide list of

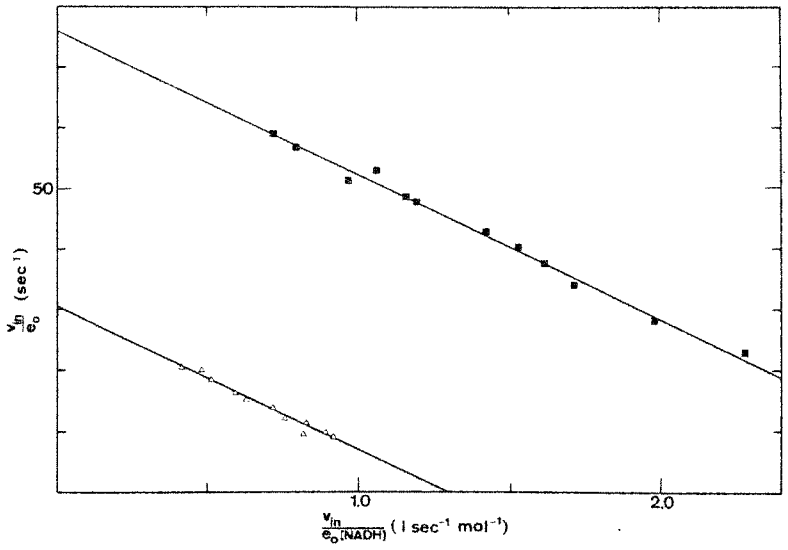

FIG. 8. Eadie plots for the reduction of acetaldehyde catalyzed by LADH in the micellar solution, at $w_0 = 19$, pH_{st} 9.2 (△), and $w_0 = 42$, pH_{st} 8.8 (■), as a function of NADH concentration and at constant acetaldehyde (1.9 mM).

LADH substrates. We have used decanal as an first simple example. This aldehyde can be added as such to the hydrogen solution, and is efficiently transformed into the corresponding alcohol (with a velocity that, at pH_{st} 9.2, is about 20 times smaller than that for acetaldehyde). Also, certain steroids (like 5-α-androstan-17β-ol-3-one) could be reduced by NADH in the micellar phase. These studies are presently in progress.

CONCLUDING REMARKS

The spectroscopic study of the micellar system presented in this paper suggests that the biomolecules NADH and LADH are essentially in an aqueous environment under the conditions investigated in this work. These data should be considered together with those obtained for ribonuclease (9), and all seems to basically confirm the model presented in the preceding paper (18). Accordingly, the protein is confined in the water pool of the reverse micelle, and is protected by a layer of water molecules from the hostile hydrocarbon phase. This model should, however, not be taken too literally. In fact, some small but significant differences from a normal water solution appear at a closer analysis. Thus water molecules have a larger quenching efficiency toward the exposed tryptophan residues of LADH, and, furthermore, there are some small alterations of the main chain peptide conformation in the micellar solution. The physical origin of these structural changes is not clear at the moment. It is possible that, at least partly, this is connected with the particular physical state of water inside the micelles. Whether the physical state of water is in turn connected with the modulation of the catalytic activity of the enzyme remains to be seen.

More generally, these studies show that dehydrogenases are able to maintain their activity in a milieu containing as little as 1% water. The activity profile appears to be rather complex, and at present the various factors that influence such a behavior are not completely clarified. The actual pH_{wp} value, the w_0 value, the change in the enzyme's pK, and perhaps the anomalous properties of water are likely to be among the most important determinants of the micellar enzymatic solutions. More studies are needed in order to sort out their individual effects.

Finally, we comment on $(K_m)_{ov}$ and $(K_m)_{wp}$. Since the enzyme is localized in the water pool, one should compare K_m in water with $(K_m)_{wp}$. By doing so, a large apparent decrease in affinity would appear in the micelle with respect to water. This smaller affinity might be rationalized on the basis of the particular interactions acting among the reaction partners in the micelles (e.g., because of the particular charge distribution). However, we feel that clarification of the physical meaning of the kinetic parameters in the micellar solution will require further conceptual elaboration.

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