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Effects of bile salts on the solubility and activity of yeast alcohol dehydrogenase in AOT reversed micelles

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Abstract

The effects of two bile salts, sodium taurocholate (NaTC) and 3-[(3-cholamidylpropyl) dimethylammonio]-1-propane sulfonate (CHAPS), on the solubility, stability and catalytic activity of yeast alcohol dehydrogenase (YADH) in bis(2-ethylhexyl) sodium sulfosuccinate (AOT) reversed micelles are reported. Spectroscopic measurements of extrinsic fluorescent probes and intrinsic fluorescent residues of YADH indicate that adding bile salt to AOT reversed micelles changes the relative polarity of the interfacial region and the rotational freedom of the solubilized enzyme. Activity measurements were performed using ethanol as the substrate and β-nicotinamide adenine dinucleotide (β-NAD) as the coenzyme. YADH activity in the reversed micelles is optimal at pH 8.0 and increases with increasing ω (ω = [water]/[AOT]), presumably due to larger water pools in the micellar interior. Activity is lower in the AOT micelles than in buffer solution but some activity is regained upon addition of bile salt. Addition of bile salt also increases the stability of YADH in the reversed micelles. Kinetic analysis of the enzymatic reaction data shows that YADH has a lower affinity for ethanol in reversed micelles than in buffer, likely due to partitioning of the ethanol among the different phases in the reversed micellar media. Bile salts increase both the affinity of YADH for ethanol and the reaction velocity in the reversed micelles. The effects of bile salt are attributed primarily to an increase in the size of the water pool in the reversed micelle in the presence of bile salt, which allows the enzyme to assume a more active conformation.

Keywords: Micelles; Buffer solution; Bile salt

1. Introduction

Reversed micelles have been used as novel systems for enzyme catalysis for decades. These colloidal microsuspensions of water in organic solvent offer the possibility of studying enzymatic reactions of both water-soluble and water-insoluble substrates. Studies generally have focused on enzymes in traditional, three-component reversed micellar systems containing surfactant and water in the organic solvent. Extensive research has shown that adding a fourth component as a cosurfactant can modify and, in some cases, stabilize the reversed micellar structure [1].

Recent work has investigated the effects of bile salt cosurfactant on AOT reversed micelles in a novel, four-component microreactor for enzyme catalysis. It was shown that the activity of the water-soluble, globular enzyme chymotrypsin is greater in reversed micelles than in buffer [2–4], and that addition of bile salt further increases the activity by as much as 200%

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[4]. Dramatic improvements in the stability and activity of lipase, a surface-active enzyme, were obtained in the same system [5]. It was proposed that the superactivity of these enzymes in the four-component system is due to modification of the microenvironments inside the water pool and in the surfactant layer through bile salt interactions [3,4].

We report here on the effects of solubilization in the four-component system on yeast alcohol dehydrogenase (YADH). Alcohol dehydrogenases comprise an important group of enzymes that react with a wide variety of alcohols, aldehydes and ketones. The catalytic centers of these enzymes are similar but the size and number of active groups differ depending on the source of the enzyme. YADH is a tetrameric enzyme with eight zinc ions and a total molecular mass of 150 kD. The active site of each subunit contains one zinc ion, which is necessary for enzyme activity. A second zinc ion present in each subunit is thought to play a conformational role by stabilizing the tertiary structure of YADH.

Compared to other alcohol dehydrogenases, such as Horse Liver alcohol dehydrogenase, much less is known about the catalytic properties of YADH in reversed micelles. YADH is of interest to us for characterization of the four-component microreactor for several reasons. Unlike chymotrypsin and lipase, YADH is composed of several subunits and its catalytic activity requires a coenzyme as well as the presence of a metal ion in its active site. The coenzyme is treated as a second substrate in kinetic analysis, which is a less investigated area in micellar enzymology. Furthermore, since YADH has affinity for a wide range of alcohols, substrates of varying lipophilicity could be used in order to study the effects of diffusion and partitioning of substrate on the overall rates of enzymatic catalysis in the reversed micelles and the effects of bile salts on these processes.

2. Experimental section

2.1. Materials

AOT (MicroSelect, ≥99% (TLC), Fluka), isooctane (99%, spectrophotometric grade, Fluka), NaTC (ULTROL grade, >99.5%, Calbiochem), CHAPS (>99%, Calbiochem), yeast alcohol dehydrogenase (from Baker Yeast, >90%, Sigma), β-NAD (Sigma), fluorescein (sodium salt, Sigma), 2,6-anilinonaphthalene sulfonate (2,6-ANS, Molecular Probes), Tris/HCl buffer (Sigma) and absolute ethanol (Sigma) were all used as received.

Unless otherwise noted, the reversed micellar system to which NaTC (or CHAPS) was added is isooctane/100 mM AOT/0.1 M Tris/HCl buffer. The water:AOT ratio (ω) is 13. It should be noted that the ω value refers to the ratio of the total concentrations of water and AOT that are added to the system, and do not necessarily describe the actual ratios of water:AOT in each of the individual reversed micelles. NaTC (or CHAPS) concentration ranged from ≤1 to 20 mM, which is the limit of NaTC (or CHAPS) solubility in this particular reversed micellar phase.

2.2. Spectroscopic studies

Fluorescent probes were dissolved in absolute ethanol. Samples were prepared by evaporating the ethanol using ultra pure nitrogen, followed by the addition of NaTC or CHAPS, then the AOT/isooctane solution, and finally buffer. The resulting solution was stirred by a magnetic stir bar at room temperature.

For spectroscopic measurements of intrinsic absorbance or fluorescence of YADH, a stock solution of YADH in buffer was injected into AOT/isooctane solutions (with or without bile salt) as the aqueous phase. Buffer was added to adjust the final ω value. The solution was then stirred at room temperature until clear.

Absorption spectra were measured using a Perkin-Elmer Lambda 6 double-beam spectrophotometer. Samples were contained in quartz cuvettes and maintained at $25.0 \pm 0.1^{\circ}$C in the thermostatted sample compartment. Spectra were collected at scan speed of 120 nm/min.

Fluorescence emission spectra were obtained using a spectronic instruments model 48000 multifrequency spectrofluorometer with 450 W xenon arc lamp as the excitation source. The instrument is equipped with single grating monochromators for excitation and emission wavelength selection, and a photomultiplier tube for detection. A reference channel is used for ratiometric correction of intensity fluctuations. Samples were contained in quartz cuvettes and equilibrated at $25.0 \pm 0.1^{\circ}$C for 10 min in the thermostatted sample compartment.

The intrinsic fluorescence emission spectra of YADH were measured over the range of 300–400 nm using an excitation wavelength of 275 nm. Fluorescein and 2,6-ANS were excited at 488 and 320 nm, respectively, and emission spectra were measured over the ranges of 500–600 and 350–600 nm, respectively. In order to observe variations in fluorescence intensity due to quantum yield only without influence of varying molar absorptivity, the emission spectra were divided by the absorbance at the excitation wavelength.

Fluorescence anisotropy was measured using an SLM 48000 spectrofluorometer in the L-format. Excitation wavelength was selected using the monochromator, and emission wavelength was selected by a 10 nm bandpass interference filter (520 nm and 400 nm for fluorescein and 2,6-ANS, respectively) or a 345 nm longpass filter for YADH. Data were acquired using in-house software in which the polarizers alternate between horizontal and vertical orientations so that the fluorescence anisotropy can be calculated using an instrumental correction factor. Anisotropy values are reported as the average of triplicate determinations with error bars representing the standard deviation.

2.3. Measurements of enzyme activity

The activity of YADH was measured in aqueous buffer and in the reversed micelle system. Aqueous buffers and the final aqueous pool of the reversed micelles consisted of 0.1 M Tris/HCl buffer.The pH variations in the reversed micelles were accomplished by altering the pH of the stock solution prior to addition to the reversed micellar solution. Direct measurement of the pH of the aqueous pool of the reversed micelles was not attempted. Varying amounts of ethanol substrate and β-NAD stock solutions were added to volumetric flasks containing solid NaTC or CHAPS. A stock solution of AOT/isooctane was then added to the volumetric flasks and stirred until the bile salt was solubilized and the solution became clear. A final ω value of 13 was obtained by the addition of enzyme solution.

After equilibration of sample solutions in cuvettes at $25.0 \pm 0.1^{\circ}$C in the spectrophotometer for 10 min, a small aliquot of YADH in reversed micelles was injected into the sample cuvette to initiate the reaction. The accumulation of product, β-NADH, was monitored through the absorbance change at 340 nm. The molar absorptivity (ε) was determined to be $5500\,M^{-1}cm^{-1}$ in the reversed micelles. Absorbance data were collected at 2 s intervals for a period of 5 min. The initial reaction velocity was calculated using linear regression of the change in absorbance during the first 60 s after initiation of the reaction.

2.4. Kinetic analysis

YADH typically operates by an ordered mechanism with one ternary complex [6,7]. The common rate equation can be expressed as:

$$\frac{1}{\nu} = \left(\frac{K_m^A}{V_{max}} + \frac{K_s^A K_m^B}{V_{max}[EtOH]}\right)\frac{1}{[\beta\text{-NAD}]} + \left(\frac{1}{V_{max}} + \frac{K_m^B}{V_{max}[EtOH]}\right)$$

where K_m^A, K_m^B are Michaelis constants for β-NAD and ethanol, respectively. K_s^A is the dissociation constant of YADH-β-NADH, V_{max} is the maximum specific reaction rate and ν is the initial velocity of ethanol oxidation.

Kinetic parameters were determined from the initial velocities of the enzymatic reaction by varying β-NAD concentration at each of five different concentrations of ethanol substrate. Plots of $1/\nu$ versus 1/[β-NAD] for each ethanol concentration generated a set of straight lines in the primary plot. The slopes and the intercepts of these lines were plotted versus 1/[EtOH] in the secondary plot, yielding two straight lines from which the kinetic parameters (K_m^A, K_m^B, K_s^A, V_{max}) could be calculated.

3. Results and discussions

3.1. Spectroscopic characterization of AOT/isooctane reversed micelles with bile salt

In previous work, fluorescent probes were used to characterize microenvironmental heterogeneity in the four component system consisting of heptane/AOT, NaTC (CHAPS)/water at $\omega = 10$. This system was studied as a microreactor for chymotrypsin [2] and lipase [5]. YADH is a larger, heavier enzyme than chymotrypsin or lipase, which necessitated some modification of the reversed micellar system. A

higher AOT concentration was used in order to form an aqueous pool large enough to accommodate the YADH enzyme and isooctane was used instead of heptane to increase the aggregation number, i.e. the average number of detergent molecules per micelle. The higher AOT concentration reduced the amount of bile salt that could be dissolved and therefore reduced the maximum bile salt:AOT molar ratio that could be achieved. Because of these modifications, we performed fluorescent probe studies on the new system for comparison with the previous system.

Fluorescein is hydrophilic and resides at the AOT/water interface [2] where it can respond to microenvironmental changes at the interface and in the aqueous core. The emission maximum of fluorescein is shifted to the red as NaTC or CHAPS are added, which is indicative of increasing microenvironmental polarity. The same effect was observed in the previous system. The fluorescence anisotropy of fluorescein increases as NaTC or CHAPS is added to the system. The anisotropy is a measure of the average angular displacement of a fluorophore in the excited state; an increase in anisotropy indicates a decrease in rotational diffusion of the fluorophore. Thus, the increase in the anisotropy of fluorescein indicates a decrease in rotational freedom due to interactions with bile salt at the micellar interface. This, combined with the increase in overall size of the micelles in the presence of bile salt, cause the increase in anisotropy. In previous studies [2,5], the anisotropy consistently passed through a minimum at the lower bile salt concentrations which was ascribed to a disruption of the micellar organization. No such minimum is observed in the current system.

The lipophilic probe 2,6-ANS is located near the AOT/organic interface [2]. Addition of NaTC or CHAPS causes a significant increase in the absorbance-adjusted fluorescence intensity of ANS. A blue shift in the emission maximum indicates decreasing polarity of the microenvironment. The anisotropy of 2,6-ANS stays fairly constant across the bile salt concentration range, showing only a slight increase at high bile salt concentrations. This is similar to the results in the previous system [2,5], but the magnitude is much smaller here, probably because the concentration ratio of bile salt to AOT is smaller and has less effect on the interfacial structure.

3.2. Solubility of YADH in AOT/isooctane reversed micelles

3.2.1. Effect of ω on enzyme solubility

The molar ratio of water to surfactant (ω) affects most of the structural and physical features of reversed micelles and is one of the most important parameters affecting enzyme solubility and activity in reversed micelles. Under the present experimental conditions, the microemulsions were isotropically clear, stable, transparent solutions within an ω range of 5–20. Above this range, the microemulsion becomes unstable. Below this range, solutions become cloudy upon addition of enzyme because the water pools are too small to completely contain a YADH. Based on fluorescence intensity of the enzyme (Fig. 1), maximum solubility is at $\omega = 13$. According to the literature [8,9], most of the water in the reversed micellar core is bound to the polar headgroups of the surfactant at ω values up to 13, above which free water exists inside the core.

The anisotropy (Fig. 2) of the intrinsic fluorescent residues of YADH is higher in the reversed micelles than in buffer, which indicates that the rotational freedom of the fluorescent residues becomes more restricted in the reversed micelles. Anisotropy decreases as ω is increased from 5 to 10 and then

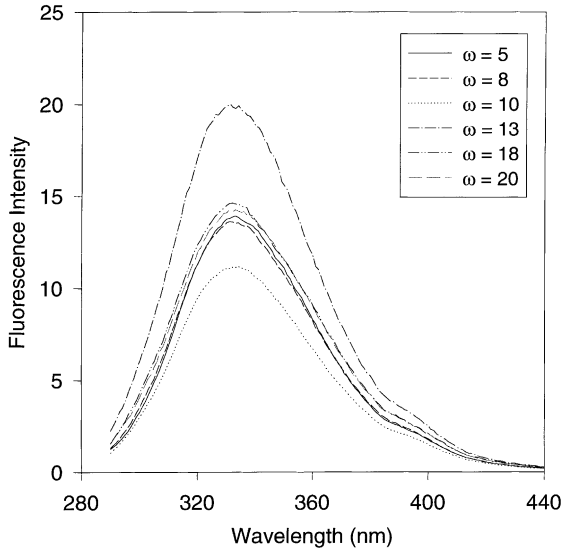

Fig. 1. Fluorescence emission spectra of YADH in reversed micelles at different ω values.

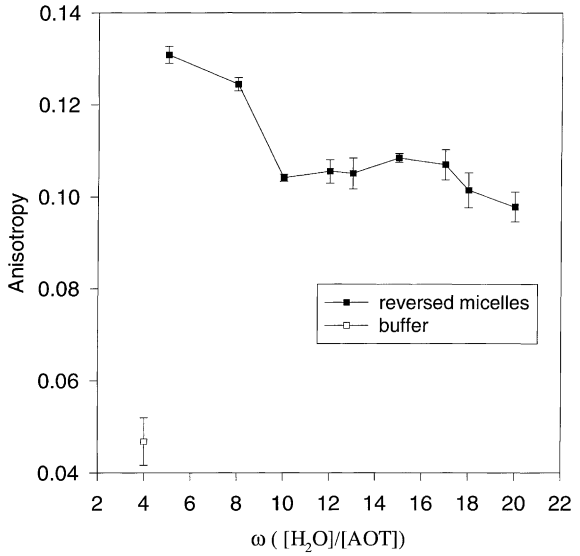

Fig. 2. Anisotropy of the intrinsic fluorescence of YADH vs. ω in reversed micelles; anisotropy in buffer is also shown.

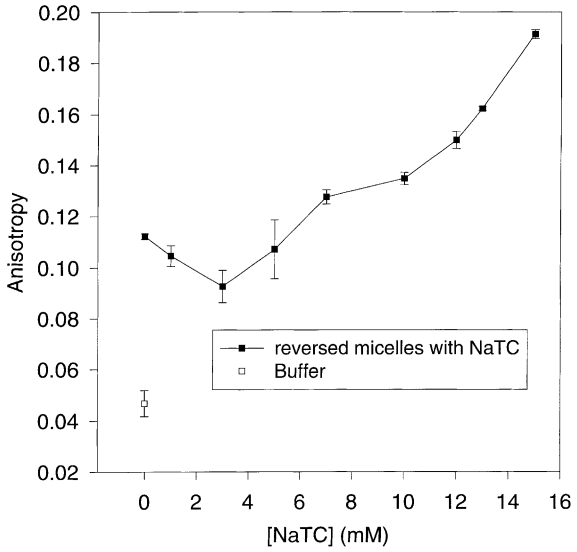

Fig. 3. Anisotropy of the intrinsic fluorescence of YADH vs. [NaTC] in reversed micelles ($\omega = 13$); anisotropy in buffer is also shown.

levels off, indicating that the fluorescent residues are freer to rotate in the larger water pools.

3.2.2. Effect of bile salt on enzyme solubility

UV–VIS absorption spectra of YADH in the reversed micelles were obtained for different concentrations of bile salt. The presence of 3 mM NaTC lowers the baseline of the absorption spectrum of YADH, indicating reduced light scattering, and the fluorescence emission intensity of YADH increases approximately 10-fold. These results indicate increased solubility of YADH. Further addition of NaTC decreases the intensity, falling back to the original intensity at 18 mM NaTC. Similar results were obtained for CHAPS.

The anisotropy of the fluorescent residues in YADH decreases for NaTC concentrations up to 3 mM and then increases (Fig. 3). The decrease at low NaTC may reflect disorganization of the detergent layer, perhaps facilitating reorganization of the surfactants about the protein. At higher concentrations, NaTC increases the organization and rigidity of the surfactant layer and the protein must reconfigure its own structure in order to fit into the reversed micelle.

Conformational changes of proteins, such as those that occur upon solubilization of the protein in a reversed micelle, can often be monitored by using circular dichroism (CD). Unfortunately, CD could not be used in this work because the strong absorbance of AOT masked the CD signal of YADH.

3.3. Enzyme activity

3.3.1. Effect of ω

Fig. 4 shows β-NADH absorbance versus time for different ω values. Results for the same reaction in buffer are also shown. Absorbance increases as β-NAD is reduced to form β-NADH during the YADH-catalyzed oxidation of ethanol. YADH activity is highest in buffer. The activity in the reversed micellar solutions approaches that in buffer as ω is increased and the properties of the water pool approach those of bulk water. There is a big increase in going from $\omega =$ 10 to 13 due to the dramatic increase in solubilization over this range, after which smaller increases are observed. An ω value of 13 was chosen for subsequent activity studies since it is optimal for YADH solubilization and provides high activity without compromising the stability of the reversed micellar solution.

3.3.2. Effect of pH

Buffer stock solutions of pH ranging from 7 to 9 were prepared for studying the effect of pH on the

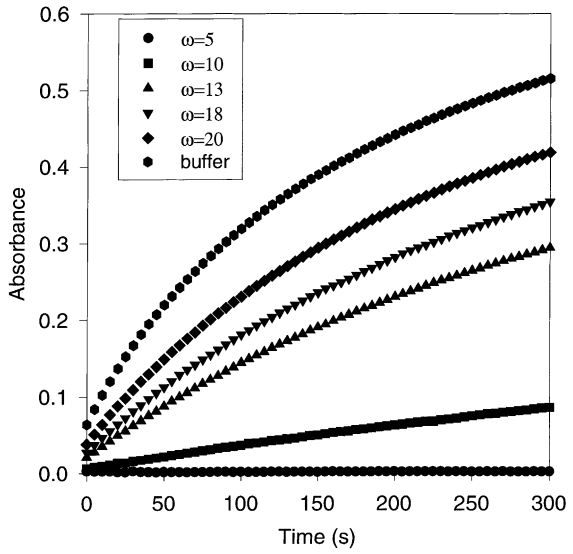

Fig. 4. β-NADH absorbance vs. time during YADH-catalyzed oxidation of ethanol in reversed micelles with various ω and in buffer.

enzymatic reaction in the reversed micellar system. As described previously [5], the pH of stock buffer solution (pH_{ST}) was used to approximate the pH inside the water pool (pH_{WP}). It has been reported that pH_{WP} is generally within 0.4 pH unit of pH_{ST} [10], thus this is a reasonable assumption.

YADH-catalyzed oxidation of ethanol in AOT reversed micelles with varying NaTC or CHAPS concentration was measured at various pH. Results show that the enzyme is quite active between pH_{ST} 7.5 and 8.5, but exhibits diminished activity outside this range. This may be due to conformational changes as the pH is varied. The fluorescence emission spectrum from the tryptophan residues in YADH is sensitive to the degree of exposure of the residues to the external solution. As pH_{ST} is decreased below 7, the emission maximum is blue shifted, indicating a more hydrophobic environment for the tyrosine and tryptophan residues, probably due to folding of the protein. These results are similar to the effects of pH in bulk aqueous solution, in which extreme acidic conditions induce the folding of protein while alkaline conditions lead to unfolding [11,12].

In contrast to previous results for chymotrypsin [4], addition of bile salt did not shift the optimum pH for YADH activity in the reversed micelles. Maximum initial velocity was obtained at pH 8.0 in all cases. As mentioned above, the lower concentration ratio of bile salt to AOT in the present system may reduce perturbation of the AOT reversed micellar structure. Also, compared to chymotrypsin, YADH has a more compact conformation that may afford better shielding from external influences.

3.3.3. Effects of bile salts

YADH activity was studied in the reversed micellar system with Tris buffer at pH 8.0 and containing 3 mM bile salt (NaTC or CHAPS). As expected, increasing the coenzyme concentration increases the slope and magnitude of the reaction curve. Table 1 lists the kinetic parameters of YADH obtained from kinetic analysis of the data.

K_m^A and K_m^B are the affinities of YADH for coenzyme and substrate, respectively [7]. An increase in these values indicates a decrease in affinity. YADH has less affinity for ethanol in the AOT reversed micelles without bile salt compared to aqueous buffer solution. This is consistent with published reports [13,14],

Table 1
Kinetic parameters for YADH-catalyzed oxidation of ethanol substrate in either reversed micellar solutions (isooctane/100 mM AOT/1300 mM water) with no bile salt, 3 mM NaTC, or 3 mM CHAPS, or in 0.10 M Tris/HCl buffer[a]

Parameters	Solution media			
	AOT	AOT/NaTC	AOT/CHAPS	Buffer
V_{max} (mM/s)	3.1×10^{-4}	3.5×10^{-4}	3.3×10^{-4}	5.6×10^{-4}
K_m^A (mM)	0.097	0.076	0.075	0.056
K_m^B (mM)	0.43	0.30	0.28	0.021
K_s^A (mM)	0.16	0.21	0.22	0.24

[a] Standard deviations were estimated to be <0.4% for the linear regression analysis of the initial velocity curves and <3% for the kinetic parameters.

although the experimental conditions varied among the different studies. Addition of 3 mM bile salt to the reversed micelles decreased K_m^A and K_m^B, indicating increased affinity of YADH for both coenzyme and substrate.

V_{max} for ethanol oxidation is larger in aqueous buffer solution than in the reversed micelles without bile salt, which agrees with a previous report on a similar reaction catalyzed by YADH [14,15]. Bile salt increases V_{max} in the reversed micelles, possibly due to the increased affinity for substrate as well as changes in the enzyme conformation and the water pool. Also, it is possible that bile salts change the partitioning of ethanol, increasing its accessibility to YADH.

3.3.4. Enzyme stability

The stability of YADH in the reversed micelles, expressed as the change in the initial velocity of the catalyzed reaction over time upon addition of the YADH stock solution in buffer to the reversed micelles, is lower than the stability of YADH in bulk buffer and slightly increased by addition of bile salt.

3.3.5. Octanol substrate

The activity of YADH was lower when octanol replaced ethanol as the substrate. This is expected since octanol is essentially insoluble in water and partitions into the bulk organic phase in the reversed micellar solution. Consequently, the reaction depends upon diffusion of substrate and reversed micelles, which governs the rate of collisions between them. The octanol must then penetrate the surfactant layer in order to come into contact with the YADH, which has a small substrate binding pocket [16] that does not favor binding of alcohols with long hydrocarbon chains. It is possible that penetration is facilitated by interactions of the hydrocarbon chain of octanol with the hydrocarbon tails of the AOT, and further by interactions of the polar hydroxyl group with the bile salt molecules.

3.3.6. Binding of substrate and coenzyme to YADH

The binding of YADH to the coenzyme and to the substrate in the reversed micellar media was studied by titrating the enzyme with β-NAD, ethanol, or octanol. β-NAD significantly quenched the intrinsic fluorescence of YADH, and the quenching increased with increasing concentration of β-NAD. The results indicate that in reversed micelles, the coenzyme directly binds to the amino acid residues of YADH in the reversed micelles, as has been previously observed for YADH in aqueous solution [17].

In contrast to the coenzyme, titration with alcohol substrate had little effect on the fluorescence intensity of YADH, indicating that the alcohol does not bind directly to the enzyme, at least not in the vicinity of the tryptophan residues or in such a way as to affect their fluorescence through conformational changes. It has been proposed that, in aqueous solution, alcohol binds directly to the zinc ion center as a ligand [18]. Based on the fluorescence emission spectra, it appears that this is the binding situation in reversed micelles as well.

Since alcohols are often used as cosurfactants in reversed micelle formation, it is likely that the alcohol substrate influences the structure of the reversed micelles. To explore this, fluorescein and 2,6-ANS were used to probe the effects of ethanol and octanol on the detergent layer. Addition of either alcohol shifted the absorption and emission spectra of fluorescein towards those observed in bulk ethanol, indicating a decrease in microenvironmental polarity. The magnitude of the effect was larger with ethanol. The emission intensity of 2,6-ANS showed a similar, small decrease upon addition of either alcohol, indicating increased polarity in the microenvironment of the probe. These effects are opposite to those observed for bile salt discussed above, from which it can be concluded that bile salt and ethanol have very different effects when serving as cosurfactants in AOT reversed micelles.

4. Conclusion

The concentration ratio of water to AOT (ω) and the bile salt concentration are key factors that can affect enzyme solubility and activity in the four component reversed micellar system. Both ω and bile salt affect the size of water pool, which changes the solubilization and conformation of the enzyme. Bile salt also modifies the structure of the detergent layer in the reversed micelle, which may affect the

enzyme through specific molecular interactions as well as changes in local pH and polarity. Changes in the interfacial structure may also affect the partitioning and accessibility of substrate and coenzyme.

For YADH in the AOT reversed micelles, increasing the size of the water pool by either increasing ω or adding bile salt increases enzyme solubility and activity. Reversed micelles with relatively low water content, i.e. small ω, do not provide sufficient space to completely encapsulate YADH in the water pool, presumably forcing YADH into a less active conformation. Bile salts increase the uptake of water in AOT reversed micelles and allow the formation of larger water pools. In addition, bile salt decreases the micropolarity at the detergent/isooctane interface and increases the micropolarity at the detergent/water interface. These effects favor the native state of YADH and significantly increase its affinity for both substrate and coenzyme.

The effects of bile salt are smaller than those previously observed for chymotrypsin in a similar four-component system. These differences likely originate from a combination of two factors. First, the effects of the alcohol substrate in the YADH system are opposite to those of the bile salts, which would reduce the impact of bile salts on enzyme conformation and activity. Second, the bile salt:AOT ratio was lower here than in the previous systems, diluting the effects of bile salt on the reversed micellar structure. This leads us to conclude that the effectiveness of bile salt cosurfactant to increase the activity and/or stability of enzymes in AOT reversed micelles will be greatest for enzymes that can be solubilized without pushing the stability limits of the reversed micellar system itself.

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