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Dissociation of lactate dehydrogenase in aqueous and reversed micellar solutions

A time-resolved polarized fluorescence study

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Dissociation behavior of lactate dehydrogenase from hog muscle, both in aqueous solution and reversed micelles of sodium bis(2-ethylhexyl sulfosuccinate) in octane was studied using time-resolved polarized fluorescence spectroscopy. It was found that, in aqueous solutions, the enzyme underwent partial dissociation with the formation of isolated subunits at enzyme concentrations below 8 nM. Dissociation of the enzyme also took place upon entrapment of lactate dehydrogenase into reversed micelles under conditions of low surfactant hydration, when micelles were not large enough to accomodate a whole protein molecule.

Dissociation behavior of oligomeric enzymes in general and lactate dehydrogenase (LDH) in particular has received much attention because of the close interrelation existing between functional properties and the association state of these complex proteins [1]. Analysis of available experimental data shows that, despite considerable progress in this field, certain aspects of the dissociation phenomenon still have to be elucidated. For example, although it is generally recognized that the dilution of aqueous LDH solutions leads to the dissociation of the enzyme, different reports give quite contradictory estimates of the actual lower concentration limit of existence of the tetrameric form, ranging from 0.7 nM [2] to 1 μM [3] and 7 μM [4]. One of possible reasons for these discrepancies is that the investigations mentioned above were performed using ultracentrifugation or gel-permeation chromatography. An inherent feature of these experimental techniques is that they inevitably generate a gradient of the protein concentration, which, in turn, could affect the concentration-dependent association state of the enzyme.

Another interesting aspect of the dissociation behavior of oligomeric enzymes has been recently revealed within the framework of micellar enzymology, which deals with enzymes entrapped in surfactant reversed micelles in organic solvents [5, 6]. Several oligomeric enzymes, including LDH, have been reported to dissociate into monomers or dimers during the entrapment into reversed micelles [7, 8]. The existence of reversed micelles containing monomers and dimers of oligomeric enzymes was derived from measurements of catalytic activity and ultracentrifugation studies, performed at varying surfactant hydrations [7, 8], and has not been yet confirmed using independent direct physical techniques.

The aim of the present work was twofold: first, to clarify the dissociation behavior of LDH in aqueous solutions at high dilutions, and second, to investigate the possibility of dissociation of the enzyme upon entrapment into reversed micelles. The experimental technique used in this study was time-resolved polarized fluorescence spectroscopy of tryptophan residues, which are in abundance in LDH (each of four subunits of the enzyme contains six tryptophan residues [9]). This method represents a powerful tool to assess structural characteristics of fluorescent species in solution by measuring their rotational correlation times which are directly related to the hydrodynamic radius, provided that a sufficiently long-lived fluorescence lifetime component is present to carry the fluorescence anisotropy signal which contains the rotational diffusion information. Such a long fluorescence-lifetime component is present in LDH [9]. Although multiple tryptophan-containing proteins may be considered as complex macromolecular systems to be studied with time-resolved fluorescence, the presence of more tryptophan residues can also be an advantage in measuring rotational correlation times of proteins. The majority of globular proteins cannot be considered as spherical rotors; the shape is better described by ellipsoids of revolution. Consequently, when using time-resolved fluorescence anisotropy, the rotational properties depend on the orientation of the transition dipoles relative to the main symmetry axis [10]. The presence of many randomly distributed, fluorescing tryptophan residues within one protein implies that all orientations are sampled and therefore one can obtain the average overall rotational correlation time of the globular macromolecule from time-resolved fluorescence anisotropy. An important feature of this technique is that it is essentially nonperturbing with respect to the protein structural organization. Time-resolved polarized fluorescence spectroscopy has been successfully

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Abbreviations. LDH, lactate dehydrogenase; AOT, sodium bis(2-ethylhexyl sulfosuccinate); MEM, maximum entropy method.

Enzyme. Lactate dehydrogenase (EC 1.1.1.27).

applied for structural characterization of different proteins both in aqueous [11, 12] and reversed micellar systems [13, 14], including concentration-dependent dissociation of a fluorescamine-labelled bacterial LDH [15].

MATERIALS AND METHODS

Materials

LDH from hog muscle (molecular mass 140 kDa) was obtained from Boehringer in the form of a suspension in ammonium sulfate solution. Prior to use, the enzyme was dissolved in 20 mM potassium phosphate, pH 7.0, and purified by repeated (threefold) ultrafiltration in a Centricon centrifuge tube (Amicon) with molecular mass cutoff of 30 kDa. The enzyme concentration in the resulting stock solution was determined by the absorption at 280 nm using the molar absorption coefficient of 1.96×10^{-5} M^{-1} cm^{-1} [16]. The enzyme stock solution was stored at room temperature to prevent cold inactivation [17]. 20 mM potassium phosphate, pH 7.0, was used as an aqueous component throughout the work. Sodium bis(2-ethylhexyl sulfosuccinate) (AOT) (Sigma) and *n*-octane (Merck) were used as received. The water content of the AOT preparation was determined to be 0.4 mol H_2O/mol AOT, using Karl Fischer titration performed on a Mettler DL 18 automatic titrator. This value was taken into account during calculations of the water content of reversed micellar systems, expressed as the ratio of molar concentrations, $w_o = [H_2O]/[AOT]$.

Preparation of reversed micellar solutions containing solubilized LDH

In a typical experiment, 6–13 µl stock enzyme solution with concentrations 8.2–30.7 µM and a required amount of buffer were added to the spectrophotometric cell containing 2 ml 0.1 M solution of AOT in octane, followed by vigorous shaking to achieve solubilization. Fluorescence measurements were performed with 5–10 min after solubilization.

Time-resolved polarized fluorescence measurements

Polarized fluorescence-decay curves were measured by the time-correlated single-photon-counting technique. The excitation source consisted of the frequency-doubled output of a cavity-dumped DCM dye laser which was synchronously pumped by a mode-locked Nd-YLF laser (Coherent, Inc.). The excitation pulse frequency was 600 kHz, the pulse duration was less than 4 ps and the excitation light was vertically polarized. Further technical details are given in [18, 19]. Two excitation wavelengths were used, 295 nm and 302 nm. The former wavelength was selected to enable measurements to be made at extremely high enzyme dilution. The latter wavelength was chosen for red-edge excitation. Emission was measured through a combination of a WG335 cutoff filter (Schott) and a 348.8-nm interference filter of 5.4 nm bandpass (Schott). The instrumental response function, corresponding to the laser pulse convoluted with the detection response, was determined by measuring the fluorescence decay of *p*-terphenyl (BDH) in ethanol (fluorescent grade, Merck) having a lifetime of 1.05 ns [11]. Tryptophan fluorescence in LDH was sampled during 20 cycles of 10 s in each polarization direction where the detection frequency of the parallel polarized component was set to 30 kHz (5% of 600 kHz) to prevent pulse pile-up. The reference compound (*p*-terphenyl) was sampled during two cycles of 10 s in each polarization direction. Background (same solution but without protein) was sampled either at 20% or, in case of 30–40% background, at 50% of the sample acquisition time. One complete measurement consisted of measuring the polarized fluorescence decays of the reference compound, the sample, the background and again the reference compound. Especially at very dilute enzyme solutions, it turned out that the fluorescence intensity was fading during the measurement cycle. This was due to irreversible photobleaching in which the illuminated volume of the sample could not be replenished with fresh, non-irradiated material. Note in this respect that the laser intensity was increased to reach the same 30-kHz photon frequency. This complication could be overcome by gently stirring the enzyme and background solutions, thereby maintaining a constant fluorescent intensity during the measurement cycle.

All measurements were performed at 20°C.

Data analysis

Analysis of the total fluorescence decay $I(t)$ and fluorescence anisotropy decay $r(t)$ were performed with the commercially available maximum entropy method (MEM; Maximum Entropy Data Consultants Ltd.). The principle of MEM has been described in the literature [20] and full details, related to the experiments on LDH, can be found in [19]. In the analysis of the total fluorescence decay of tryptophans in LDH, 150 equally spaced values on a $\log(\tau)$ scale over 0.01–20 ns were used. This yielded an image $\alpha(\tau)$ of the fluorescence decay which was introduced as a fixed image in the analysis of the anisotropy decay. As a starting model, 100 equally spaced values on a $\log(\phi)$ scale (0.1–1000 ns) were used in the analysis and a limiting anisotropy was encoded to allow for enzyme which is immobilized during the experimental time span, which was typically 80 ns.

In all cases the χ^2 values were close to 1.0, and the weighted residuals between experimental and calculated decay curves and the autocorrelation function of the residuals [11] were randomly distributed around zero, indicating an optimal fit to the data. Reproducibility was found to be satisfactory in duplicate experiments, and the standard errors in the parameters presented in the results section were based upon multiple determinations.

The hydrodynamic radius of fluorescent species, R_h, was calculated using the Stokes-Einstein equation

$$\phi = 1/6D_{rot} = 4\pi R_h^3\eta/3kT, \quad (1)$$

where ϕ is the rotational correlation time, D_{rot} is the rotational diffusion coefficient, η is the viscosity of the medium (1.00 c*P* for water and 0.54 c*P* for *n*-octane at 20°C [21]), k is the Boltzmann constant, and T is the absolute temperature.

RESULTS AND DISCUSSION

LDH fluorescence in water

A typical example of the experimental total decay of LDH fluorescence in aqueous solution is shown in Fig. 1 (curve 1). The fluorescence decay was found to be highly heterogeneous. The fluorescence lifetime distribution of LDH (Fig. 2A) consists of four well-resolved peaks at 0.12 ± 0.03, 1.0 ± 0.1, 3.7 ± 0.3 and 6.8 ± 0.1 ns, independently of the enzyme concentration. The three latter values

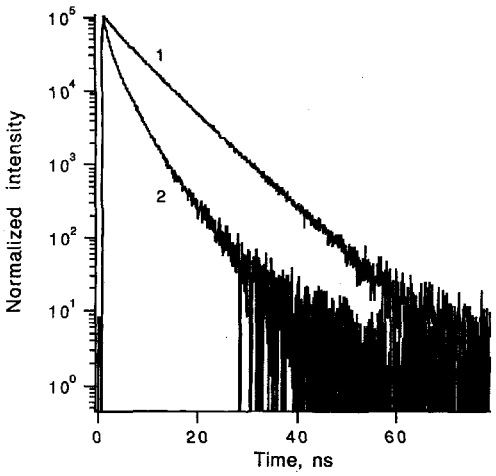

Fig. 1. Experimental total decay of LDH fluorescence in aqueous solution at the enzyme concentration of 70 nM (1), and in AOT reversed micellar system at $w_o = 23.0$ and overall enzyme concentration of 0.38 μM (2).

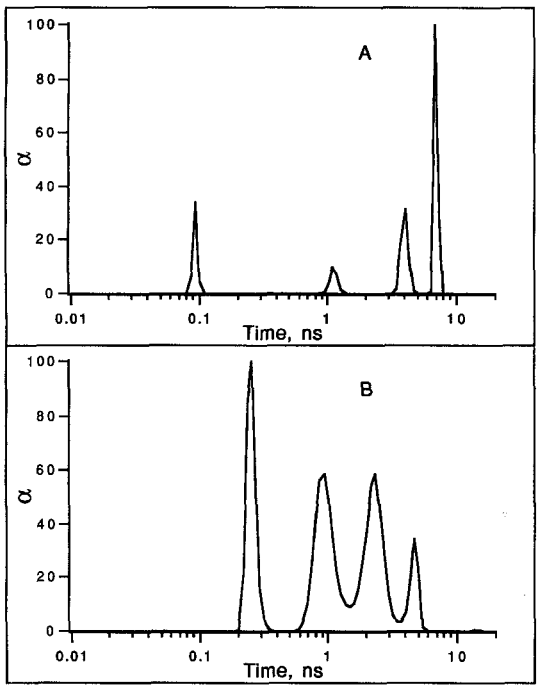

Fig. 2. Fluorescence lifetime distribution of LDH in aqueous solution at enzyme concentration of 70 nM (A), and in AOT reversed micellar system at $w_o = 23.0$ and overall enzyme concentration of 0.38 μM (B).

are in good agreement with lifetimes of 1, 4 and 7.6 ns, reported for LDH from pig heart [9].

The fluorescence anisotropy decay of LDH was measured in the enzyme concentration range 1.0−330 nM. As an example, Fig. 3 A shows the experimental polarized intensity decay curves (parallel and perpendicular) obtained at the enzyme concentration of 70 nM. The two curves are initially clearly different, but they merge together at longer times (around 50 ns), indicating that the polarization of the fluorescence vanishes because of slow rotational diffusion of the enzyme. Both curves were globally analyzed by MEM to obtain the distribution of correlation times. The correlation

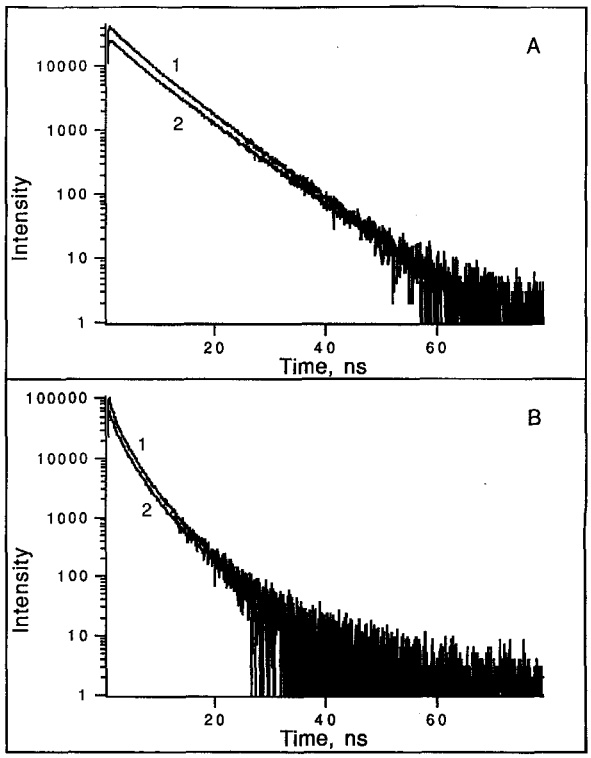

Fig. 3. Parallel (curve 1) and perpendicular (curve 2) fluorescence intensity decay curves of LDH in aqueous solution at enzyme concentration of 70 nM (A), and in AOT reversed micellar system at $w_o = 23.0$ and overall enzyme concentration of 0.38 μM (B). Intensity is expressed in photon counts.

time distribution pattern contained a peak at 0.69 ± 0.17 ns which appeared at all enzyme concentrations (Fig. 4). This short correlation time can be assigned either to fast internal motions of tryptophan residues in the polypeptide chain or to inter-tryptophan energy transfer. The latter process can be ruled out because of the following arguments. If energy transfer is a depolarizing mechanism, then this effect will be diminished or disappear when excitation is at the red-edge of the absorption band [12]. When excitation was at 302 nm corresponding to red-edge excitation, the same peak at 0.7 ns appeared with similar amplitude (data not shown). The shorter component of the correlation time distribution must therefore be ascribed to rapid internal flexibility of tryptophan residues in the protein.

On the contrary, the longer component of the correlation time distribution, which corresponds to the rotation of the protein molecule as a whole, showed a pronounced dependence on the enzyme concentration. At LDH concentrations above 8 nM, the long correlation time remained unchanged and was equal to 59.6 ± 4.2 ns (Fig. 4 A), which corresponds to the hydrodynamic radius of 3.86 ± 0.16 nm. The latter value is in a good agreement with dimensions of the tetrameric LDH molecule in solution, which can be approximated by an ellipsoid of revolution with semiaxes 3.7×3.7×4.2 nm [22]. However, at decreasing LDH concentrations, a new peak appeared in the distribution profile at 10.5 ± 1.0 ns (Fig. 4 B). This correlation time corresponds to the hydrodynamic radius of 2.16 ± 0.01 nm and can be assigned to the isolated subunit of LDH, whose radius was estimated to be 2.3 nm [8]. It must be pointed out that LDH dimers, possessing the minimal estimated radius of 2.9 nm [8], would

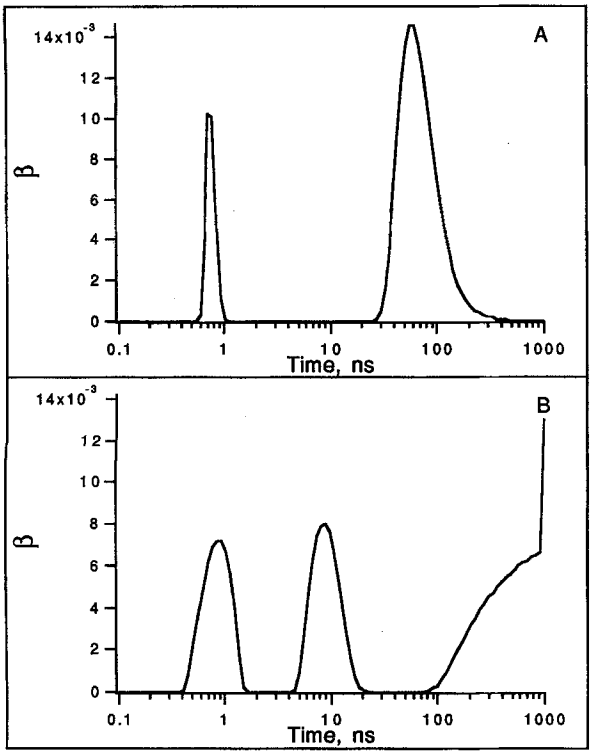

Fig. 4. Correlation time distribution patterns of fluorescence anisotropy decay of LDH obtained in aqueous solutions at different enzyme concentrations. (A) LDH concentration 70 nM. Similar profiles were obtained also at other six different enzyme concentrations of 8.1−330 nM; (B) LDH concentration, 1.0 nM. Similar profiles were obtained also at enzyme concentrations of 2.0 nM and 4.1 nM. The peak at ϕ = 1000 ns corresponds to the infinite anisotropy value.

give rise to the correlation time of at least 22 ns, which is clearly distinguishable from the actually observed value of 10.5 ns. These results unambiguously show that LDH undergoes dissociation with the formation of isolated subunits at concentrations below 8 nM. One should note, however, that correlation time-distribution profiles, observed at low enzyme concentrations, invariably contained considerable unresolved contributions of much longer correlation times (Fig. 4B), implying that the dissociation was far from being complete even at the lowest enzyme concentration (1 nM) we could reach without an unacceptable deterioration of the signal to background ratio.

LDH fluorescence in reversed micelles

A typical example of the experimental total decay of LDH fluorescence obtained in AOT reversed micellar system at w_o = 23.0 is shown in Fig. 1 (curve 2). The fluorescence decay is shortened as compared to that in aqueous solution (Fig. 1, curve 1), indicating that the tryptophan fluorescence exhibits dynamic quenching in reversed micelles. The fluorescence decay was again highly heterogeneous and did not show any consistent dependence either on w_o or on the overall enzyme concentration, which were varied in the range 14.3−31.0 and 0.10−0.38 μM, respectively. The fluorescence lifetime distribution of LDH entrapped into AOT reversed micelles (Fig. 2B) consists of four peaks at 0.31 ± 0.05, 1.1 ± 0.2, 2.7 ± 0.5 and 5.3 ± 0.8 ns. One can observe that the longer lifetimes (2.7 ns and 5.3 ns) are shortened as compared to their counterparts observed in buffer. In addition, the contribution of the emitter with the longest lifetime to the total fluorescence is considerably reduced (Fig. 2 A and B). These results indicate that the microenvironment of LDH inside reversed micelles significantly differs from that in aqueous solution at all studied values of w_o.

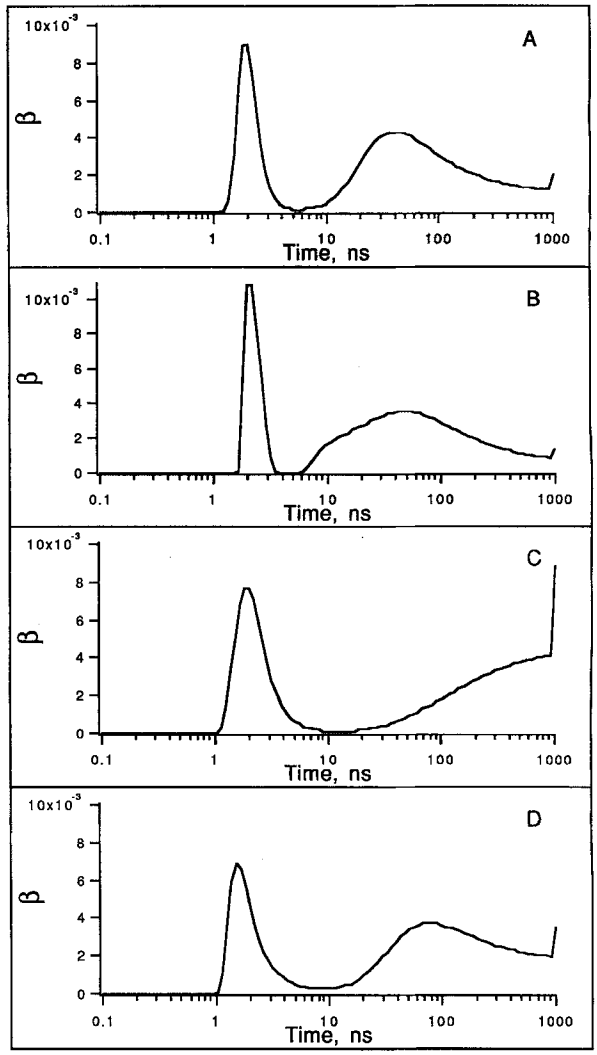

Fig. 5. Correlation time distribution patterns of fluorescence anisotropy decay of LDH obtained in AOT reversed micellar systems at different w_o and overall enzyme concentrations. (A) w_o = 14.3, [LDH] = 0.15 μM; (B) w_o = 17.8, [LDH] = 0.15 μM; (C) w_o = 23.0, [LDH] = 0.15 μM; (D) w_o = 14.3, [LDH] = 0.38 μM. The peak at ϕ = 1000 ns corresponds to the infinite anisotropy value.

Fig. 3B shows an example of two experimental polarized fluorescence intensity decays (parallel and perpendicular) of LDH entrapped into AOT reversed micelles, obtained at w_o = 23.0. Although not as clear as in the case of the aqueous solution, the difference between parallel and perpendicular components persists over 3−4 decades of intensity. The curves were subjected to MEM analysis as described above. At all water contents studied, w_o = 14.3, w_o = 17.8, w_o = 23.0 and w_o = 31.0, the distribution of rotational correlation times revealed a peak at 1.9 ± 0.2 ns (Fig. 5). The latter value is more than twice as high as the short correlation time observed in water, indicating that internal motions of the pro-

tein are slower as compared to those in aqueous solution. This conclusion is in line with results of previous studies which showed that the protein conformation could become more rigid upon entrapment into reversed micelles [23, 24].

Unlike the short correlation time, the longer component of the correlation time distribution, which can be assigned to the rotation of the protein-containing micelle, showed a pronounced dependence on w_o. Since the workable time span was clearly less than for LDH in aqueous solution (40 ns and 60 ns, respectively; Fig. 3 A and B), the distribution of the longer correlation time was broader than in the case of LDH in aqueous solution (Fig. 4 A and Fig. 5 A). The rising water content resulted in a rapid increase of the longer correlation time, so that at $w_o > 17.8$, the corresponding peak in the distribution profile could not be resolved (see Fig. 5 A–C). The increase in the apparent correlation time indicates that enzyme-containing micelles grow in size with increasing w_o until, at a certain point, they become so large that very long correlation times, corresponding to their slow rotation, cannot be resolved any more on the time scale of anisotropy-decay measurements. The peak values of the long correlation time, determined at the overall LDH concentration of 0.15 μM, were found to be 38 ns at $w_o = 14.3$ and 46 ns at $w_o = 17.8$, which correspond to the radius of protein-containing micelles equal to 4.1 nm and 4.4 nm, respectively. Since the thickness of the surfactant shell is approximately 1 nm [25], the radii of the core of protein-containing micelles existing at $w_o = 14.3$ and $w_o = 17.8$ do not exceed 3.1 nm and 3.4 nm, respectively. Clearly, these micelles are not able to accommodate much larger tetrameric LDH molecules (see above), implying that the entrapment of the enzyme into AOT reversed micelles at these w_o values is accompanied by its dissociation into smaller species, although based on the present results it is hardly possible to distinguish whether these species represent dimers or monomers. It is to be stressed that the concentration of LDH in the aqueous stock solution used for solubilization was in the micromolar range, where neither monomers nor dimers could be detected (see Fig. 4 A). The reason for the micellar-induced dissociation probably lies in the fact that the size of LDH molecules considerably exceed that of aqueous cores of initial empty micelles existing at $w_o = 14.3$ and $w_o = 17.8$ (the radius of aqueous cores is 2.5 nm and 3.1 nm, respectively [25]). The size difference means that the enzyme cannot be fitted freely into the micellar interior, and therefore it is forced to disintegrate into smaller species in the process of solubilization. The radius of LDH-containing micelles is larger than that of initial empty micelles (4.2 nm compared to 3.5 nm at $w_o = 14.3$ and 4.5 nm compared to 4.1 nm at $w_o = 17.8$), implying that during solubilization under these hydration conditions the enzyme tends to create its own tailor-made reversed micelle around itself, in competition with forces causing its dissociation. These results are in full agreement with previously reported findings that LDH as well as other oligomeric enzymes can undergo dissociation during entrapment into reversed micelles under conditions of low surfactant hydration, when micelles are not large enough to accommodate a whole protein molecule [7, 8]. The occurrence of dissociation was derived from a thorough analysis of the dependence of catalytic activity of entrapped enzymes on w_o (i. e. on the size of inner micellar cores) in combination with ultracentrifugation studies [7, 8].

The increase of the overall enzyme concentration from 0.15 μM to 0.38 μM produced, at $w_o = 14.3$, a shift of the longer correlation time from 38 ns to 74 ns (Fig. 5 A and D). The latter value corresponds to the radius of the micellar core of approximately 4.1 nm, which is close to the longest dimension of LDH tetramer. These findings imply that LDH exhibits the tendency to preserve its tetrameric structure at increasing enzyme concentration. A similar effect was observed also at $w_o = 17.8$, when the same change in the overall LDH concentration resulted in an increase of the radius of protein-containing micelles from 4.4 nm (correlation time 46 ns) to 5.3 nm (correlation time 81 ns). These results are in line with earlier findings that increasing concentrations of proteins can cause their association inside reversed micelles [25].

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REFERENCES

1. Jaenicke, R. (1982) *Biophys. Struct. Mech. 8*, 231–256.
2. Bartholmes, P., Durchschlag, H. & Jaenicke, R. (1973) *Eur. J. Biochem. 39*, 101–108.
3. Cho, I. C. & Swaisgood, H. (1973) *Biochemistry 12*, 1572–1577.
4. Griffin, J. H. & Criddle, R. S. (1970) *Biochemistry 9*, 1195–1205.
5. Martinek, K., Levashov, A. V., Khmelnitsky, Yu. L., Klyachko, N. L. & Berezin, I. V. (1986) *Eur. J. Biochem. 155*, 453–468.
6. Luisi, P. L., Giomini, M., Pileni, M. P. & Robinson, B. H. (1988) *Biochim. Biophys. Acta 947*, 209–246.
7. Klyachko, N. L., Merker, S., Vakula, S. V., Ivanov, M. V., Berezin, I. V., Martinek, K. & Levashov, A. V. (1988) *Dokl. Akad. Nauk SSSR (Russ.) 298*, 1479–1481.
8. Kabanov, A. V., Klyachko, N. L., Nametkin, S. N., Merker, S., Zaroza, A. V., Bunik, V. I., Ivanov, M. V. & Levashov, A. V. (1991) *Protein Eng. 4*, 1009–1017.
9. Torikata, T., Forster, L. S., O'Neal, C. C. & Rupley, J. A. (1979) *Biochemistry 18*, 385–390.
10. Visser, A. J. W. G., van Hoek, A., O'Kane, D. J. & Lee, J. (1989) *Eur. Biophys. J. 17*, 75–85.
11. Vos, K., van Hoek, A. & Visser, A. J. W. G. (1987) *Eur. J. Biochem. 165*, 55–63.
12. Bastiaens, P. I. H., van Hoek, A., Wolkers, W. F., Brochon, J. C. & Visser, A. J. W. G. (1992) *Biochemistry 31*, 7050–7060.
13. Vos, K., Laane, C., van Hoek, A., Veeger, C. & Visser, A. J. W. G. (1987) *Eur. J. Biochem. 169*, 275–282.
14. Marzola, P. & Gratton, E. (1991) *J. Phys. Chem. 95*, 9488–9495.
15. Clarke, A. R., Waldman, A. B. D., Munro, I. & Holbrook, J. J. (1985) *Biochim. Biophys. Acta 828*, 375–379.
16. Jaenicke, R. & Knof, S. (1968) *Eur. J. Biochem. 4*, 157–163.
17. King, L. & Weber, G. (1986) *Biochemistry 25*, 3637–3640.
18. van Hoek, A. & Visser, A. J. W. G. (1992) *Proc. SPIE 1640*, 325–329.
19. Bastiaens, P. I. H., van Hoek, A., Benen, J. A. E., Brochon, J. C. & Visser, A. J. W. G. (1992) *Biophys. J. 63*, 839–853.
20. Livesey, A. K. & Brochon, J. C. (1987) *Biophys. J. 52*, 693–706.
21. Weast, R. C., ed. (1987) *CRC Handbook of Chemistry and Physics*, 68th edn, CRC Press, Boca Raton.
22. Squire, P. G. & Himmel, M. E. (1979) *Arch. Biochem. Biophys. 196*, 165–177.
23. Klyachko, N. L., Bogdanova, N. G., Koltover, V. K., Martinek, K. & Levashov, A. V. (1989) *Biokhimiya 54*, 1224–1230.
24. Levashov, A. V., Klyachko, N. L., Bogdanova, N. G. & Martinek, K. (1990) *FEBS Lett. 268*, 238–240.
25. Levashov, A. V., Khmelnitsky, Yu. L., Klyachko, N. L., Chernyak, V. Ya. & Martinek, K. (1982) *J. Colloid Interface Sci. 88*, 444–457.