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FORMATION OF HOMO- AND HETEROOLIGOMERIC SUPRAMOLECULAR STRUCTURES BY D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND LACTATE DEHYDROGENASE IN REVERSED MICELLES OF AEROSOL OT IN OCTANE

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Summary: The supramolecular structure of oligomeric enzymes can be specifically regulated by changing the size of an inner cavity of Aerosol OT reversed micelles in octane. Both D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) reveal an ability to exist and function in monomeric, dimeric and tetrameric forms (homooligomers). Various heterooligomeric complexes, in particular, GAPDH monomer - LDH monomer, GAPDH dimer - LDH tetramer were detected in reversed micelles.

Keywords: D-glyceraldehyde-3-phosphate dehydrogenase, Lactate dehydrogenase, Homo- and Heterooligomeric forms, Reversed micelles, Microemulsions, Aerosol OT, Micellar enzymology, Enzyme design.

INTRODUCTION

The systems of surfactant reversed micelles have opened a new direction in enzymology, micellar enzymology, which is aimed at revealing potential opportunities of biocatalysis in a membrane-like environment (see, for example, reviews [1-7]). At the same time micellar systems should be considered both as media for enzymatic reactions, and also as nanoreactors of molecular sizes under control [8]. By simply changing the surfactant hydration degree (w_o= [H_2O] / [Surfactant]) the size of an inner cavity of micelles where protein molecules are entrapped can be varied, which provides protein chemistry and enzymology with a perfect tool for purposeful design of macromolecular associates [8-10]. Most successfully this approach was applied in the case of oligomeric enzymes. Thus, using reversed micelles of Aerosol OT in octane (the well documented system from the point of view of micellar size dependencies on w_o), the possibility for regulation of supramolecular structure of enzymes has been shown on a number of examples: lactate dehydrogenase (LDH) from rabbit muscle [11], D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle [12], ketoglutarate dehydrogenase from pigeon breast muscle [9], pigeon liver malic

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enzyme [13], alkaline phosphatase from calf intestinal mucosa [14-15], γ-glutamyltransferase from hepatoma [16,17], penicillin acylase from E.coli [18], human lysosomal galactosidase [19], angiotensin-converting enzyme [20]. The main feature of oligomeric enzymes functioning in reversed micellar systems is a "wave-like" dependence of their catalytic activity on surfactant hydration degree (micelle sizes) with several maxima. Each optimum represents formation and functioning of the appropriate oligomeric form. The same principle of geometric fit [6-10,21-23] as in the case of simple (protomeric) enzymes is valid for oligomeric ones under optimal conditions. The principle states that the enzyme catalytic activity is maximal when the size of an inner cavity of reversed micelle is equal to that of solubilized protein species (one or another oligomeric form). The structure of protein-containing micelles can be also controlled by an independent non-kinetic method - ultracentrifugation. The description of a procedure for the determination of molecular weight of proteins included into reversed micelles of Aerosol OT (AOT) in octane can be found in [9,24,25].

The purpose of the present work was to establish the ability of oligomeric enzymes in the system of reversed micelles of AOT in octane to form heterooligomeric polyenzymic complexes. Two earlier studied [11,12] enzymes, GAPDH and LDH, were chosen as objects for this research. Both enzymes are involved in the system of glycolysis, thus possessing natural potential to complex formation. In our opinion, the developed procedure can contribute to understanding the principles of organization of natural polyenzymic systems (metabolons).

MATERIALS AND METHODS

Enzymes

Preparations of GAPDH and LDH from rabbit skeletal muscle were obtained as described [26], their specific activity was 77 and 450 units/mg, respectively. Molecular weights of enzymes were accepted equal to 144 and 140 kDa, accordingly [27]. The crystal preparations of GAPDH and LDH were stored in a solution of ammonium sulfate, having saturation 0.67 and containing 5 mM EDTA and 5 mM dithiothreitol. The suspension of enzymes was centrifuged before experiment, the pellet dissolved in the appropriate buffer and desalted on Sephadex G-25 column (1x10cm).

Other reagents

NAD, NADH, NBD-chloride (7-chloro-4-nitrobenzo-2-oxy-1,3-diazole) were from Sigma (USA), barium and calcium salts of fructose 1,6-diphosphate, EDTA, sodium arsenate ($Na_3AsO_4 \cdot 12H_2O$) and glycine were from "Reanal" (Hungary), dithiothreitol from "Serva" (Germany). Preparation of bis(2-ethylhexyl)sulfosuccinate sodium salt (Aerosol OT) was from "Merck" (Germany). According to IR data, the preparation contained 0.5 mol of water per 1 mol of surfactant, that was taken into account when the total amount of water in the micellar system was calculated.

The experiments with GAPDH were carried out in 50 mM glycine buffer, pH 8.5, with LDH - in 20 mM imidazole buffer, pH 7.0.

Chemical synthesis of glyceraldehyde 3-phosphate (GAP) from fructose 1,6-diphosphate was carried out by the method [28]. The complete oxidation of the aldehyde in the presence of GAPDH has shown that the resulting preparation contained 30% GAP.

GAPDH labeled by fluorescent reagent, NBD-chloride, was obtained as described [29], leading to modification of eight SH-groups on tetrameric enzyme.

Catalytic activity of GAPDH in reversed micelles of AOT in octane

The catalytic activity of GAPDH was assayed spectrophotometrically by following the absorbance of NADH at 340nm using $\varepsilon_{340} = 6.22 \cdot 10^3$ $M^{-1}cm^{-1}$. The experiments were carried out at 26°C using a spectrophotometer Beckman-25 (USA) with a thermostated cell holder. Arsenate instead of inorganic phosphate was used to make the reaction of GAP oxidation irreversible. In a typical kinetic experiment 10 μL of 0.12 M GAP was added to 2 mL of 0.1 M AOT in octane; then, certain amount of a buffer solution was added to achieve the desired hydration degree, further - 5 μL of 40 mM NAD solution and 5 μL of 80 mM sodium arsenate solution. After addition of each reagent the mixture was vigorously shaken to achieve an optically transparent solution, 10 μL of acetonitrile was added to the final mixture of the substrates. The reaction was initiated by addition of 1-5 μL of GAPDH (1-5μg). To determine the kinetic parameters of the enzymatic reaction, the final GAP concentration in the system was varied from 10 to 600 μM and NAD concentration - from 10 to 100 μM.

Catalytic activity of LDH in reversed micelles of AOT in octane

The catalytic activity of LDH was assayed spectrophotometrically by following the absorbance of NADH at 366nm using $\varepsilon_{366} = 3.3 \cdot 10^3$ M^{-1} cm^{-1}. The experiments were carried out at 26°C using a spectrophotometer Beckman-25 (USA) with a thermostated cell holder. In a typical kinetic experiment required (10-120 μL) amount of a buffer solution was first added to 2 μL of 0.1 M AOT solution in octane, the system was vigorously shaken; then, 5 μL of 240 μM NADH solution and, the last, the enzyme solution (2 μg) was added. A final mixture was shaken and incubated at 26°C during 10 min. After the incubation 5 μL of 240 μM pyruvate solution and then 10 μL of acetonitrile were added to the system. To determine the kinetic parameters of the enzymatic reaction, the pyruvate concentration was varied from 30 to 600 μM, the concentration of NADH - from 30 to 150 μM.

Catalytic activity of GAPDH in the presence of LDH was assayed by the same procedure as described above for GAPDH itself. A final mixture contained 2.5 μg GAPDH and LDH quantity was varied from 2.5 to 240 μg.

Stability of GAPDH and LDH in reversed micelles of AOT in octane

In a special experiment the stability of GAPDH and LDH in micellar systems was studied. As it was found, the catalytic activity of LDH in reversed micelles containing NAD did not change, at least, during 30 min, and the GAPDH activity did not vary during an hour both in the presence and absence of the cofactor.

Sedimentation analysis

Sedimentation coefficients were determined from the analysis of sedimentograms obtained at 26°C on a Beckman E (USA) analytical ultracentrifuge equipped with a photoelectric scanning device, a monochromator, a multiplexor using 12-mm two-section cells, and a An-G-Ti rotor at 20 000 and 30 000 rpm. The samples were preincubated for 1-2 h. The scanning was performed at 280 nm for reversed micelles containing sole GAPDH or LDH. The scanning was carried out at 420 nm when GAPDH labeled by NBD-chloride was used in the presence, as well as in the

absence of LDH. Theoretical values of the sedimentation coefficients for different oligomeric GAPDH, LDH forms and their heterooligomeric complexes formed in reversed micelles were calculated according to the procedure described in [9,25].

Fluorescence study

Fluorescence measurements were made with a spectrofluorimeter Hitachi F-3000 with a thermostated cell holder at 26°C.

RESULTS AND DISCUSSION

Fig. 1 represents the profiles of the catalytic activity of GAPDH (Fig.1a) and LDH (Fig.1b) on the parameter w_o (surfactant hydration degree). As it was shown earlier [11,12], the character of dependencies reflects formation and functioning of various homooligomeric forms of enzymes, namely monomers, dimers and tetramers (see also pairs of data on Fig.1 a and d, b and e, respectively). The addition of LDH into the system of reversed micelles containing GAPDH brings about the dramatic changes in the profile of the GAPDH catalytic activity. Starting from the equal concentrations of enzymes, LDH concentration was varied (increased up to 100-fold excess). The GAPDH catalytic activity profile in the presence of LDH (62-fold excess) is given in Fig. 1c.

The effect of bovine serum albumin on the catalytic activity of GAPDH was studied as a control experiment. In this case no changes in the profile of GAPDH activity was observed even with bovine serum albumin concentration being hundred times higher than that of GAPDH. In other words, one can conclude that the GAPDH activity profile observed in Fig.1c, does represent the functioning of the enzyme complexes with LDH. It can be clearly seen that at least at hydration degree 34 where maximum is normally observed when both GAPDH and LDH are present in the system (Fig. 1c), and no such a peak on the catalytic activity profiles can be found when the enzymes are studied separately (see Fig. 1a and b). At the same time, although the position of the first peak of the GAPDH activity remains practically unchanged (or even shifted towards more "dry" small micelles), the catalytic activity of GAPDH is far more pronounced in the presence of LDH. Comparing the structures shown in Fig.1g and h, one can assume the formation of associates containing GAPDH and LDH monomers in micelles of small size (Fig. 1i). It could be possible due to the fact that the monomer GAPDH itself has peculiar "bean-like" form. However, additional experimental data are required for strict conclusion about the phenomenon observed.

For this purpose we use traditionally reliable sedimentation analysis (see, for example, [9,12]). However, it is obvious that the excess of one of the components in the mixture of two almost identical molecular weight enzymes limits the reliability of the results of sedimentation analysis. To solve this problem we used a "coloured" GAPDH preparation. For this purpose, the NBD (as a chloride) chromophore label-reporter was

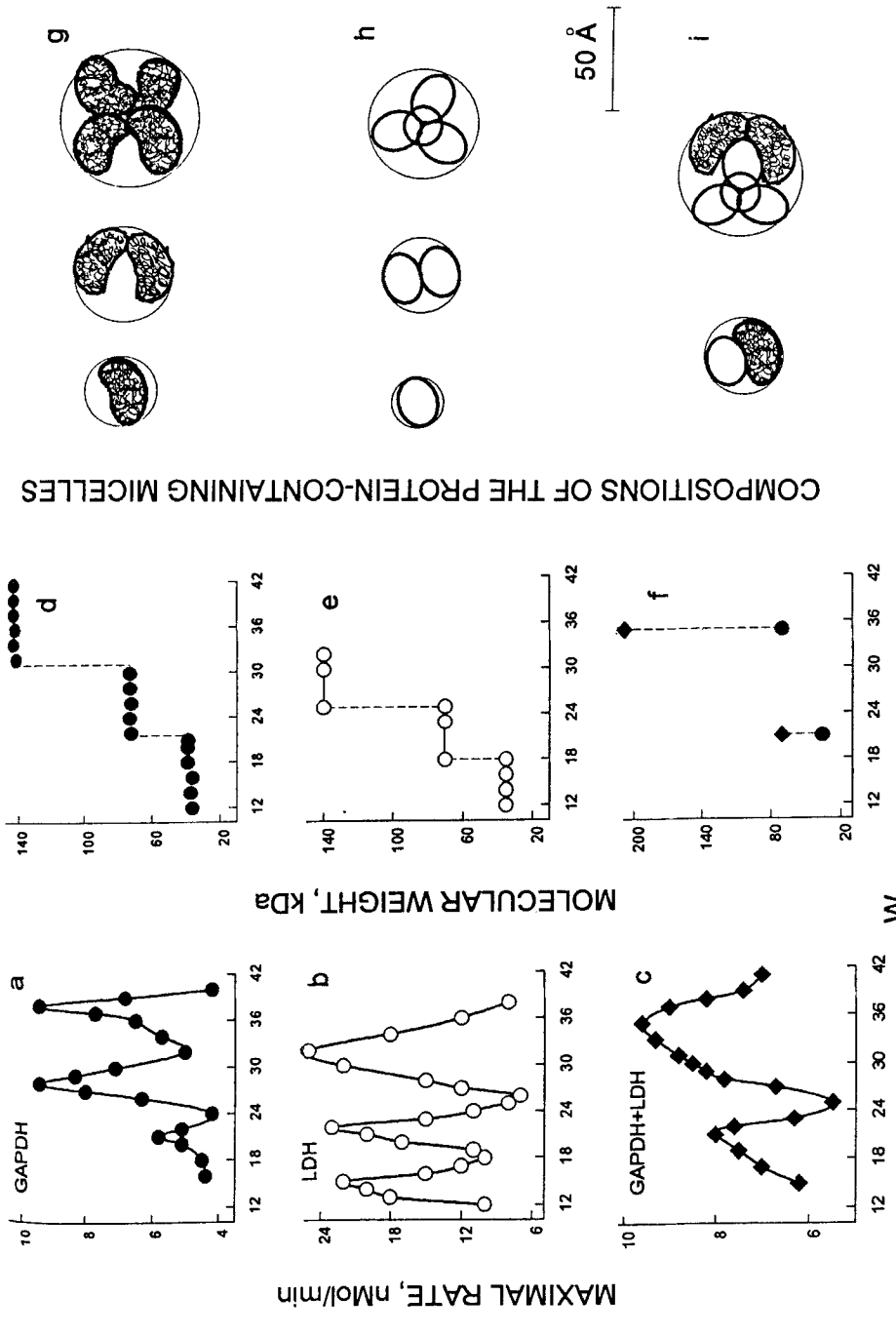

Fig. 1. Properties of GAPDH, LDH and polyenzyme complexes in the system of reversed micelles of AOT in octane. Catalytic activity (Vmax) (a-c), molecular weights - (d-f), and schematic representation of protein-containing reversed micelles (g-i) in dependence on surfactant hydration degree, $w_o=[H_2O]/[AOT]$ (characterizing micelle size). Experimental conditions are given in METHODS. Lactate dehydrogenase and D-glyceraldehyde-3-phosphate dehydrogenase molecular dimensions and forms are given according to their X-ray structures from [30] and [31], respectively.

attached to the GAPDH molecule. It enabled us to follow the sedimentation of GAPDH-containing reversed micelles at λ=420 nm (where LDH and other components of the mixture have no absorbance). In a special experiment we made sure that modification of GAPDH by NBD-chloride did not provoke any changes in sedimentation properties of the enzyme-containing micelles. It was found out that the entrapment of LDH (even only 5-10 times in excess) into GAPDH-containing reversed micelles resulted in the appearance of two types of GAPDH-containing micelles: light (black dots on Fig. 1f) and heavy (black rhombes on Fig. 1f) populations, both at w_0=34 and 21. The molecular weights of aggregates in these fractions calculated from the sedimentation analysis data are given in Fig. 1f. As it is seen, a dimeric aggregate from monomers of GAPDH and LDH appeared at w_0=21, while a hexameric one formed from the GAPDH dimer and LDH tetramer at w_0=34 (black dots and rhombes in Fig. 1f show molecular weights of aggregates).

It is possible that the structure of GAPDH monomers or dimers undergoes some conformational changes, at least being "pressed" by LDH spherical monomer or tetramer in a "wall" of an inner cavity of micelle (see schematic representation on Fig. 1i). The similar situation of close (extended) contact of the GAPDH homodimer with micelle was realized, and it was manifested in a significant increase in an intrinsic GAPDH fluorescence intensity in comparison with that of monomeric or tetrameric forms of the protein [12]. In the case of heterodimeric structure formed at w_0=21 by GAPDH and LDH monomers we also found the fluorescence intensity increasing. This fact proves our suggestion concerning the possibility of such "monomer-monomer" structure shown in Fig. 1i. We admit also that in the system studied some other types of structures can be formed. In this respect we are planning to carry out a sedimentation analysis of GAPDH-LDH-containing micelles at various AOT hydration degrees, and to study the influence of GAPDH present in excess on the structure and activity of LDH. However, even now on the basis of the experiment described it is possible to make a definitive conclusion about the formation of heterooligomeric GAPDH and LDH complexes which is of potential for designing polyenzyme assemblies. We especially would like to draw attention to the fact that such complex formation is accompanied by increasing the catalytic activity of the enzyme which could be the result of protein-protein interactions.

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