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Regulation of Catalytic Activity of Acid Phosphatase by Lipids in a Reverse Micellar System

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Abstract—The influence of biomembrane lipids on the catalytic activity of a peripheral membrane enzyme, acid phosphatase (AP), was studied in a reverse micellar system. It was found that the interaction of AP with lipids led to a number of kinetic effects depending on lipid nature on enzyme function. The observed effects might be caused by the formation of lipoprotein complexes as well as by the influence of lipids on structure and properties of the micellar matrix. The results are important for clear understanding of molecular mechanisms of regulation of the catalytic activity of the membrane-associated enzyme *in vivo*. These data can also be used as a physicochemical basis for application of AP in medical fields as a diagnostic tool for diseases caused by changes in lipid metabolism, e.g. urinary, orthopedic, and allergic diseases.

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Key words: acid phosphatase, reverse micelles, lipids, catalytic activity, secondary structure

Acid phosphatase (AP) is used in applied medicine as a marker for diagnostics of several diseases. AP most precisely reflects the clinical course of orthopedic and urinary diseases [1-6]. Recently data correlating AP activity and degree of severity were obtained for allergic diseases [7]. Diagnostic methods are based on the fact that in pathological states connected with these diseases there are dysfunctions in lipid metabolism that result in changes in activity of membrane-associated enzymes including AP. Acid phosphatase is a peripheral membrane protein and functions *in vivo* mainly in association with the membrane. Therefore, the catalytic properties of AP are largely defined by the lipid content of biomembranes [8]. Lipids play a key role in protein association with membrane, regulation of protein conformation, and enzymatic activity [9]. Kotrikadze et al. [10] found that in a reverse micellar system of surfactants used to model membrane environment, AP was sensitive to changes in lipid composition of the system.

The present work is devoted to the investigation of molecular mechanisms of regulation of the catalytic activity of AP by lipids. For this purpose, the influence of individual lipids on functional properties of AP is considered. In the work a "micellar approach" is used, based on the fact that the surfactant hydration degree, W_0 = $[H_2O]/[Surfactant]$, alters the size of the polar internal cavity of micelles. Each functional form of an enzyme has its optimal hydration degree where the catalytic activity is maximal. Maximum catalytic activity is reached at geometrical coincidence of sizes of internal micellar cavity and solubilized enzyme or its complex [11-15]. This method has been applied for the analysis of changes in catalytic, membrane, and structural properties of AP and also to study the influence of the nature and content of lipids on oligomeric structure of the enzyme. It was demonstrated that AP has a high sensitivity to lipid composition of a system. Functional properties of the enzyme can be regulated by the formation of lipoprotein complexes, and can also be influenced by lipid structure of the membrane, which is defined by the nature of the lipids.

The results of this work can be applied as a physical and chemical basis for the development and improvement of diagnostic methods for some diseases associated with dysfunctions in lipid metabolism.

Abbreviations: AOT (aerosol OT), 1,4-bis(2-ethylhexyl)sulfosuccinate, sodium salt; AP, acid phosphatase; AU, specific activity unit (μmol substrate hydrolyzed by 1 mg enzyme in 1 min); CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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MATERIALS AND METHODS

Preparation of reverse micelles with chosen hydration degree. The necessary amount of AOT (bis(2-ethylhexyl)sulfosuccinate) (Sigma, USA) was dissolved in octane. The final concentration of AOT was varied in the range of 0.03-0.3 M. The solution thus obtained was mixed for 12 h at room temperature. Mixed reverse micelles with included lipid were prepared similarly, dissolving a mixture of AOT and lipid in octane in the necessary proportion. The solution was then mixed at least for 12 h at room temperature. To prepare a solution of reverse micelles of given hydration degree (W_0), 2 ml of micellar solution (V_{mic}) was supplemented with specified amount of aqueous buffer solution (V_{aq}), calculated according to the formula $V_{aq} = 36C_{AOT}W_0$ (μl), where C_{AOT} is AOT concentration (M) [13]. The resulting mixture was vigorously shaken until the formation of a homogenous optically transparent solution.

Determination of catalytic activity of AP in reverse micelles. The catalytic activity of acid phosphatase from wheat sprouts (EC 3.1.3.2) (Sigma) was determined using a Hitachi MPF-4 fluorimeter (Japan) with a thermostatted cuvette unit. The reaction rates were measured registering an accumulation of fluorescent reaction product with the excitation wavelength of 350 nm and emission wavelength of 450 nm. Depending upon the needed hydration degree, 2 ml of 0.03-0.3 M AOT solution in octane was supplemented with 30-200 μl of potassium acetate-citric acid (20 mM each) buffer, pH 5.0, 10 μl AP in the same buffer (initial enzyme concentration was 0.2 mM), and 2 μl of 4-80 mM solution of 4-methylumbelliferyl phosphate (Sigma) in dimethylsulfoxide–dimethylformamide mixture (1 : 1 v/v). The resulting mixture was shaken until the formation of a homogenous optically transparent solution, whereupon the catalytic activity was measured at 37°C. The relative molar fluorescence of 4-methylumbelliferone (Sigma) at 450 nm was determined for all systems studied. Catalytic constants k_{cat} and K_m were determined from the analysis of initial rates of the enzymatic reaction by nonlinear regression. Rate of catalytic reaction was expressed in activity units (AU) of specific enzyme activity (μmol substrate hydrolyzed by 1 mg enzyme in 1 min).

Sedimentation analysis. Sedimentation analysis was performed on a Beckman E analytical centrifuge (USA) equipped with photoelectric scanning device with monochromator and multiplexer using 12 mm bisector cells, using an An-G-Ti rotor at 30,000 rpm. Aqueous buffer solution of AP and micellar solutions with and without lipids added were studied at several hydration degrees. Micellar solutions were incubated for 1-2 h before measurements. Protein-containing micelles were scanned at 280 nm. Sedimentation coefficients of empty micelles were determined in an independent experiment at 405 nm adding 2,4-dinitrophenol for coloring of the micelles. The coefficients were calculated according to the standard procedure [16].

Intrinsic fluorescence spectra of AP. The intrinsic fluorescence spectra of AP in aqueous buffer and reverse micellar solutions were registered using a Hitachi 650 10-S fluorimeter (Japan) at 22°C. The excitation wavelength was 295 nm. The spectrum was scanned in the wavelength range 310-380 nm with slit width corresponding to 5-10 nm resolution. The enzyme concentration was 10 μM. Spectra registered in the corresponding solutions without the protein were used as controls. All solutions were preincubated at room temperature for at least 1 h.

Fluorescence polarization of AP. The fluorescence polarization of AP in aqueous buffer and reverse micellar solutions was determined using a Hitachi F-4000 fluorimeter (Japan) at 22°C. The excitation wavelength was 295 nm, and the emission wavelength was 345 nm. Values of I_{VV} and I_{VH} (fluorescence intensity with parallel and perpendicular positions of excitation and emission polarizers, respectively) were registered. The intensities of parallel and perpendicular components of fluorescence were registered in enzyme solution ($I_{VV}^{(E)}$ and $I_{VH}^{(E)}$) at 10 μM protein and in solutions without protein added ($I_{VV}^{(0)}$ and $I_{VH}^{(0)}$). All solutions were preincubated at room temperature for at least 1 h.

Fluorescence anisotropy value (r) was calculated taking into account the G-factor ($G = I_{HV}/I_{HH}$, a value characterizing the system sensitivity for vertically and horizontally polarized light) according to the equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}),$$

where I_{VV} and I_{VH} are intensities of parallel and perpendicular components of protein fluorescence background subtracted ($I = I^{(E)} - I^{(0)}$) [17].

Circular dichroism spectra of AP. Circular dichroism spectra of AP in aqueous solution and reverse micelles were obtained using a Jasco 720 spectropolarimeter (Great Britain) in a 1 mm cuvette. All solutions were preincubated for 0.5-1 h to achieve equilibrium. Spectra were obtained by 5-fold scanning in the wavelength range of 200-260 nm with 1 nm steps. The concentration of enzyme preparation in the final system was 0.1-0.2 mg/ml. Solutions without the protein added were used as controls. Secondary structures (α-helices, β-sheets, and loops) were quantitatively calculated using the CD Spectra Deconvolution program (version 2.1).

RESULTS AND DISCUSSION

In the initial model system of reverse AOT micelles, the dependency profile of AP catalytic activity on hydration degree is characterized by two optimum of enzyme activity at hydration degrees of 20 and 24 (Fig. 1). Sedimentation analysis of the micellar system showed

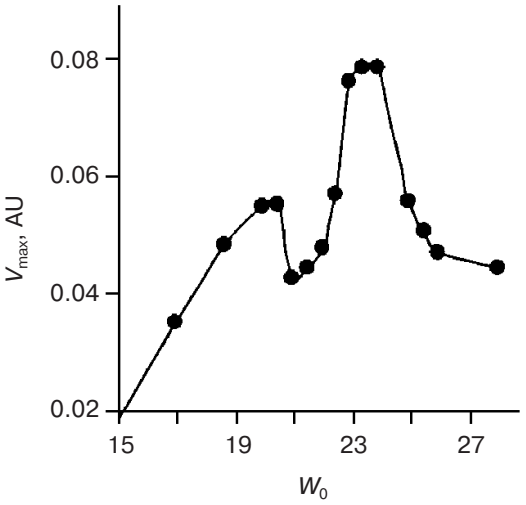

Fig. 1. Dependence of catalytic activity of AP on surfactant hydration degree in the reverse micellar system of 0.1 M AOT in octane. Experimental conditions: 37°C, pH 5.0, 1 μM AP.

that at hydration degree of 20 the enzyme functions in the form of a monomer (48 kDa), and at $W_0 = 24$ – in the form of a dimer (98 kDa). Incorporation of biomembrane lipid in the AOT reverse micellar system (up to 10% by weight) causes a number of kinetic effects on AP function: change in catalytic activity of one or both oligomeric forms of the enzyme; shift of the optimum of enzyme catalytic activity in the region of both lower and higher hydration degrees; "smoothing" of the dependency of enzyme catalytic activity upon hydration degree. The catalytic parameters of AP in the AOT reverse micellar system in the presence of lipids are summarized in Table 1.

In the case of cardiolipin (CL), an increase in AP activity is observed at a hydration degree corresponding to the monomeric form of the enzyme (Fig. 2). Thus, significant (almost 2-fold) increase in the activity of the monomeric form is observed in the system already at 5% CL. Excess CL apparently interferes with dimer formation and therefore causes a decrease in its activity that is observed upon increasing CL content to 10% (Fig. 2b). In micelles containing phosphatidylcholine (PC), a decrease in the activity of both monomeric and dimeric forms of AP was observed in comparison with the AOT reverse micellar system (Fig. 3). And increasing PC content to 10% in the system leads to a shift of the optima of enzyme catalytic activity in the region of lower hydration degrees. In contrast, phosphatidylinositol (PI) incorporation in the AOT reverse micellar system shifts AP optimum activity to the region of higher hydration degrees (Fig. 4). This effect is observed at the lipid content of only 2%.

Unlike the AOT reverse micellar system where two optimum of catalytic activity are observed, the enzyme activity is virtually unchanged with surfactant hydration degree on incorporation of cholesterol and phosphatidylethanolamine (PE) (Figs. 5 and 6). This effect is observed already at 2.5% cholesterol in the system. Sedimentation analysis showed the presence of only the monomeric form of the protein at hydration degrees of $W_0 = 20$ and 24, corresponding to the monomer and dimer in the AOT reverse micellar system. A similar effect was found when the micellar system contained PE, where smoothing of activity peaks both for monomers and dimers of AP was also observed. This effect is amplified on increasing PE content in the system. Thus, sedimentation analysis of the system at hydration degree of 24 (corresponding to the dimer functioning) showed the presence of AP dimers at 2.5% PE, and a mixture of dimer and monomer at 5% PE. This fact indicates that PE hinders dimer formation while interacting with the monomeric form of AP.

It should be noted that the observed effects might be caused by the influence of the lipid on the structure and properties of micelles as well as being a consequence of protein–lipid interactions. In the case of PC-containing

Table 1. Kinetic parameters of the reaction catalyzed by AP in reverse micellar system

Lipid	Lipid content, % (w/w)	W_0 for maximum activity, %		V_{max}, AU		K_m*, μM
		monomer	dimer	monomer	dimer	
Not present	–	20	24	0.055	0.078	20-30
Cardiolipin	2.5	20	24	0.075	0.082	30-60
The same	5	20	24	0.116	0.110	40-60
–"–	10	20	24	0.110	0.078	40-70
Phosphatidylcholine	5	20	24	0.039	0.048	20-30
The same	10	18	22	0.039	0.048	20-30
Phosphatidylinositol	2	23	28	0.055	0.078	20-60
Cholesterol	2.5-10	20-24	30	0.045	0.045	20-30
Phosphatidylethanolamine	2.5-5	20	24	0.050	0.060	20-40

* K_m values are indicated in the concentration range since Michaelis constant is dependent upon AOT hydration degree.

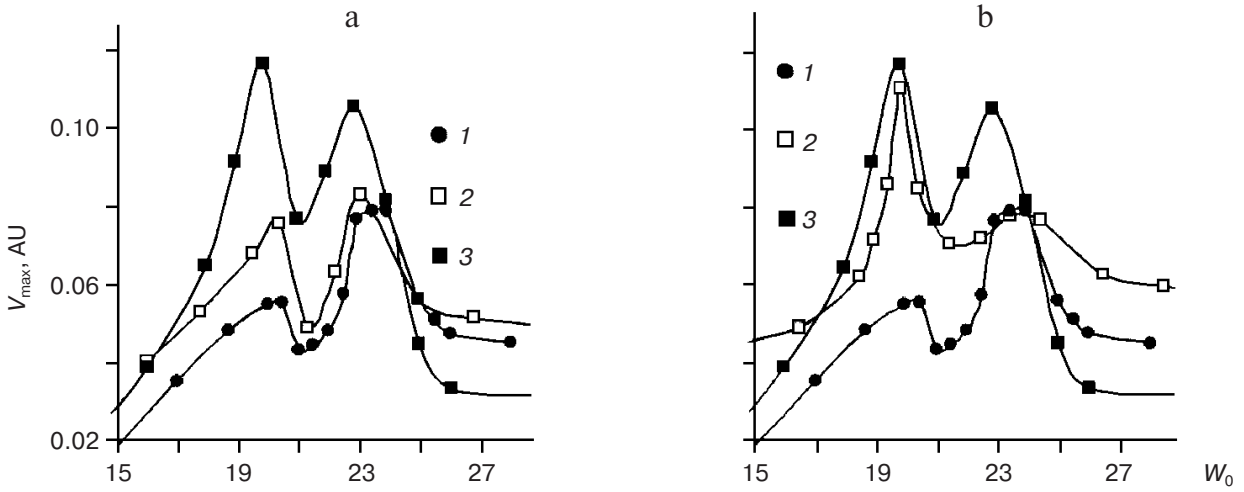

Fig. 2. Dependence of AP catalytic activity on surfactant hydration degree in reverse micellar system of 0.1 M AOT in octane with cardiolipin incorporation of 2.5 and 5% (w/w) (*2* and *3*, respectively) (a) or 5 and 10% (w/w) (*2* and *3*, respectively) (b); *1*) reverse AOT micelles without cardiolipin.

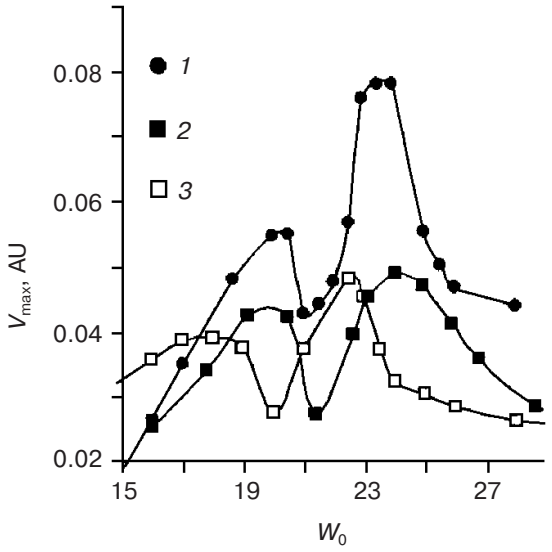

Fig. 3. Dependence of AP catalytic activity on surfactant hydration degree in reverse micellar system of 0.1 M AOT in octane with phosphatidylcholine incorporation of 5 and 10% (w/w) (*2* and *3*, respectively); *1*) reverse micelles of 0.1 M AOT.

micelles, the shift of enzyme activity optimum in the region of lower hydration degrees is most likely to be connected with an increase in micelle size in presence of PC, as demonstrated earlier for PC-containing micelles [18, 19]. The opposite effect is observed for PI: according to the data of sedimentation analysis, PI-containing empty micelles are significantly lighter than those of AOT, and this difference grows upon increase in hydration degree, which could cause a shift of activity profile. This possibility is also supported by sedimentation analysis data for enzyme-containing micelles, which showed the presence of enzyme monomers at hydration degree $W_0 = 23$ and dimers at $W_0 = 28$. On the other hand, it is known that PI can form complexes with proteins [20], which also could cause a shift in AP activity profile. To investigate the possible complex formation of the enzyme with lipids, the enzyme activity was measured in the presence of PI in aqueous buffer solution. It appears that even low (50 μM) PI content in the system increases the enzyme activity two-fold compared with that in the absence of PI. When glucose was added to the system the enzyme activity returned to its initial value because excess glucose interferes with complex formation. It is likely that besides the reduction of micelle size, the complex formation of AP with PI also contributes to the shift in dependency profile of AP catalytic activity towards higher hydration degrees.

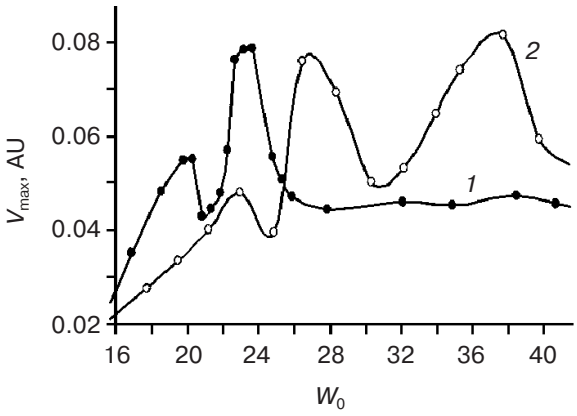

Fig. 4. Dependence of AP catalytic activity on surfactant hydration degree in reverse micellar system of 0.1 M AOT in octane with phosphatidylinositol incorporation of 2% (w/w) (*2*); *1*) reverse micelles of 0.1 M AOT.

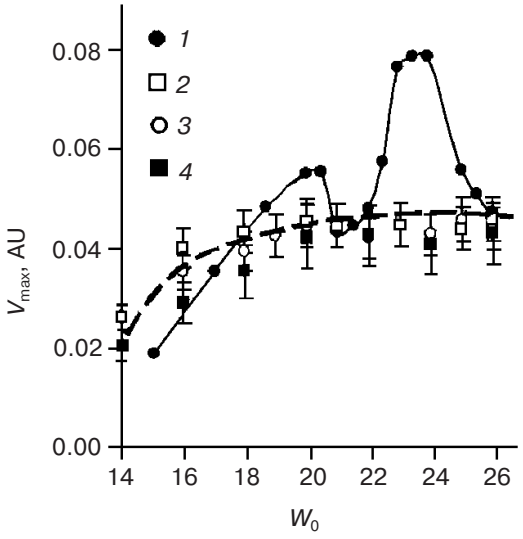

Fig. 5. Dependence of AP catalytic activity on surfactant hydration degree in reverse micellar system of 0.1 M AOT in octane with cholesterol incorporation of 2.5, 5, and 10% (w/w) (*2-4*, respectively); *1*) reverse micelles of 0.1 M AOT.

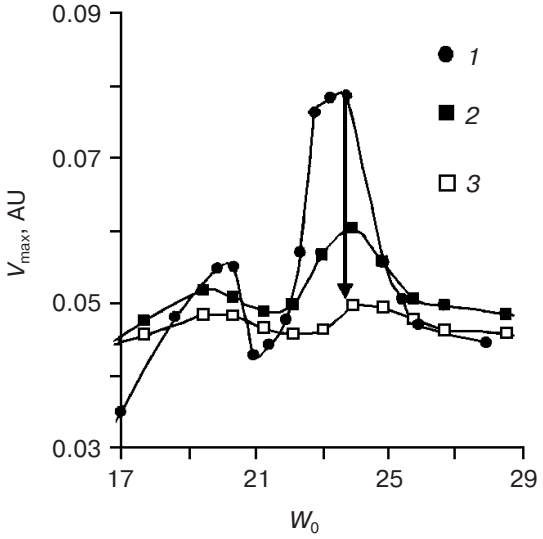

Fig. 6. Dependence of AP catalytic activity on surfactant hydration degree in reverse micellar system of 0.1 M AOT in octane with phosphatidylethanolamine incorporation of 2.5 and 5% (w/w) (*2* and *3*, respectively); *1*) reverse micelles of 0.1 M AOT.

In the presence of cholesterol and PE, an independence of AP activity on hydration degree of micelles can be connected with the formation of enzyme–lipid complex that is not sensitive to the change in micelle size since it is known that both lipids are capable of complex formation with proteins [9]. On the other hand, it can also indicate that PE and cholesterol interact directly with the micelle, "loosening" its structure and hindering the geometrical coincidence necessary for the appearance of optima. It should be emphasized that the ability of cholesterol and PE "to loosen" biomembrane structure is a characteristic feature of these lipids [9, 21-23].

Influence of lipid on protein interactions with micellar matrix. To determine the molecular reasons for the effects observed, the influence of lipids on protein interactions with micellar matrix has been studied. The dependency of the catalytic activity upon surfactant concentration in the reverse micellar system provides a test of the membrane activity of the enzyme. The appearance of the dependency of enzyme activity on surfactant concentration indicates that an enzyme has membranotropic properties. The *y*-intercept of the dependence of enzyme catalytic activity on surfactant concentration indicates the limiting activity value, which corresponds to enzyme activity in one isolated micelle with inter-micellar interaction being eliminated, and the slope reflects the enzyme membranotropic properties.

Figure 7 shows the dependencies of AP catalytic activity on the concentration of AOT. The character of the dependence indicates that both dimeric and monomeric forms of AP possess membrane-active properties. It should be noted that both the enzyme affinity to micellar matrix (slope) and the limiting value of the activity are two-fold different for monomeric and dimeric forms of the enzyme.

The dependence of AP catalytic activity on AOT concentration in reverse micellar system containing CL is presented in Fig. 8. Cardiolipin incorporation in the reverse micellar system does not change the affinities of either monomeric or dimeric forms of the enzyme to the micellar matrix AOT system. Thus, CL has an activation effect on the monomeric form of the enzyme while the limiting

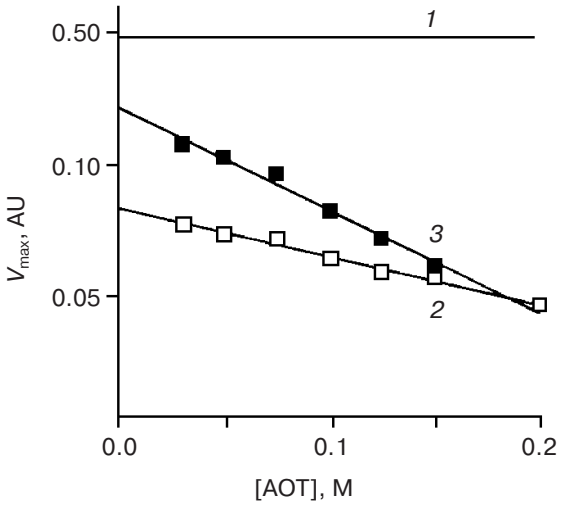

Fig. 7. Dependence of AP catalytic activity on surfactant concentration in AOT reverse micellar system at hydration degrees corresponding to monomeric (*2*) and dimeric (*3*) forms of the enzyme; *1*) AP activity in aqueous buffer solution.

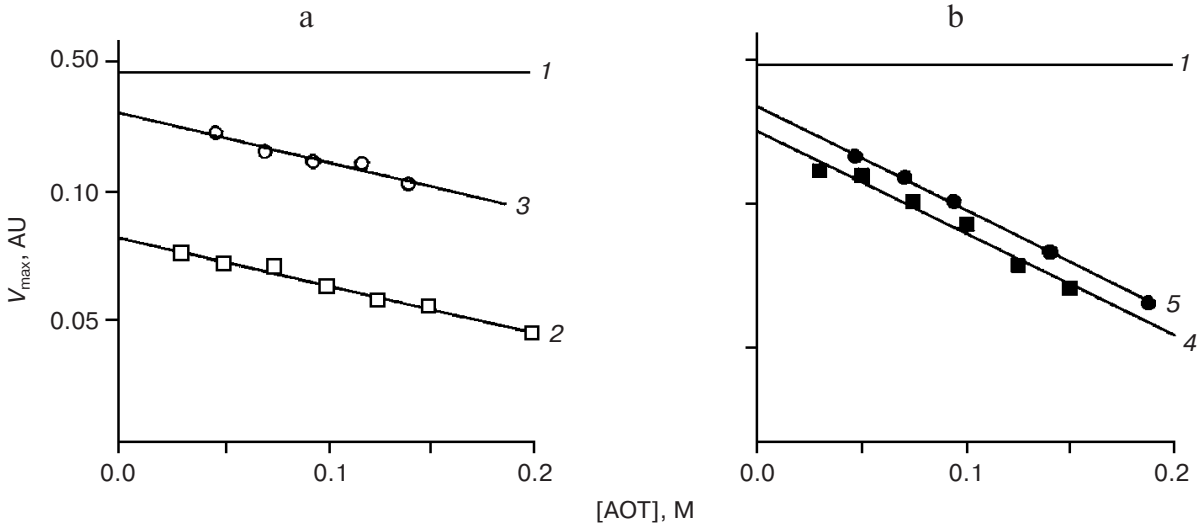

Fig. 8. Dependence of AP catalytic activity on surfactant concentration in reverse micellar system of AOT with cardiolipin incorporation (10%, w/w) at hydration degrees corresponding to monomer (a, curve *3*) and dimer (b, curve *5*) in this system. Curves *2* and *4* correspond to reverse micelles of 0.1 M AOT without lipid incorporation for monomers (a) and dimers (b), respectively; *1*) enzyme activity in aqueous buffer solution.

activity of the dimer in this system is almost unchanged in comparison with the initial model system. Apparently, besides an activating effect, the lipid incorporation in the reverse micellar system can cause either an increase or reduction in enzyme affinity to the micellar matrix. Thus an increase in the affinity to micellar matrix was observed in the case of micelles containing cholesterol (Fig. 9). In contrast, in PI-containing micelles the interaction with micellar matrix (Table 2) was reduced, apparently due to the shielding of membrane interaction sites of the enzyme caused by complex formation with PI. The limiting activity values corresponding to the activity in one isolated micelle for AP monomers and dimers did not change in the presence of cholesterol and PI in comparison with those in AOT reverse micellar system. The data on the influence of lipids on AP membrane activity are summarized in Table 2.

Membranotropic properties of AP is also confirmed by the fluorescent anisotropy method. A significant increase in anisotropy observed during the enzyme solubilization in reverse micellar system (from 0.11 to 0.14) indicates the restriction of enzyme molecular mobility due to the linkage with micellar matrix. And the increase in anisotropy correlates with the affinity to micellar matrix. Thus, introduction of cholesterol into the system (10% by weight) results in an increase in interaction to micellar matrix, which is accompanied by an increase in fluorescent anisotropy (from 0.14 in AOT reverse micellar system to 0.15 in presence of cholesterol). In contrast, the anisotropy remains constant in comparison with the initial AOT system when the presence of lipid does not affect membrane activity of AP (for example, with CL).

Therefore, the investigation of the dependence of activity on surfactant concentration suggests that membranotropic properties of AP are sensitive to the lipid composition of the system. The introduction of lipids of different nature in the reverse micellar system (Table 2) can cause either an increase or a decrease in enzyme interaction to the micellar matrix. Possible changes in AP structure upon lipid incorporation in the reverse micellar system were studied by means of fluorescence and CD spectroscopy.

Structural properties of AP. Possible changes in the tertiary structure of the protein were investigated by fluo-

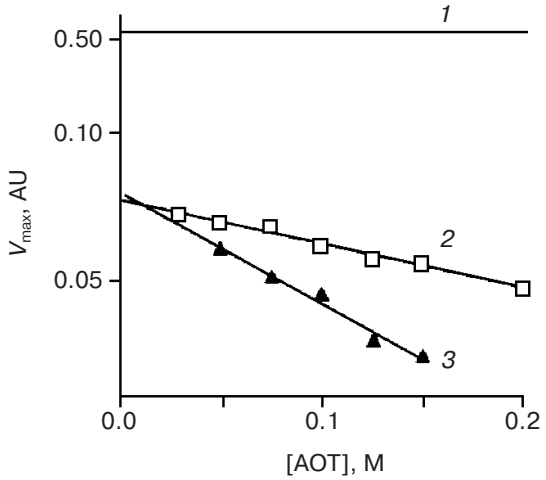

Fig. 9. Dependence of AP catalytic activity on surfactant concentration in reverse micellar system of AOT with cholesterol incorporation (10%, w/w) at hydration degree corresponding to the monomeric form (*3*) in this system; *2*) reverse micelles of 0.1 M AOT without the lipid; *1*) enzyme activity in aqueous buffer solution.

Table 2. Membrane properties of acid phosphatase

System	Affinity to micellar matrix, AU/M		Limiting value of activity, AU	
	monomer	dimer	monomer	dimer
Micelles	36	74	0.07	0.14
Micelles + 10% (w/w) CL	36	76	0.20	0.20
Micelles + 10% (w/w) cholesterol	80	–	0.08	–
Micelles + 10% (w/w) PI	36	53	0.08	0.13

Table 3. Secondary structure content in AP in aqueous buffer solution and in AOT reverse micellar system

System	W_0, %	Content*, %	
		α-helices	β-sheets
Aqueous solution	–	10.1	33.1
AOT micelles	20	8.6	35.7
The same	24	8.7	35.2
Micelles + 5% CL	20	7.9	36.8
The same	24	8.8	34.9
Micelles + 10% cholesterol	20-24	9.6	33.7
The same	30	14.9	28.0

* Error for the determination of secondary structure parameters did not exceed 5%.

rescence spectroscopy. The analysis of intrinsic fluorescence spectra of AP showed that in all systems studied (in the initial AOT reverse micellar system and in presence of lipid) monomeric and dimeric forms of AP are characterized by approximately identical wavelength of fluorescence maximum (342-343 nm) and by different fluorescence intensity (degree of quenching of tryptophan residues of the protein). The highest fluorescence intensity was observed for the dimeric forms of the enzyme. Apparently, this effect resulted from the two-fold difference in the affinity of micellar matrix to monomeric and dimeric forms of the enzyme, and also from the influence of intermolecular protein contacts formed in dimers.

It is interesting to note that unlike AOT micelles, in the presence of cholesterol, the intensity of fluorescence does not depend on hydration degree, as well as the catalytic activity of the enzyme. Thus in cholesterol-containing micelles at hydration degrees of $W_0 = 20$ and 24, AP is characterized by identical fluorescence intensity corresponding to the fluorescence intensity of monomeric form of the enzyme in the AOT system. These results indicate that the mechanism of cholesterol influence on the catalytic activity of AP is more likely to be connected with lipid influence on micelle structure rather with formation of enzyme–lipid complex. The formation of such complex would lead to a significant change in fluorescence spectra of the protein in comparison with that in AOT system. Thus, in the presence of PI quenching of fluorescence was observed for both monomeric and dimeric forms of AP as compared to the AOT system. This effect reflects the formation of AP complex with PI, being one of the reasons for the shift of catalytic activity profile towards higher hydration degrees in PI-containing micelles.

Detailed information on the secondary structure of AP was obtained by CD spectroscopy. Table 3 shows the results of CD spectral analysis of AP secondary structures in aqueous buffer solution and in AOT reverse micellar system. It was found that enzyme solubilization in reverse micelles led to increase in β-sheet content and a decrease in α-helix. The introduction of lipids in the micellar system leads to structural reorganization of AP. Moreover, the influence of lipid on AP structure depends upon the oligomeric structure of the enzyme. Thus, CL incorporation in reverse micellar system does not cause changes in the secondary structure of dimers. However, a decrease in α-helices and an increase of β-sheets were observed for the monomeric form in comparison with the AOT system, which was accompanied by a two-fold increase in the catalytic activity. Moreover, the affinity degree to micellar matrix did not change in comparison with that for the AOT system (Fig. 8). In the case of cholesterol-containing micelles the secondary structures of the enzyme at hydration degrees of $W_0 = 20$ and 24 (both corresponding to the monomeric form of the enzyme) were practically identical compared to those in aqueous solution. It was found that in this system the interactions with micellar matrix do not affect the enzyme structure. This

observation is in good agreement with the hypothesis proposed above that cholesterol interacts with micelle, "loosening" its structure and hindering the realization of the principle of geometric conformity, which is necessary for the appearance of the optimum.

To summarize, our results provide evidence about the fundamental role of lipid in the regulation of catalytic activity of AP. In general, our results provide new information on the mechanisms of lipid functioning and regulation of a number of biological processes in living systems.

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