Volume 278, number 2 143 146 FEBS 09141 January 1991
1991 Federation of European Biochemical Societies 00145793/91 $3 50
(DOI 10 1016/0014 5793(91)80095E)

Regulation of the catalytic activity and oligomeric composition of enzymes in reversed micelles of surfactants in organic solvents

Alexander V Kabanov, Sergey N. Namethin, Natalya L. Klyachko and Andrey V. Levashov

Department of Chemical Enzymology, Moscow State University, Moscow, USSR

Received 26 November 1990

The phenomenon of regulation of the catalytic activity of enzymes via changing their oligomeric composition in the system of reversed micelles of sodium bis(2 ethylhexyl)sulfosuccinate (AOT) in octane was studied using α chymotrypsin (CT) from bovine brain and alkaline phosphatase (AP) from calf intestinal mucosa. The dependences of the enzyme catalytic activity on the AOT hydration degree ($W_o = [H_2O]/[AOT]$), the parameter determining the radius (r_e) of the inner cavity of micelles, usually represent the bell shaped curves. The maximal catalytic activity is observed at such W_o when r_e is equal to the size of the enzyme molecule. The position of this maximum strictly correlates with the enzyme oligomeric composition. Thus in the case of CT this is observed at $W_o \sim 12$ when r_e is equal to the radius (r_p) of the CT globule. In the case of artificially produced conjugate containing six cross-linked CT molecules, this is observed at $W_o \sim 43$ when r_e is equal to the radius of the sphere surrounding the absolute octahedron composed of six CT globules. The dependence of the catalytic activity of AP on W_o represents a curve with two maxima that are observed when r_e is equal to r_p of either AP monomer ($W_o \sim 17$) or AP dimer ($W_o \sim 25$). Ultracentrifugation experiments revealed that variation of W_o causes a change in the oligomeric composition of AP: its transition from monomeric ($W_o \sim 20$) to dimeric form ($W_o \sim 20$). Hence the observed maxima correspond to functioning of different oligomeric forms of AP.

Alkaline phosphatase, Aerosol OT; α Chymotrypsin, Reversed micelle, Micellar enzymology

1. INTRODUCTION

Study of the structure/functional relationships realized in the supramacromolecular protein complexes, particularly, in oligomeric enzymes, belongs to one of the most interesting tasks of modern molecular enzymology [1–4]. However, the progress in these studies is strongly limited by the lack of universal experimental approaches permitting purposeful variation of the supramacromolecular (oligomeric) composition of such complexes [4].

Recently we have formulated a new strategy for the study of oligomeric enzymes which is based on the use of reversed micelles of surfactants in organic solvents as matrix microreactors for the regulation of the oligomeric composition [5]. Being solubilized in the system of reversed micelles, the molecule of the oligomeric enzyme incorporates into the inner water cavity of the micelle, thus acquiring a cover of the hydrated surfactant. The size of the micelle inner cavity (r_e) can be widely varied via changing the surfactant hydration degree (W_o), i.e. the molar ratio $[H_2O]/[surfactant]$ in the system. Using homo- and heterooligomeric enzymes (lactic dehydrogenase [6]; γ-glutamyltransferase [5]) we demonstrated that one can vary the oligomeric composition of the solubilized enzyme via changing the size of the micellar matrix. The principal point here is that oligomeric forms obtained in this way are catalytically active in the micellar systems. So one can study the functional properties of the oligomeric forms artificially produced in the reversed micelles.

This paper is devoted to investigation of the phenomenon of regulation of the catalytic activity of the enzymes via changing their oligomeric composition in the reversed micelles of Aerosol OT in octane by examples of α-chymotrypsin (CT) from bovine pancrease and alkaline phosphatase (AP) from calf intestinal mucosa.

Correspondence address S. N. Namethin, Department of Chemical Enzymology, Faculty of Chemistry, Moscow State University, Leninskiye Gory, Moscow 119899, GSP, USSR

Abbreviations AOT, Aerosol OT (sodium bis(2-ethylhexyl) sulfosuccinate), AP, alkaline phosphatase, CT, α-chymotrypsin, CBZTNP, *N*-benzyloxycarbonyl L-tyrosine *p*-nitrophenyl ester, NP, *p*-nitrophenol, NPP, *p*-nitrophenylphosphate, SPDP, succinimidyl-3-(2-pyridylthio)propionate

2. MATERIALS AND METHODS

2.1. *Enzymes*

CT (EC 3.4.21.1) from bovine pancrease and AP (EC 3.1.3.1) from calf intestinal mucosa were purchased from Serva and Sigma, respectively. Titration of the active sites of CT with *N*-transcinnamoylimidazole (Sigma) [7] revealed that the content of the active enzyme in the initial CT preparation was 50%. AP was purified by gel-filtration on Toyopearl TSK-45 and hydrophobic chromatography on Octyl-silochrom. The enzyme activity was equal to 1.1 units/mg (one unit hydrolyzes 1.0 μmol of NPP per 1 min at pH 10.4, 37°C).

2.2. *Synthesis of the α-chymotrypsin conjugate*

The CT conjugate was synthesized according to [8]. The solution of 2.5 mg of SPDP (Sigma) in 150 μl of ethanol was added to the solution of 50 mg of CT in 1 ml of buffer (0.2 M H_3BO_3, 0.1 M Na_2HPO_4, 0.1 M NaCl, 50 mM acetylphenylalanine, pH 7.0). After 1 h of incubation the reaction mixture was divided into two parts. One part was fractioned on Sephadex G-10 and concentrated to 700 μl. The degree of modification of CT with SPDP, estimated spectrophotometrically by formation of pyridine-2-thiol (ε_{343} = 8080 M^{-1} cm^{-1}) after addition of dithiothreitol (Sigma) was equal to 1.8.

The second part of the solution containing modified CT was acidified to pH 4.7, and then 12.4 mg of dithiothreitol were added to it. After 0.5 h incubation this solution was fractionated on Sephadex G-10, concentrated to 700 μl and added to the first solution containing modified CT with non-reduced S-S bonds. The reaction system was incubated for 20 h and then purified on Sephadex G-10. The CT conjugate obtained was fractionated on Toyopearl HW-55.

The molecular mass of the conjugate estimated from the PAGE and gel filtration data was ≈150 kDa, which corresponded to the CT hexamer.

2.3. *α-Chymotrypsin activity in reversed micelles*

In a typical experiment, 5–130 μl of 1.25 mM Tris-HCl-carbonate buffer (pH 8.0), 5 μl of 8.5 mM solution of CBZ-TNP in acetonitrile and 2 μl of 10–30 μM enzyme solution in 1 mM HCl were solubilized in 1 ml of 0.1 M AOT. The formation of free NP was measured spectrophotometrically (400 nm) at 25°C. The coefficients of the molar absorption of NP were measured independently in the micellar systems with various W_0. The values of the catalytic constant (k_{cat}) of the reaction were determined.

2.4. *Alkaline phosphatase activity in reversed micelles*

In a typical experiment, 5–20 μl of 5–150 μM AP solution and 10–100 μl of 10–250 mM NPP solution in sodium carbonate buffer (pH 10.5) were solubilized in 2 ml of 0.1 M AOT in octane. The formation of free NP was measured as described above. The values of the specific maximal reaction rate (V/E_m) were determined.

2.5. *Sedimentation measurements*

The sedimentation coefficients (S) of the reversed micelles containing the protein were measured as described in [5] at 20°C in an analytical ultracentrifuge "Beckman L", fitted with a photoelectric scanning device with a monochromator and a multiplexor, using 12 mm bisection cells and a rotor An G-Ti at 20000 rpm [9]. The scanning was carried out at 280 nm.

The dependences of S on W_0 were analyzed as described in [5,10]. The values of the molecular masses (M_r) of the protein incorporated into the reversed micelles were calculated from the S values as previously described [10].

3. RESULTS AND DISCUSSION

3.1. *Catalytic activity of monomeric and hexameric forms of α-chymotrypsin in reversed micelles*

One of the most spectacular observations made in micellar enzymology is the catalytic activity versus hydration degree profile concept. In spite of the great variety of the enzymes under study, the observed dependences of their catalytic activity on W_0 appeared to be very similar: as a rule, they represent the bell-shaped curves (see for review [11,12]). The maximum of the catalytic activity is observed when the size of the micelle inner cavity is equal to that of the molecule of the solubilized enzyme [11,12]. The reasons for this phenomenon are discussed in [12–14].

The majority of enzymes studied in reversed micelles up to now [11,12] consist of one or several but strongly coupled subunits. Their oligomeric composition is constant as a rule. One can expect that the change of the oligomeric composition of the enzyme molecule, resulting in the change of its size, may influence the catalytic activity versus W_0 profile. We have studied this problem by examples of the monomeric enzyme CT and of its artificially produced oligomer, composed of six cross-linked CT molecules (see Materials and Methods).

The dependence of the catalytic activity of native CT (monomeric form) on W_0 is represented in Fig. 1a. The optimum at this dependence is observed at W_0 = 12 under conditions when r_e is equal to the radius (r_p) of the CT globule. In the case of the cross-linked CT hexamer (Fig. 1b) the optimum is observed at W_0 = 43. Under these conditions the value of $r_e \approx 69$ Å is approximately equal to the radius of a sphere surrounding the

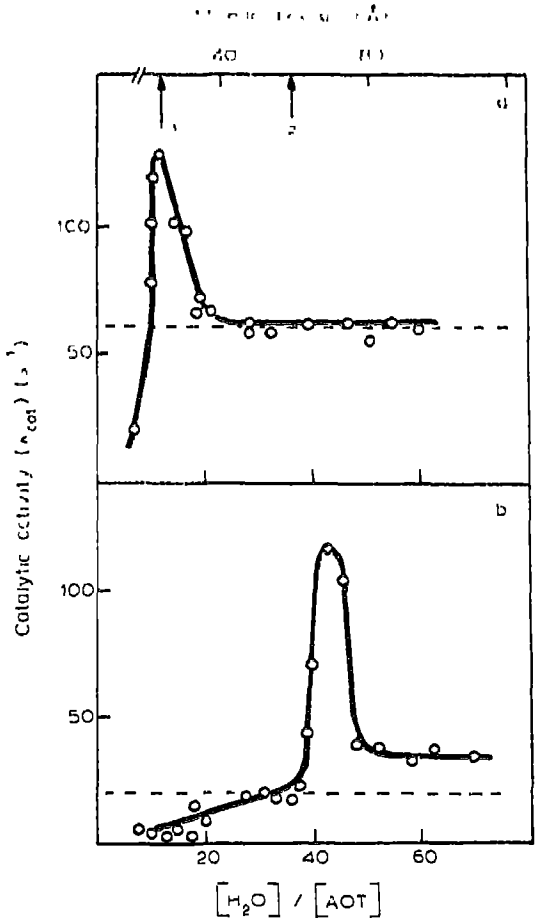

Fig. 1. The dependence of the catalytic activity (k_{cat}) of native CT (a) or of its cross-linked hexamer (b) (reaction of enzymatic hydrolysis of CBZTNP) on the hydration degree (W_0) in the system of AOT reversed micelles in octane. The k_{cat} values measured in aqueous solutions are shown by dotted lines. The scale of radii of the inner cavity of the micelles (r_e) is presented at which the arrows indicate (1) the radius of the CT globule, (2) the radius of the sphere surrounding the absolute octahedron composed of six CT molecules.

absolute octahedron composed of six CF globules ($r_{c,1}$ = 61 Å). The small difference in values between the optimal r_c and $r_{c,1}$ may result from a non-ideal packing of CF globules into octahedral hexamer and or from the fact that the CF globules in this hexamer do not contact with each other directly, but are coupled together via relatively long spacers

The experiment described provides evidence for the existence of a strict relationship between the position of the optima at the catalytic activity curve and oligomeric composition of the enzyme under study

3.2. *The dependence of the catalytic activity of alkaline phosphatase on the hydration degree*

As can be seen in Fig. 2a not one but two optima are

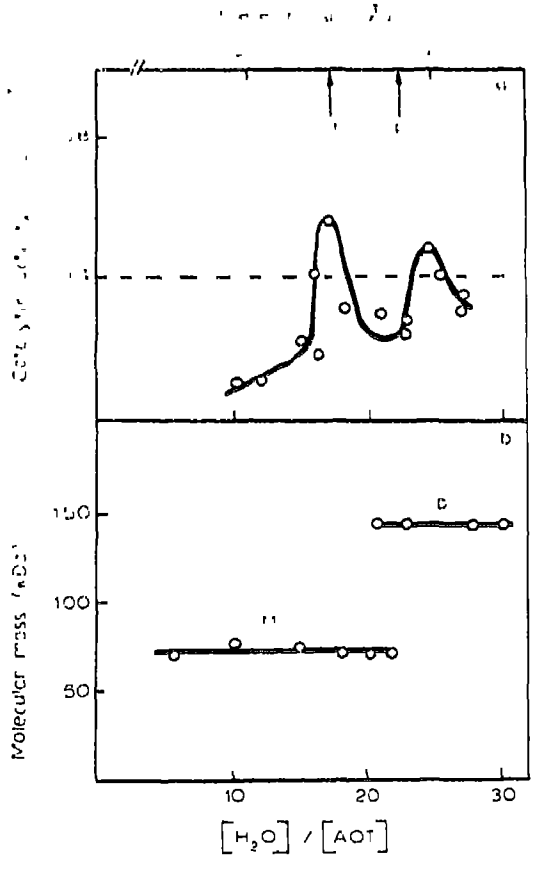

Fig. 2. (a) The dependence of the catalytic activity ($V/[E_0]$) of AP (reaction of enzymatic hydrolysis of NPP) on the hydration degree (W'_0) in the system of AOT reversed micelles in octane. The value of $V/[E_0]$ measured in aqueous solution is shown by the dotted line. The scale of radii of the inner cavity of the micelles (r_c) is presented at which the arrows indicate the radii (r_p) of the AP monomer (M) and dimer (M_2) (these radii were calculated from the values of molecular masses (M_r) of the AP monomer or dimer (the shape of which according to [18] is close to spherical) using the empirical equation $r_p = 0.7 (M_r)^{1/3}$ [13]). (b) Regulation of the oligomeric composition of AP via variation of W'_0 in the AOT reversed micelles in octane. The values of molecular masses of the protein were calculated (see Materials and Methods) from experimentally determined sedimentation coefficients of the micelles containing these proteins

observed at the profile of the AP catalytic activity at W'_0 = 17 and W'_0 = 25. Under these conditions the values of r_c are equal to r_p of the monomeric (W'_0 = 17) and dimeric (W'_0 = 25) forms of AP. Thus, following the above-formulated logics one can suppose that the observed maxima correspond to the functioning of the AP monomer and of its dimer correspondingly

This supposition was confirmed by results of the sedimentation study of the reversed micelle systems containing AP. It is known [9] that the sedimentation coefficient of the reversed micelles containing the monomeric protein (e.g. CF) increases monotonously with an increase in W'_0. On the contrary, in the case of the reversed micelles containing AP a sharp leap of the S value is observed at W'_0 = 20. Thus, two characteristic sections exist at S on the W'_0 plot. These sections are precisely described by theoretical curves [5,10] built under the assumption that either one ($W'_0 < 20$) or two ($W'_0 > 20$) subunits of AP are incorporated in one reversed micelle. Hence, one can conclude that a change of oligomeric composition of AP (its transition from the monomeric (M) to dimeric (M_2) form) takes place at W'_0 = 20, Fig. 2b

Attempts to separate the AP subunits in homogeneous aqueous solutions using conventional methods (e.g. in 6 M guanidine chloride) resulted in the loss of the enzyme activity [15–17]. This established a widespread opinion, that AP possesses catalytic activity only in the dimeric form. Our results (Fig. 2) contradict this point of view. In other words, using systems of the reversed micelles one can separate the AP subunits under non-denaturing conditions, which provides possibilities to study the catalytic and other properties of the enzyme isolated subunits.

REFERENCES

[1] Kurganov, B.I. (1978) Allosteric Enzymes (Russ.), Nauka Publ., Moscow
[2] Friedrich, P. (1984) Supramolecular Enzyme Organization, Pergamon Press, Oxford
[3] Kurganov, B.I. and Loboda, N.I. (1979) J. Theor. Biol. 111, 707–723
[4] Muronetz, V.I. and Nagradova, N.K. (1984) Immobilized Oligomeric Enzymes (Russ.), Nauka Publ., Moscow
[5] Kabanov, A.V., Nametkin, S.N., Evtushenko, G.N., Chernov, N.N., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) Biochim. Biophys. Acta 996, 147–152
[6] Klyachko, N.L., Merker, S., Vakula, S.V., Ivanov, M.V., Berezin, I.V., Levashov, A.V. and Martinek, K. (1988) Dokl. Akad. Nauk SSSR (Russ.) 298, 1479–1481
[7] Schonbaum, G.R., Zerner, B. and Bender, M.L. (1961) J. Biol. Chem. 236, 2930–2935
[8] Houston, L.L. and Nowinski, R.C. (1981) Cancer Res., 41, 3913–3917
[9] Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L., Chernyak, V.Yu. and Martinek, K. (1982) J. Colloid Interface Sci. 88, 444–457
[10] Kabanov, A.V., Levashov, A.V., Khrutskava, M.M. and Kabanov, V.A. (1990) Macromol. Chem. 191

[11] Martinek, K., Klyachko, N.L., Kabanov, A.V., Khmelnitsky, Yu.L. and Levashov, A.V. (1989) Biochim. Biophys. Acta 981, 161–172.
[12] Khmelnitsky, Yu.L., Kabanov, A.V., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) in: Structure and Reactivity in Reverse Micelles (Pileni, M.P. ed.) pp. 230–261, Elsevier, Amsterdam.
[13] Klyachko, N.L., Pshezhetsky, A.V., Kabanov, A.V., Vakula, S.V., Martinek, K. and Levashov, A.V. (1990) Biol. Membranes (Russ.) 7, 467–472.
[14] Kabanov, A.V., Levashov, A.V., Klyachko, N.L., Nametkin, S.N., Pshezhetsky, A.V. and Martinek, K. (1988) J. Theor. Biol. 133, 327–343.
[15] McCracken, S. and Meighen, E. (1979) Can. J. Biochem. 57, 834–842.
[16] McCracken, S. and Meighen, E. (1980) J. Biol. Chem. 255, 2196–2204.
[17] McCracken, S. and Meighen, E. (1983) Methods Enzymol. 133, 492–501.
[18] Sowadski, J.M., Handschumacher, M.D., Krenitsky, H.M., Murthy, H.M., Foster, B.A. and Wyckoff, H.W. (1985) J. Mol. Biol. 186, 417–433.