FEBS Letters 364 (1995) 98–100 FEBS 15416

High-pressure stabilization of α-chymotrypsin entrapped in reversed micelles of Aerosol OT in octane against thermal inactivation

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Received 15 March 1995

Abstract **α-Chymotrypsin (CT) solubilized in reversed micelles of sodium bis-(2-ethylhexyl)-sulfosuccinate (AOT) undergoes thermal inactivation and the enzyme stability decreases significantly when temperature increases (25–40°C). The half-life of CT in micelles shows a bell-shaped dependence on the degree of hydration of AOT (w_o) analogous to the previously obtained dependence on w_o for the enzyme activity. The optima of catalytic activity and thermal stability have been observed under conditions where the diameter of the inner aqueous cavity of the micelle is close to the size of the enzyme molecule (w_o = 10). Application of high hydrostatic pressure in the range of 1–1500 atm (bar) stabilizes CT against thermal inactivation at all hydration degrees (w_o) from 7 to 20; the stabilization effect is most pronounced under the experimental conditions being far from the optimum for catalytic activity.**

Key words: Thermal inactivation of enzyme; Stabilization of enzyme; Regulation of enzyme activity; High pressure; Micellar enzymology; Baroenzymology

1. Introduction

Catalysis by α-chymotrypsin (CT) solubilized in reversed micelles of AOT (Aerosol OT) is well understood and has been described in detail [1,2]. However, the problem of thermostability of this enzyme in micellar systems remains beyond the scope of systematic studies. According to literature data [3,4], a transition from room temperature to higher temperatures (>35°C) is followed by significant destabilization of CT. Reversed micelles are structurally complex and dynamic systems and the stability of enzymes entrapped in them is influenced by many different factors, which in turn are determined by characteristics of the micelles and the nature of the enzyme. In this respect, structural rigidity (mobility) of a protein molecule in relation to properties of the micellar matrix is among the most important and intriguing factors.

We carried out a systematic investigation of the thermal inactivation of CT entrapped in reversed micelles of AOT in octane as a function of the size of the spherical internal aqueous cavity, which is determined by the degree of hydration of the surfactant (the value of w_o). As a factor which is capable of modulating molecular mobility of the components of the micellar system, we used the action of high hydrostatic pressure [5,6] of up to 1.5 kbar. Under such pressures, micelles retain their integrity [7], the structure of enzymes entrapped in the reversed micelles is conserved [8], and enzymes possess high catalytic activity [9,10].

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Abbreviations: CT, α-chymotrypsin; sodium bis-(2-ethylhexyl)-sulfosuccinate; AOT, Aerosol OT; SPNA, *N*-succinyl-L-phenylalanine *p*-nitroanilide; $w_o = [H_2O]/[AOT]$, i.e. w_o is equal to the ratio between the concentrations of water and surfactant in a micellar system.

2. Experimental

2.1. Materials

Bovine pancreatic α-chymotrypsin (EC 3.4.21.1, Type 1S; the content of active sites 75 ± 5%, as determined by titration with *N-trans*-cinnamoyl imidasole [11]), *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) and AOT were all purchased from Sigma and used as received. As determined from the infrared spectra, the sample of AOT contained 0.4 mol of water per mol of surfactant; this value was taken into account when calculating the amount of water in micelles (the value of w_o). *n*-Octane was a product of Carlo Erba; salts, acids, bases and buffer components were of the highest purity grade.

2.2. Thermal inactivation of α-chymotrypsin in reversed micelles

Enzyme solution was prepared as follows: to 10 ml solution of 0.1 M AOT in octane, 0.06–0.31 ml of 0.1 M Tris-HCl buffer (pH 8.5) and 50 μl of solution of CT in 1 mM HCl were added. The concentration of CT was constant in all experiments and was equal to 11 μM with respect to the active enzyme; the degree of hydration of AOT ($w_o = [H_2O]/[AOT]$) was changed from 6 to 20.

Substrate solution was prepared as follows: to 10 ml solution of 0.1 M AOT in octane, 0.11–0.36 ml of 0.1 M Tris-HCl buffer (pH 8.5) and 50 μl of solution of SPNA in the mixture of acetonitrile and dioxane (1:1 by vol.) were added. The concentration of SPNA was kept constant in all experiments and was equal to 0.27 mM. The degree of hydration of AOT (w_o) was changed from 6 to 20.

For inactivation studies at atmospheric pressure, solutions of enzyme and substrate with the same value of w_o were prepared and incubated separately in glass vessels at 25–40°C (± 0.5°C). Periodically, equal aliquots of enzyme and substrate solutions were taken and mixed in a thermostated cuvette of spectrophotometer Varian Cary 3, and the rate of hydrolysis of SPNA by CT was detected at 380 nm (*p*-nitroaniline formation). Experiments under high pressure were carried out in a stopped-flow high-pressure apparatus developed in U 128 in Montpellier [12]. The reaction was started by mixing equal volumes (0.1 ml) of the enzyme and substrate solutions in a high-pressure cell of a spectrophotometer unit. Under all experimental conditions, the rate of substrate spontaneous hydrolysis was negligibly small in comparison with the rate of enzymatic hydrolysis. In all experiments the activity was measured at the same temperature at which enzyme and substrate solutions were incubated.

3. Results and discussion

Kinetics curves of thermal inactivation of CT in the system of reversed micelles of AOT in octane (w_o = 10) at different temperatures at atmospheric pressure are shown in Fig. 1. An increase in temperature results in a pronounced acceleration of

SSDI 0014-5793(95)00344-4

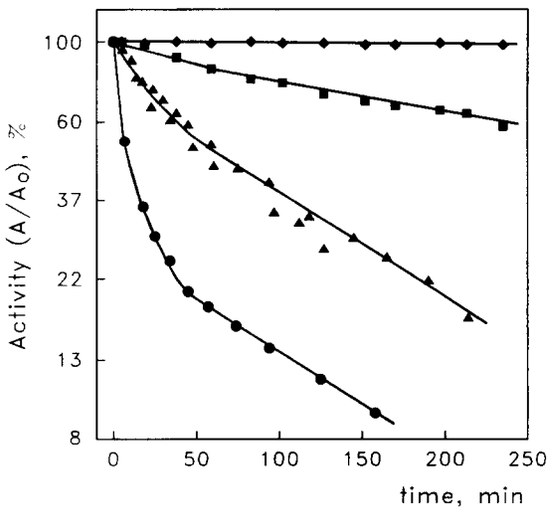

Fig. 1. Time–courses of thermal inactivation of α-chymotrypsin in the system of reversed micelles of Aerosol OT in octane (w_o = 10) at different temperatures: 25°C (◆), 30°C (■), 35°C (▲), 40°C (●). Residual enzyme activity, A (in per cent of the initial enzyme activity before thermal inactivation, A_o) is presented in logarithmic scale. Experimental conditions: 0.1 M Aerosol OT, 0.1 M Tris-HCl, pH 8.5; concentration of the active form of α-chymotrypsin is 11 μM.

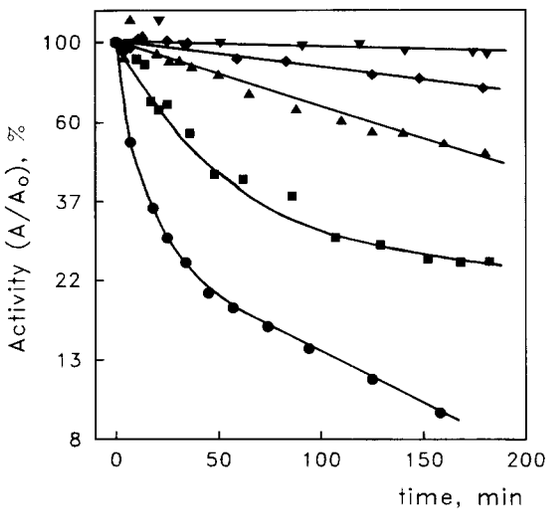

Fig. 3. Time–courses of thermal inactivation of α-chymotrypsin in the system of reversed micelles of Aerosol OT in octane (w_o = 10) at 40°C at different pressures: 1 bar (●), 275 bar (■), 500 bar (▲), 1000 bar (◆), 1500 bar (▼). Residual enzyme activity, A (in per cent of the initial enzyme activity before thermal inactivation, A_o) is presented in logarithmic scale. For other experimental conditions, see legend to Fig. 1.

inactivation of the enzyme: at 25°C, CT is almost absolutely stable whereas at 40°C, in less than an hour its activity decreases more than 5-fold. One may assume that the thermal instability of CT is connected with the mobility of the components of the micellar system. For example, this inactivation can be driven by interactions of protein molecules with molecules of the surfactant (AOT) or molecules of the organic solvent (octane) from the bulk non-polar phase, whose coming into contact with protein molecules is especially significant during collisions between micelles. Obviously, these interactions become more intensive with an increase in temperature that, in turn, gives rise to a pronounced acceleration of enzyme inactivation at high temperatures (Fig. 1). As an experimental criterion of thermal stability of CT in reversed micelles we chose the half-life of the enzyme, $t_{1/2}$ (the time at which enzyme activity is decreased 2-fold).

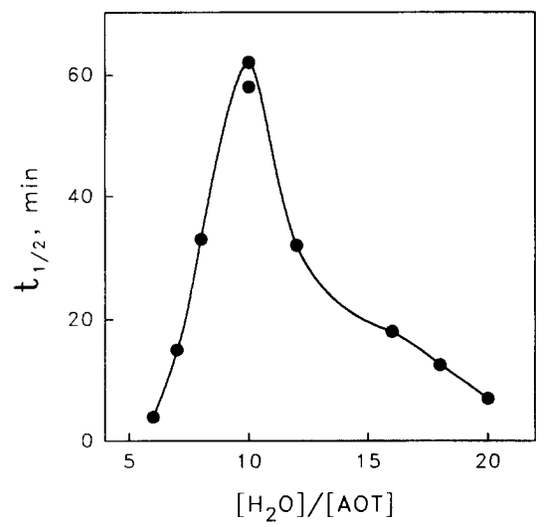

Fig. 2. Dependence of the half-life ($t_{1/2}$) of α-chymotrypsin during thermal inactivation in the system of reversed micelles of Aerosol OT in octane on w_o = [H_2O]/[AOT] at 35°C. For other experimental conditions, see legend to Fig. 1.

Fig. 2 shows the dependence of $t_{1/2}$ on the value of w_o at atmospheric pressure at 35°C; this temperature was selected for the reason of experimental convenience (see Fig. 1). The bell-shaped character in Fig. 2 is similar to the case of the dependence of catalytic activity of CT on the value of w_o [1,2]. Stability of CT is small under the conditions where the size of the micellar inner cavity is either bigger or smaller than the size of the enzyme molecule. For example, at w_o equal to 6, half of the total enzyme activity is lost during the first 4 min. The maximum of the enzyme stability is observed at w_o of 10 where the optimum of catalytic activity of CT is also found [1,2]. In these micelles, the size of the inner aqueous cavity corresponds well to the size of the enzyme molecule. In such a case, protein molecules contact most efficiently with a micellar matrix composed of the surfactant monolayer [13] and these contacts may stabilize CT from temperature inactivation. Conversely, an absence of the correspondence in the size of micelles to that of a protein (at w_o far from 10) leads to a weakening of the stabilizing action of the micellar matrix which can not oppose a destabilization effect of inter-molecular contacts.

One can assume that application of high hydrostatic pressure may augment the structural order of surfactant aggregates (micelles) that, in turn, should decelerate enzyme inactivation. Fig. 3 shows the effect of hydrostatic pressure on inactivation of CT at w_o equal to 10 at 40°C, where the enzyme stability is low. Even relatively small pressure of 275 bar produces a pronounced stabilization effect which increases progressively with a rise in pressure, and at 1.5 kbar CT is not inactivated for at least 3 h.

The high pressure stabilizing action on CT thermal inactivation in reversed micelles is observed at all w_o (Fig. 4). As a

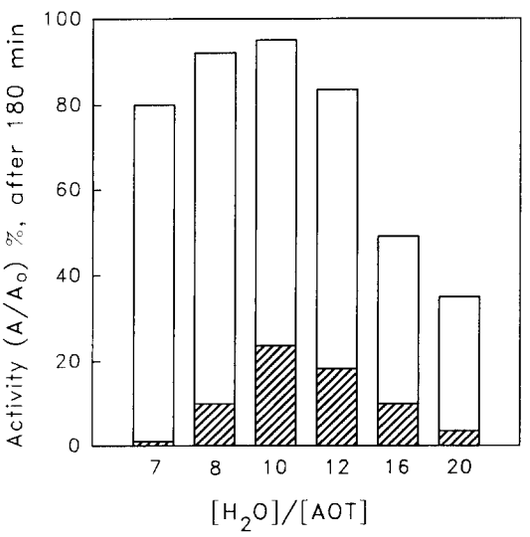

Fig. 4. Residual enzyme activity of α-chymotrypsin after 180 min of incubation in the system of reversed micelles of Aerosol OT in octane at 35°C at different values of $w_o = [H_2O]/[AOT]$. Residual enzyme activity, A, is presented in per cent of the initial enzyme activity before thermal inactivation, A_o. Total bars correspond to residual activity after incubation at 1000 bar; shaded parts correspond to residual activity after incubation at 1 bar. For other experimental conditions, see legend to Fig. 1.

quantitative criterion of this stabilizing effect, we selected a change in the level of the residual activity of CT after 3 h of incubation at 35°C at atmospheric pressure as compared to the level of activity retained after incubation under high pressure (1 kbar). At w_o of 10, where both enzyme activity and stability of CT are nearly optimal, enzyme inactivation was almost completely retarded (open bars in Fig. 4). A similar effect of complete suppression of thermal inactivation was also observed at w_o smaller than 10, when the diameter of the inner micellar cavity is smaller than the size of the molecule of CT. This stabilization does not depend on whether the enzyme possesses high (at w_o of 10) or low (at $w_o < 10$) stability at atmospheric pressure (shaded bars in Fig. 4). The stabilizing effect, as it was determined above, can be very significant and reach up to nearly 100-fold at, for example, a w_o of 7. Under conditions of high values of w_o, the stabilizing effect is not in an excess of 10-fold and it is also not possible to increase to 100% the level of the activity retained after incubation at high temperature even by applying pressure of 1 kbar. The molecular reasons for the protein stabilization by high pressures will be studied by us by using different physical methods adjusted to the conditions of high pressure, such as NMR [14], different spectroscopies [15], IR and vibrational spectroscopy [16].

Acknowledgements: The authors thank Dr. R. Lange for helpful advice during experimental work and stimulating discussions. This work was supported in part by grants from INTAS 93-38 (staying and work in Montpellier of R.V.R. and A.V.L.) and INSERM (for S.N.N.). V.V.M. is grateful to INSERM for a long-term fellowship.

References

[1] Klyachko, N.L., Levashov, A.V., Kabanov, A.V., Khmelnitsky, Yu.L. and Martinek, K. (1991) in: Kinetics and Catalysis in Microheterogeneous Systems, Surfactant Science Series vol. 38 (Gratzel, M. and Kalyanasundaram, K., eds.) pp. 135–181, Marcel Dekker, New York.
[2] 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.
[3] Matveeva, E.G., Levashov, A.V., Schegolev, A.A., Ryzhova, V.V., Polyakov, V.A. and Martinek, K. (1985) Biotekhnologiya (Russian) 1, 84–93.
[4] Levashov, A.V., Klyachko, N.L., Khmelnitsky, Yu.L. and Martinek, K. (1987) Ann. NY Acad. Sci. 501, 205–209.
[5] Mozhaev, V.V., Heremans, K., Frank, J., Masson, P. and Balny, C. (1994) TIBTECH 12, 493–501.
[6] Gross, M. and Jaenicke, R. (1994) Eur. J. Biochem. 221, 617–630.
[7] Smith, R.D., Fulton, J.L., Blitz, J.P. and Tingey, J.M. (1990) J. Phys. Chem. 94, 781–787.
[8] Affleck, R., Clark, D.S., Kamat, S. and Russel, A.J. (1994) Biotechnol. Bioeng. 43, 342–348.
[9] Mozhaev, V.V., Bec, N. and Balny, C. (1994) Biochem. Mol. Biol. Int. 34, 191–199.
[10] Mozhaev, V.V., Bec, N. and Balny, C. (1995) Ann. NY Acad. Sci. 995, 94–96.
[11] Schonbaum, G.R., Zerner, B. and Bender, M. (1961) J. Biol. Chem. 236, 2930–2938.
[12] Balny, C., Saldana, J.-L. and Dahan, N. (1987) Anal. Biochem. 163, 309–315.
[13] Klyachko, N.L., Bogdanova, N.G., Koltover, V.K., Martinek, K. and Levashov, A.V. (1989) Biokhimiya (Russian, Engl. translation) 54, 1004–1009.
[14] Jonas, J. and Jonas, A. (1994) Annu. Rev. Biomol. Struct. 23, 287–318.
[15] Silva, J.L. and Weber, G. (1993) Annu. Rev. Phys. Chem. 44, 89–113.
[16] Wong, P.T.T. and Heremans, K. (1988) Biochim. Biophys. Acta 956, 1–9.