Thermobarostability of α-Chymotrypsin in Reversed Micelles of Aerosol OT in Octane Solvated by Water-Glycerol Mixtures

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Abstract: Thermostability of α-chymotrypsin at normal pressure in reversed micelles depends on both an effective surfactant solvation degree and glycerol content in the system. The difference in α-chymotrypsin stability in reversed micelles at various glycerol concentrations [up to 60% (v/v)] was more pronounced at high surfactant degrees of solvation, $R \geqslant 16$. After a 1-h incubation at 40°C in ''aqueous'' reversed micelles (in the absence of glycerol), α-chymotrypsin retained only 1% of initial catalytic activity and 10, 22, 59, and 48% residual activity in glycerol-solvated micelles with 20, 30, 50, and 60% (v/v) glycerol, respectively. The explanation of the observed effects is given in the frames of micellar matrix structural order increasing in the presence of glycerol as a water-miscible cosolvent that leads to the decreasing mobility of the α-chymotrypsin molecule and, thus the increase of its stability. It was found that glycerol or hydrostatic pressure could be used to stabilize α-chymotrypsin in reversed micelles; a lower pressure is necessary to reach a given level of enzyme stability in the presence of glycerol. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 552–556, 1998.

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INTRODUCTION

The effect of high pressure is a field that is now well exploited in biotechnology and successfully applications in food science (Balny et al., 1992; Hayashi and Balny, 1996). Moreover, recent years have witnessed a development of methods of micellar enzymology in connection with applied baroenzymology (Affleck et al., 1994; Mozhaev et al., 1994a; Rariy et al., 1995). These new trends give us the ways to modulate the enzyme activity, opening the route of potential use in biotechnology, for example, such properties as enzyme selectivity or such phenomenon as protein extraction. If the effects of pressure on proteins are well documented in recent books and reviews (see, e.g., Markley et al., 1996; Mozhaev et al., 1996a), enzymology in extreme conditions (e.g., in such experimental media as micelles and organic solvents under high pressure) requires up to date extended experimental data, mainly on the enzyme stability that is a fundamental problem for the practical application in biocatalysis (Clark et al., 1996).

Catalysis by enzymes solubilized in reversed micelles of surfactants is of interest to many research groups (for review see, e.g., Bru et al., 1995; Klyachko et al., 1991; Luisi et al., 1988; Martinek et al., 1989). α-Chymotrypsin catalysis, a well known model enzyme, in reversed micelles has been described in detail (see, e.g., Bru et al., 1995; Fletcher et al., 1985b; Khmelnitsky et al., 1989; Klyachko et al., 1991). Nevertheless, the systematic study of thermostability of α-chymotrypsin in the system of reversed micelles has been started recently (Rariy et al., 1995). It was shown that the stability of the enzyme decreases essentially when the temperature increases (in the range of 25–40°C). The half-life of α-chymotrypsin in reversed micelles of aerosol OT (AOT, dioctyl sulfosuccinate) reveals a bell-shaped dependence on the surfactant hydration degree (w_o) analogous to the previously obtained dependence on w_o for the enzyme activity. The optima of the catalytic activity and thermostability have been observed under the conditions where the diameter of the inner aqueous cavity of the micelle was close to the size of the enzyme molecule ($w_o = 10$).

As a hypothesis, a fundamental factor affecting the α-chymotrypsin stability in reversed micelles is an enhanced mobility and/or flexibility of the components of the micellar matrix that destabilize the protein structure when temperature increases. For this reason we used high pressure as a factor capable of modulating the enzyme catalytic activity both in micelle free solutions (Gross and Jaenicke, 1994; Mozhaev et al., 1994b) and in the micellar system (Clery et

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al., 1995; Mozhaev et al., 1994a, 1995). It was shown that α-chymotrypsin stability rose significantly when a hydrostatic pressure (up to 150 MPa) was applied to the protein-containing micellar system (Rariy et al., 1995). It is known that under such pressure micelles retain their integrity (Smith et al., 1990) and the structure of enzymes entrapped into reversed micelles is conserved (Affleck et al., 1994). In our opinion, the application of high pressure increases the structural order of surfactant aggregates (micelles), this being the main factor affecting α-chymotrypsin stabilization.

It is well known that the use of organic cosolvents instead of water in the inner cavities of micelles leads to an increase of the time one molecule of surfactant exists in one micelle (Fletcher et al., 1984, 1985a). AOT is more closely packed at the glycerol interface (Fletcher et al., 1984): the area occupied by one AOT molecule on a surface of a micellar inner cavity is 0.41 ± 0.07 and 0.51 ± 0.08 nm^2 in glycerol–containing and ''aqueous'' micelles, respectively (Nicholson and Clarke, 1983).

Thus, one could expect that the effect caused by glycerol on the α-chymotrypsin stability in reversed micelles would be similar to that caused by pressure. The purpose of the work presented was to study glycerol and pressure joint influence on the α-chymotrypsin stability in reversed micelles of AOT in octane.

MATERIALS AND METHODS

Materials

Bovine pancreatic α-chymotrypsin [EC 3.4.21.1, type 1S, containing 75 ± 5% of the active enzyme, as determined by titration with *N-trans*-cinnamoyl imidazole (Schonbaum et al., 1961)], *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA), glycerol, 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 water/mol surfactant; this value was taken into account for the calculation of the total water quantity in micelles. *N*-Octane was from Carlo Erba; salts, acids, bases, and buffer components were of the highest purity grade.

Thermal Inactivation of α-Chymotrypsin in Reversed Micelles

Enzyme solution was prepared as follows: 0.06–0.31 mL of 0.1*M* Tris-HCl buffer (pH 8.5) and 50 μL of a solution of α-chymotrypsin in 1 m*M* HCl were added to 10 mL of 0.1*M* AOT solution in octane. Tris-HCl buffer was chosen because of its pressure-invariant p*K* value (Neumann et al., 1973). When the effect of glycerol on the enzyme behavior was studied, 0.1*M* Tris-HCl buffer was prepared on the basis of water–glycerol mixtures of a certain volume/volume ratio. In this case a stock solution of α-chymotrypsin was also prepared on the basis of water–glycerol mixtures at pH 3.0. Concentration of α-chymotrypsin was constant in all experiments and equal to 11μ*M* (calculated using the percentage of the active enzyme in the preparation).

In order to be able to compare results obtained for various concentrations of glycerol in the buffer introduced into the system, the parameter *R*, termed the surfactant solvation degree, was employed in the present work. The *R* value is calculated similarly to the well known value of the surfactant hydration degree (i.e., molar ratio of water to surfactant), where the volume of water brought into the system of reversed micelles was substituted for an identical volume of the water–glycerol mixture. The degree of solvation of AOT (*R*) was changed from 6 to 20.

Substrate solution was prepared as follows: 0.11–0.36 mL of 0.1*M* Tris-HCl buffer (pH 8.5) and 50 μL of SPNA in acetonitrile/dioxane mixture (1:1 by volume) were consecutively added to 10 mL of 0.1*M* AOT in octane. When the effect of glycerol on the enzyme behavior was studied, 0.1*M* Tris-HCl buffer was prepared on the basis of water–glycerol mixtures of a certain volume/volume ratio. The concentration of SPNA was kept constant in all experiments and was equal to 0.27 m*M*. The degree of solvation of AOT (*R*) was changed from 6 to 20.

For inactivation studies at atmospheric pressure, solutions of enzyme and substrate with the same *R* value were prepared and incubated separately in glass vessels at 40°C (±0.5°C). Periodically, equal aliquots of enzyme and substrate solutions were taken and mixed in a thermostated cuvette of a Varian Cary 3, spectrophotometer and the rate of hydrolysis of SPNA by α-chymotrypsin was detected at 380 nm (*p*-nitroaniline formation). Typical curves of thermal inactivation of α-chymotrypsin in reversed micelles of AOT in octane are presented on Figure 1. (A log scale was used to show complex kinetics.)

Experiments under high pressure were carried out in a stopped-flow high-pressure apparatus constructed in U 128 in Montpellier (Balny et al., 1984). The reaction was started by mixing equal volumes (0.1 mL) of the enzyme and substrate solutions in a thermostated high-pressure cell of a spectrophotometer unit. The reaction rate was measured at 380 nm (*p*-nitroaniline formation). 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.

RESULTS AND DISCUSSION

We studied the stability of α-chymotrypsin in the system of reversed micelles at normal pressure as a function of glycerol concentration in the system. The dependence of α-chymotrypsin residual activity after 1 h of incubation (the parameter chosen as a criterion of the enzyme stability) in reversed micelles on surfactant solvation degree at various glycerol concentrations is given on Figure 2. As shown earlier (Rariy et al., 1995), α-chymotrypsin in aqueous re-

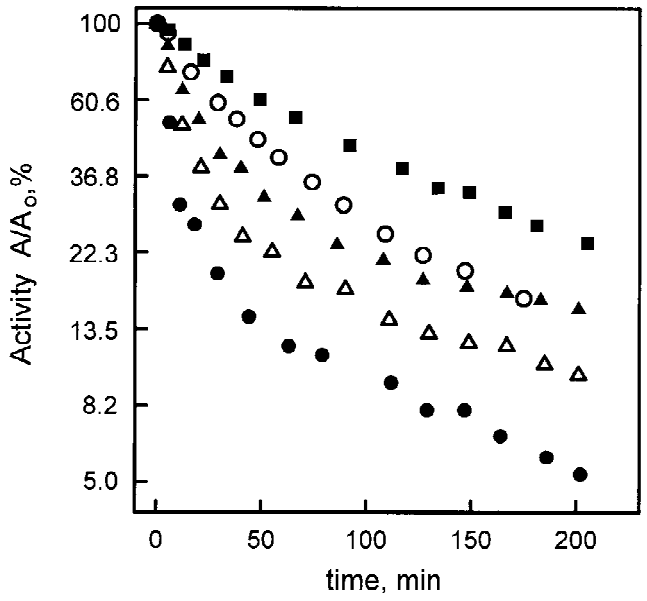

Figure 1. Time courses of thermal inactivation of α-chymotrypsin in the system of reversed micelles of aerosol OT in octane ($R = 10$) at 40°C for various concentrations of glycerol in the buffer brought into the system: (●) 0%, (△) 10%, (▲) 20%, (■) 50%, (○) 60%. Residual enzyme activity, A (in percent of the initial enzyme catalytic activity before thermal inactivation, A_o), is presented in logarithmic scale. Experimental conditions: 0.1*M* aerosol OT, 0.1*M* Tris-HCl buffer on the basis of water–glycerol mixtures, pH 8.5; concentration of the active form of α-chymotrypsin is 11 μ*M*.

versed micelles (without adding glycerol) is stable at 25°C (the enzyme does not lose the catalytic activity during 3 h) and becomes extremely unstable at 40°C (half-life of inactivation, $t1/2$, is equal to 6 min). Thus, 40°C was chosen for the work presented.

The curve of α-chymotrypsin stability in reversed micelles not containing glycerol (black circles on Fig. 2) is analogous to that found by us earlier (Rariy et al., 1995). The dependence of the residual activity is represented by a bell-shaped curve, and the optimum of the α-chymotrypsin stability found at $R = 10$ coincides with that of the enzyme catalytic activity. In such micelles the radius of the inner aqueous cavity (20 Å) corresponds to the size of the enzyme molecule (21 Å). In such a case, protein molecules contact most efficiently with a micellar matrix composed of the surfactant monolayer (Klyachko et al., 1989) and these contacts may stabilize α-chymotrypsin from temperature inactivation. Conversely, an absence of correspondence in the size of micelles to that of a protein (at R far from 10) leads to a weakening of the micellar matrix stabilizing action that then cannot resist a destabilization effect of intermolecular contacts.

Addition of the water-miscible organic cosolvent glycerol solubilized in the inner cavity of reversed micelles leads to the pronounced increase of the α-chymotrypsin stability at all values of solvation degree. Dependencies of the enzyme residual activity on R in the presence of 20 and 30% (v/v) glycerol have the same bell-shaped form as in the absence of glycerol (Fig. 2). It is known (Klyachko et al., 1989) that the addition of glycerol into the reversed micelles results in a decrease of conformational mobility of a spin label attached in the region of the active site of α-chymotrypsin. It is considered that such addition also leads to a decrease of the conformational mobility of a protein globule as a whole due to the micellar matrix becoming more ''rigid.'' Such ''rigidification'' leads to an increase of α-chymotrypsin stability in reversed micelles.

As seen from Figure 2, at 50 and 60% (v/v) glycerol the dependence of residual activity on R changes its form significantly. At low R the stability grows sharply (10 times from $R = 6$ to $R = 12$), and then practically does not change. It should be noted that at all surfactant solvation degrees, α-chymotrypsin appeared to be more stable in 50% glycerol in reversed micelles than in 30%.

To give a reasonable explanation, let us refer to the fact that increasing glycerol concentrations from 0 to 50% results in changing of the micellar matrix physical state, namely, in increasing the AOT molecule lifetime in the micelle and in retarding the intermicellar exchange at constant number of micelle collisions, the micellar matrix itself becomes more stable (fixed) and thus more rigid (Fletcher et al., 1984, 1985a). It could be possible that the enzyme entrapped into reversed micelles, containing glycerol, becomes more stable due to such rigidity.

A 50% glycerol solution possesses considerable viscosity so that any small layer of water–glycerol mixture between the protein surface and the micelle wall excludes the influ-

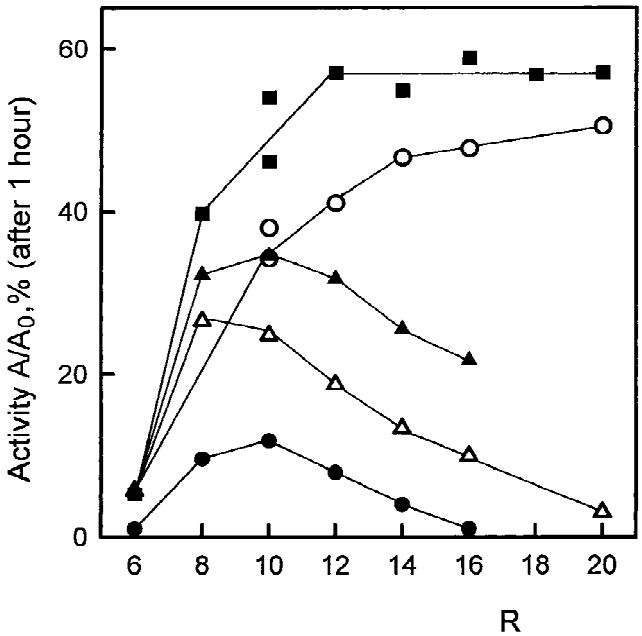

Figure 2. Residual α-chymotrypsin catalytic activity after 60 min of incubation in the system of reversed micelles of aerosol OT in octane at 40°C at different values of R and at various concentrations of glycerol in the buffer brought into the system: (●) 0%, (△) 20%, (▲) 30%, (■) 50%, (○) 60%. Residual enzyme activity, A, is presented as a percent of the initial enzyme activity before thermal inactivation, A_o. For other experimental conditions, see legend to Figure 1.

ence of a matrix on the protein stability. (The difference between radii of micelles is ~3 Å when R changes from 10 to 12.)

Further increasing the glycerol concentration [up to 60% (v/v)] results in decreasing the level of α-chymotrypsin residual activity in comparison with the 50% (v/v) glycerol observed. Such a reduction of protein stability at high glycerol concentrations can be caused by the fact that glycerol can participate in protein structure changes (unfavorable in this case), replacing water molecules in the protein structure. The similar picture of α-chymotrypsin stabilization at low concentrations of glycerol (up to 50%) and reducing the stabilizing effect at high concentrations was also observed in bulk water–glycerol mixtures (in the absence of micellar matrix) (Mozhaev et al., 1996b). One of the possible reasons can be replacement of water molecules on the protein surface by glycerol molecules that destroy (disturb) the structure of the protein globule (Khmelnitsky et al., 1991).

To illustrate a scale of changes of α-chymotrypsin stability in the system of reversed micelles, the dependence of the half-life of inactivation ($t_{1/2}$) on glycerol concentration at a constant solvation degree ($R = 10$) is given in Figure 3. The half-life of the enzyme inactivation increases 12.7 times (from 6 to 76 min) with an increasing content of glycerol from 0 to 50%, respectively. At 80% glycerol the enzyme is even less stable than in the absence of glycerol ($t_{1/2}$ in aqueous micelles is equal to 6 min, whereas in 80% glycerol it is only 4 min).

Our previous results (Rariy et al., 1995) show that essential stabilization of α-chymotrypsin in the aqueous reversed micelles can be reached by applying high pressure (up to 150 MPa) to the system. In this connection, an interesting question arises concerning joint influence of glycerol and pressure (both factors increasing the structural order of surfactant aggregates, i.e., micelles) on α-chymotrypsin stability in reversed micelles. Figure 4 presents profiles α-chymotrypsin residual activity in reversed micelles for 20 and 50% glycerol and 0.1 and 50 MPa pressure. As seen, even the moderate pressure of 50 MPa can produce a significant stabilization effect on α-chymotrypsin in reversed micelles containing 50% glycerol. Even at the low solvation degree of $R = 6$, the level of residual activity is 84%, becoming close to 100% at a higher R. The stabilizing effect of pressure is more pronounced in the region of low solvation degrees. If we compare the curves in Figure 4, one can see that in 50% glycerol the pressure effect on the enzyme residual activity at $R = 6$ is more than 17 times, and only 1.7 times at $R = 20$. It should be noted that such a high stability of α-chymotrypsin at all R values cannot be reached in the absence of glycerol, even when applying pressure up to 150 MPa (Rariy et al., 1995). For lower glycerol concentrations (20%) it is also possible to increase the stability of α-chymotrypsin in reversed micelles by application of hydrostatic pressure.

To answer the question of whether the phenomenon we observed reflects a specific role of glycerol in the α-chymotrypsin stability or if it is a general effect of polyols, we studied glucose influence on the stability of α-chymotrypsin in reversed micelles. We compared the sta-

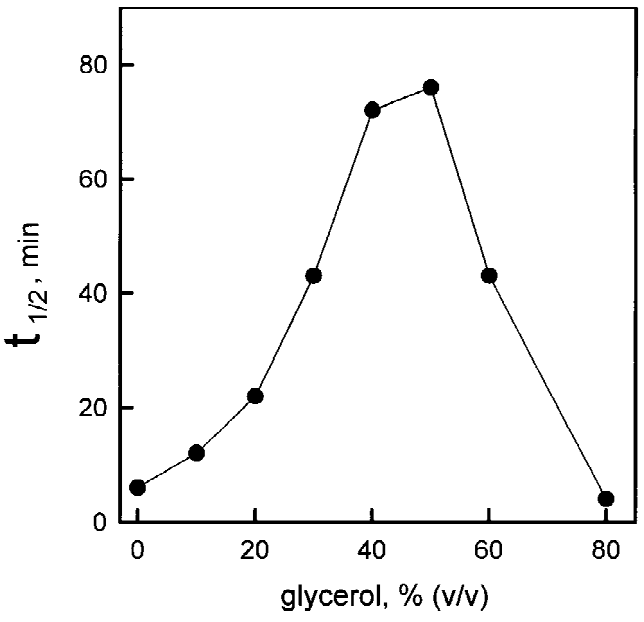

Figure 3. Dependence of the half-life ($t_{1/2}$) of α-chymotrypsin during thermal inactivation in the system of reversed micelles of aerosol OT in octane on glycerol concentration in the buffer brought into the system at $R = 10$ and 40°C. For other experimental conditions, see legend to Figure 1.

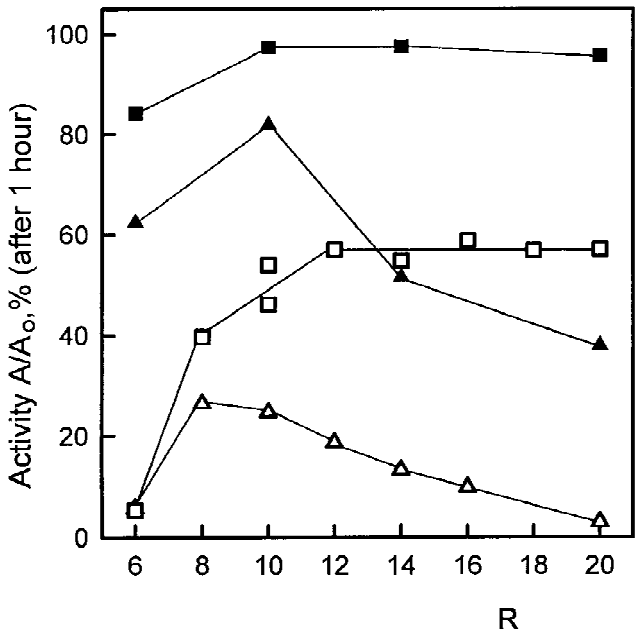

Figure 4. Dependences of the residual α-chymotrypsin catalytic activity after 60 min of incubation in the system of reversed micelles of aerosol OT in octane at 40°C on surfactant solvation degree, R, at various glycerol concentrations in the buffer brought into the system and pressure (P) applied to the system: (△) 20% glycerol, $P = 0.1$ MPa; (▲) 20% glycerol, $P = 50$ MPa, (□) 50% glycerol, $P = 0.1$ MPa; (■) 50% glycerol, $P = 50$ MPa. Residual enzyme catalytic activity, A, is presented as a percent of the initial enzyme activity before thermal inactivation, A_o. For other experimental conditions, see legend to Figure 1.

bility of α-chymotrypsin in reversed micelles in the presence of 10% glycerol with that in the presence of 1*M* glucose. (The concentration of OH groups in solutions of both reagents in this case is equal). It is shown that at $R = 10$ and $T = 40$°C the stability of α-chymotrypsin in reversed micelles containing glycerol or glucose is the same ($t_{1/2} = 12$ min). Moreover 50-MPa pressure also equally affects the stability of α-chymotrypsin in the presence of both reagents; that allows us to make a suggestion about the generality of polyol influence. Indeed, polyols easily form the system of hydrogen bonds and are able to fix the protein structure through rigidifying the matrix structure.

Therefore, physical chemical factors like glycerol or pressure, which are capable of increasing the rigidity of the micellar matrix, might increase the thermostability of a protein entrapped into such a micelle.

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