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Regulation of the supramolecular structure and the catalytic activity of penicillin acylase from *Escherichia coli* in the system of reversed micelles of Aerosol OT in octane

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The properties of penicillin acylase from *E. coli* solubilized by hydrated reversed micelles (RM) of Aerosol OT in octane were studied. The dependence of catalytic activity on the hydration degree, a parameter which determines the size of the micelle inner cavity, has a curve with three optima, each one corresponding to the enzyme functioning either in a dimer form (w_o = 23) or in a form of separate subunits, a heavy one, β, and a light one, α (w_o = 20 and 14, respectively). The reversible dissociation of the enzyme was confirmed by ultracentrifugation followed by electrophoresis.

Penicillin acylase; Protein subunit; Micellar enzymology; Reversed micelle

1. INTRODUCTION

Penicillin acylase (PA) from *E. coli* (EC 3.5.1.11) consists of two non-covalently bound subunits (M_r 23,000 and 62,000, respectively), which are processed from the common polypeptide precursor [1,2]. The role of such structural organization is not yet clear, but the active site of the enzyme is thought to be located on the heavy β-subunit, while the light α-subunit is responsible for substrate specificity and participates in binding penicillin side chains [3]. The suggestion that the formation of the active site should involve the association of both subunits has been formally approved by experiments on subunit separation which resulted in the entire loss of enzyme activity [3,4]. Moreover, a fact which should be taken into account is that according to the traditional methods of enzymology the dissociation of oligomeric enzymes (the first step in the separation of subunits) is usually accomplished under strongly denaturing conditions [5], and the above attempts [3,4] were not an exception.

A new approach to the analysis of structure–function relationships in oligomeric enzymes, which includes a 'soft' disassembling of subunit complexes in non-denaturing conditions, has been developed and has already found practical application [6–9]. The main idea of this method is the use of reversed micelles (RM) of a surfactant in organic solvent as the reaction medium in enzymology (for review see [10–12]). The size of the inner water cavity of RM can be widely varied by changing the surfactant hydration degree, w_o = [H_2O]/[surfactant]. This allows for conditions which promote the formation of certain protein associations or, vice versa, facilitate protein complex dissociation into components, or subunits, in the oligomeric enzymes selected. Interestingly, the maximum catalytic activity for the enzymes solubilized in the RM system is observed at a particular hydration degree, namely when the size of the protein molecule is equal to that of the micellar matrix [13]. In relation to this principle for oligomeric enzymes the dependence of their catalytic activity on w_o usually gives several optima, which indicates the functioning of different supramolecular forms, as well as discrete subunits [6-9]. The stoichiometry of protein incorporation into RM can be followed by using ultracentrifugation techniques [13].

We have applied this method to PA in order to solve the problem of soft dissociation of the enzyme and to study the catalytic properties of the subunits.

2. MATERIALS AND METHODS

2.1. *Enzyme*

PA (EC 3.5.1.11) from *E. coli* was purified by ion exchange chromatography on DEAE-Sepharose and gel filtration on Sephadex G-200. The enzyme activity was equal to 26 U/mg (1 U hydrolyzes 1 μm of PAANA at pH 7.5, 25°C). The purity control of the obtained preparations was performed by SDS-PAGE.

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Abbreviations: RM, reversed micelles; PA, penicillin acylase; AOT, Aerosol OT (sodium bis-(2-ethyl)-sulfosuccinate); PMSF, phenylmethanesulfonilfluoride; PAANA, phenylacetic acid *p*-nitroanilide.

2.2. *Enzymatic activity in the reversed micelles*

In a typical experiment 25–70 μl of 50 mM Tris-HCl buffer, 3-7 μl of 0.4 M PAANA solution in a mixture of acetonitrile with dioxane (1:1) and 2-5 μl of enzyme solution with a known concentration of active sites were solubilized in 1.4 ml of 0.1 M Aerosol OT (AOT, Merck) in octane. The substrate (PAANA) was a kind gift from A.A. Shegolev. The accumulation of free *p*-nitroaniline was determined spectrophotometrically (410 nm) at 25°C using a PHILIPS PU 8630 instrument with the thermostatic vessel section. The coefficients of the molar absorption of *p*-nitroaniline were measured independently in the micellar system at various w_o. The k_{cat} values were calculated using Lineweaver–Burk double-reciprocal plots. For all w_o parameters the pH optimum values of k_{cat} were found at 7.5.

2.3. *Sedimentation measurements*

The sedimentation coefficients (S) of the RM containing the protein were measured in an analytical ultracentrifuge (Beckman E), the measurements were performed and the dependence of S on w_o was analyzed as described in [13].

2.4. *Separation of light and heavy PA subunits*

440 μl of 50 mM PA solution and 800 μl of 50 mM Tris-HCl buffer (pH 7.5) were solubilized in 140 ml of 0.1 M AOT in octane (w_o = 5). The micelle fraction containing the heavy subunit was precipitated at 100,000 × *g* for 60 min in an MSE Superspeed 65 centrifuge. The supernatant containing the light subunit was separated from the sediment: the sediment was dissolved in 200 μl of 50 mM Tris-HCl buffer and solubilized in 20 ml of 0.1 M AOT in octane. To isolate the α-subunit the micellar solution of the enzyme, prepared in the same way, was centrifuged at 125,000 × *g* for 60 min.

2.5. *Electrophoresis of PA preparations*

Purity control of enzyme preparations, molecular mass determinations and evaluation of the quantitative ratio of PA subunits in the sediment after centrifugation, were accomplished electrophoretically according to Laemmli [14] in the presence of SDS and 2-mercaptoethanol. Before the electrophoretic procedure, PA was extracted from RM system by shaking in a separating funnel equal volumes of micellar and 1.5 M NaCl solutions. Then the water phase was concentrated up to 1 ml and dialyzed. The preparation was applied to the concentrating gel as a solution in sample buffer.

2.6. *Inhibition of PA in the AOT RM system in octane by PMSF*

15-30 μl of 50 mM Tris-HCl buffer (pH 7.5), 2 μl of 30 μM PA solution and 0–10 μl of 10 μM PMSF solution in acetonitrile were solubilized in 0.7 ml of 0.1 M AOT in octane. After 10 min incubation, 4 μl of 0.4 M PAANA solution was added and the rate of the reaction was measured. The quantity of PMSF needed for the complete inhibition of all the enzyme available was calculated from the dependence: Remaining activity vs. [PMSF]/[PA]

3. RESULTS AND DISCUSSION

3.1. *The dependence of the catalytic activity of PA on the hydration degree, w_o*

The profile of PA activity in AOT RM reveals at least three maxima observed at w_o = 14, 20 and 23 (Fig. 1A). Following the line of argument as mentioned above, we assume these three maxima to correspond to functioning of the light (w_o = 14) and heavy (w_o = 20) subunits and their dimer (w_o = 23). Theoretical calculations carried out according to the model described earlier [11], with the α- and β-subunits of PA, as well as their dimer, being considered spherical particles with M_r's of 26,000, 63,000 and 89,000, respectively, give w_o values of a few units less than the experimental ones. Such a discrepancy may well be accounted for by a number of reasons, for example, the presence of a mixture proteins and/or because of their non-spherical shape. In view of this, some additional methods were employed to confirm the effect under investigation, one of which was sedimentation analysis [11].

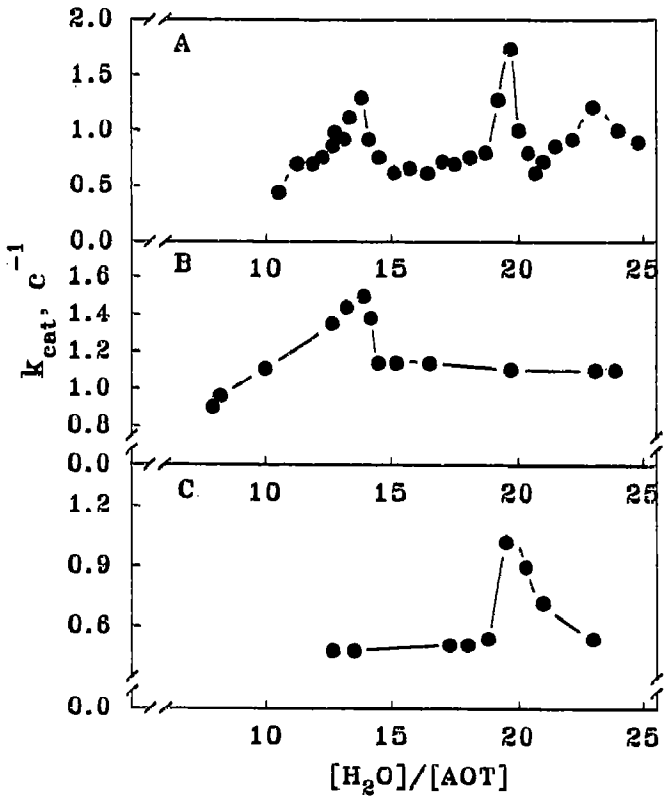

Fig. 1. The dependence of the catalytic activity (k_{cat}) of PA on the hydration degree, w_o, in the AOT RM system in octane. (A) Intact preparation of PA. (B) α-subunit preparation. (C) β-subunit preparation.

3.2. *Ultracentrifugation of AOT RM containing solubilized PA*

The sedimentation coefficients of protein-containing RM are presented in Fig. 2. When the value of w_o exceeded 22, the sedimentation data revealed the exis-

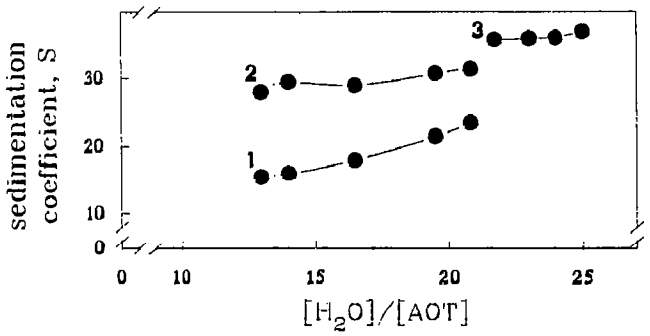

Fig. 2. The dependence of the sedimentation coefficients (S) of RM containing PA on the hydration degree. (1) α-subunit, (2) β-subunit, (3) αβ-dimer.

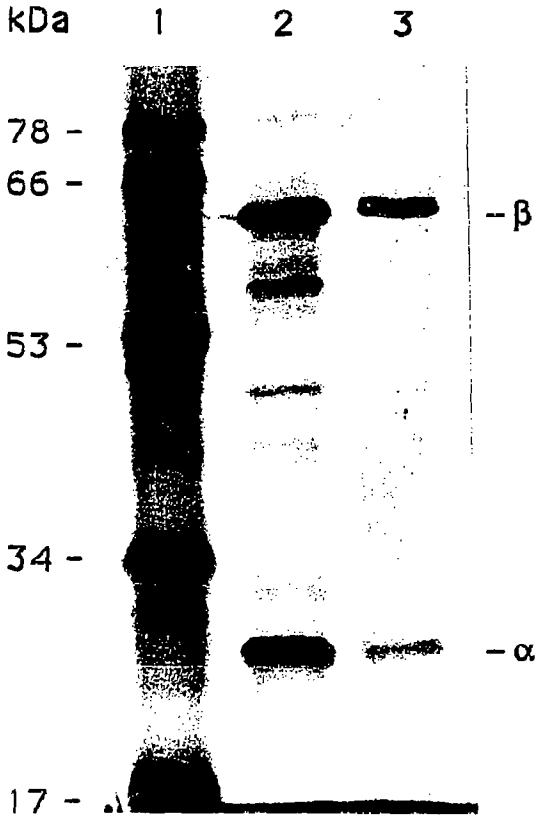

Fig. 3. SDS-PAGE of PA preparations. Lanes: (1) molecular mass standards (kit LKB 1860-102), (2) the intact preparation of the enzyme, (3) sediment after 60 min centrifugation at 100,000 × g.

tence of micelles of a single type, namely, that with the enzyme in a dimer form. At lesser hydration degrees, two types of protein-containing micelles were detected, micelles with α- and β-subunits. A considerable difference in the sedimentation coefficients of these two types of micelles permits separation of the light and heavy subunits. The micelles containing β-subunits can be precipitated via preparative centrifugation, while the α-subunit micelles remain in solution.

The enzyme preparation obtained by this method was analyzed using SDS-PAGE. Fig. 3 shows that there are two major bands with M_r's of 26,000 and 63,000 (i.e. α- and β-subunits) in the intact enzyme preparation. In contrast, the precipitate contains predominantly the β-subunit (Fig. 3, lane 3).

The sedimentation analysis, together with SDS-PAGE data, strengthen the assumption that in the AOT RM system in octane a reversible dissociation of PA into individual subunits occurs, and this process is controlled by the value of w_o.

Support for this hypothesis comes from the experiment which reconstructs the Fig. 1A profile, step by step, using preparations of partially separated subunits (supernatant and sediment after centrifugation). Fig. 1B shows that in the case of the α-subunit preparation

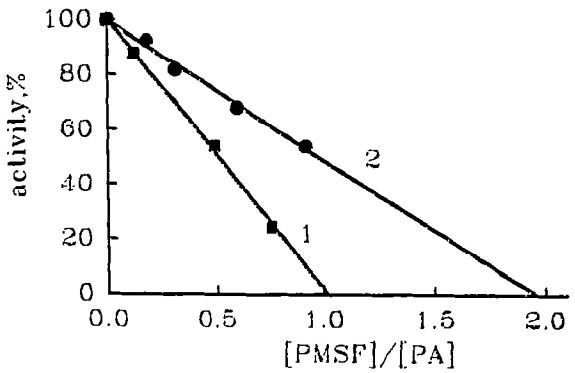

Fig. 4. PMSF titration of PA active sites in the system of AOT RM. (1) w_o = 23, (2) w_o = 14. Before activity measurements were carried out the enzyme was incubated for 10 min in a range of volumes (0–10 μl) of 10 μM PMSF solution.

solubilized by RM, only one optimum can be seen when studying the dependence of catalytic activity on the hydration degree, and its position corresponds to the first optimum in Fig. 1A. For the β-subunit fraction in the same experiment (Fig. 1C), there is also only one optimum, but the location coincides with the β-subunit optimum in Fig. 1A. When both micellar solutions are mixed all the maxima, including the one corresponding to the dimer at w_o = 23, appear, analogously to the dependence in Fig. 1A.

The fact that both subunits of PA turned out to be catalytically active makes us acknowledge the existence of two independent active sites on the subunits. That may seem surprising, because in the experiments on active site titration with PMSF in water solutions (for PA in a dimer form) only one active site has been found [1,2,15]. To answer this question the influence of PMSF on the activity of PA in reversed micelles was examined.

3.3. *Inhibition of PA with PMSF*

The titration of the active sites of the enzyme in RM was carried out at two different values of w_o, 14 and 23, which correspond to the conditions at which PA exists in the forms of the individual subunits and the dimer, respectively. We have found that the concentration of PMSF needed for the inactivation of a mixture of the subunits is approximately two-times higher than that needed for dimer inactivation (Fig. 4).

In addition, on solubilizing the enzyme, which was previously inactivated by PMSF in aqueous solution, catalytic activity with an optimum at w_o = 14 can be observed. It is likely that the active site located on the light subunit is screened in the dimer form and so does not work. Under the conditions of subunit dissociation this site becomes accessible to the inhibitor, which results in the increase in stoichiometry of the enzyme–inhibitor interaction.

The result that both subunits appear to possess catalytic activity brings about a number of additional ques-

tions concerning the causes of such structural organization, as well as the possible distinctions in substrate specificity of the active sites on the α- and β-subunits. We are planning to continue the experiments in this direction.

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