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Homogeneous solutions of hydrophilic enzymes in nonpolar organic solvents

New systems for fundamental studies and biocatalytic transformations

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A typical hydrophilic enzyme, CT, can be dissolved in nonpolar organic solvents (*n*-octane, cyclohexane and toluene) up to μM concentrations. In the homogeneous solution obtained, the enzyme possesses catalytic activity and enormously high thermostability. It does not lose this activity even after several hours refluxing in octane (126°C) or cyclohexane (81°C).

Enzyme solution; Organic solvent; Activity; Stability

1. INTRODUCTION

Biocatalysis in nonaqueous media is an emerging challenge of present-day biochemistry and biotechnology [1]. Systems in which enzymes function in the presence of only small amounts of water (less than several percent) have been recently described [1–4].

Among the less-developed systems are the homogeneous solutions of enzymes in organic solvents. The reason is that in polar organic solvents (alcohols, formamides, etc.) most hydrophilic proteins lose their unique structure and activity [5]. The inactivation is explained by the 'stripping off' of essential water molecules by solvent molecules from the aqueous shell noncovalently bound to a protein [6]. On the other hand, it was considered impossible to dissolve in appreciable amounts a hydrophilic protein in a nonpolar solvent (e.g. hydrocarbon) [5,7]. Thus, the few experimental indications that enzymes work dissolved in organic solvents are the exceptions rather than the rule [8-10].

In this paper, we present the first strong experimental evidence that a typical hydrophilic enzyme, CT, can be dissolved in nonpolar organic solvents in μM concentrations, where it works as a catalyst and possesses high thermostability.

Abbreviations: CT, α-chymotrypsin; CbzTNE, *N*-carbobenoxy-L-tyrosine *p*-nitrophenyl ester; PrOH, propanol-1.

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2. MATERIALS AND METHODS

2.1. *Labelling of CT by thermal activation of gaseous tritium [11]*

Walls of a 50 ml glass vessel were covered by a thin layer of 1 ml solution (1 mg/ml) of CT (α-chymotrypsin, EC 3.4.21.1, Sigma) and lyophilized. The vessel was vacuumized (10^{-2} Pa) and gaseous tritium was passed through during a short (10 s) contact with a tungsten thread at 1800 K. The protein was then dissolved in 1 mM HCl and dialyzed up to a constant level of radioactivity in the preparation (a test for the absence of labile tritium in labelled CT) equal to 2×10^8 dpm/mg lyophilized powder. The labelling did not change electrophoretic patterns or enzymatic activity of CT.

2.2. *Preparation of enzyme powders for dissolution*

Commercial or tritium-labelled CT was dissolved in distilled water (1 mg/ml), the pH adjusted to 8.0 by 5 M KOH and the solution lyophilized. For the preparations used in enzyme activity assays, one of the enzyme effectors, i.e. H_3BO_3 (Reakhim), *N*-acetyl- or *N*-benzoyl-L-tyrosine (Sigma) was added before lyophilization in at least 100 molar excess in relation to the enzyme.

2.3. *Dissolution of CT in organic solvents*

Method 1. A suspension of the enzyme powder in an organic solvent was placed in a stoppered flask and shaken at 100 rpm, 20°C. Before use, cyclohexane, octane and toluene (all from Reakhim) were purified by distillation under Na. The solution was finally passed through a Millipore 0.22 μm filter or centrifugated at 13 000 rpm for removal of solid particles.

Method 2. 1 ml acetone was added to 2 ml suspension and evaporated off under vacuum with intensive stirring at 25°C. The solution was thereafter separated from the particles by filtration.

Method 3. The suspension was refluxed in the vessel connected with second containing the solvent refluxing under Na, in such a way that the vapors were in contact. The solution of CT was thereafter obtained by filtration. In the case of cyclohexane and toluene, the solution was additionally evaporated off under vacuum with ca. 1.5-fold reduction in volume and without perturbing optical transparency.

2.4. *Catalytic activity of CT in organic solutions*

0.1–1 ml organic solution of CT was placed in a 3 ml cuvette of a spectrophotometer (Beckman 25) with thermostatic control (+0.5°C).

A substrate, CbzTNE (5–200 μM, Serva), was then introduced by diluting a concentrated stock solution in acetonitrile. The concentration of the latter in the cuvette was always 1% v/v. The reaction was initiated with PrOH (Reakhim, distilled under Na) added to a concentration of 7% (v/v). The rate of *trans*-esterification was followed at 310 nm, 20–85°C (the difference extinction coefficient was equal to ca. 10^4 $M^{-1}·cm^{-1}$).

2.5. *Study of the thermostability of CT in organic solutions*

1–5 ml solution of CT in the organic solvents was refluxed, aliquots were taken after certain time intervals, cooled to room temperature and the residual catalytic activity determined.

3. RESULTS AND DISCUSSION

3.1. *Dissolution of CT in organic solvents*

CT powder is dissolved in octane during a prolonged (weeks) shaking at room temperature (*Method 1*) to a concentration of 0.1–0.3 μM as detected by radioactivity (bar 1, Fig. 1). Both the kinetics of dissolution and the final concentration of CT depends on a number of factors. The solubility increases with an enzyme-to-octane ratio, and with temperature, and strongly depends on the humidity of both the powder and the solvent but not on the presence of effectors of CT. Two more methods were developed which significantly accelerated the dissolution.

According to *Method 2*, acetone (or diethyl ether) is added to a suspension of CT in octane and then is evaporated at room temperature under vacuum. The concentration of CT in the octane solution after 30 min is higher than for a 4-week shaking without acetone (bars 1 and 2, Fig. 1). The acceleration is probably due to a removal of detrimental water by aseotrope with acetone.

In *Method 3*, a favorable action of heating and drying is combined (at the expense of constant contact of solvent vapors with Na). By this method, concentration of CT increases up to 0.6 μM in octane and exceeds 1 μM in cyclohexane and toluene (bars 3–5, Fig. 1). For the latter two, concentration of CT is additionally increased 1.5-fold by evaporating off a homogeneous solution (Fig. 1). We made sure that the procedure did not perturb the optical transparency and homogeneity of the solutions. Similar results were obtained for peroxidase, lysozyme, albumin and covalently modified CT.

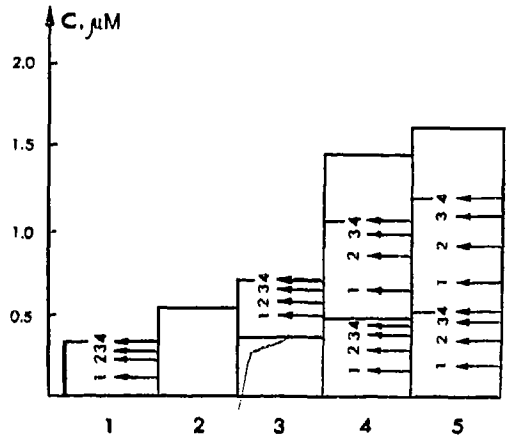

Fig. 1. Concentration of CT (C, in μM) in nonpolar solvents as a function of the method of preparation of solutions. Bars 1–3 correspond to *Methods 1–3* with octane as solvent (see Materials and Methods), and bars 4 and 5 to *Method 3* with cyclohexane and toluene as solvents, respectively. The arrows show the concentration of CT after 1, 2, 3 and 4 weeks of dissolution by *Method 1* (bar 1 and lower parts of bars 4 and 5) and after 1, 2 and 3 h of dissolution by *Method 3* (bar 3 and middle parts of bars 4 and 5).

3.2. *Physical properties of CT in nonpolar solvents*

Preliminary light-scattering experiments show that there are no particles larger than 100 Å in the solution. Sedimentation also proves the absence of larger aggregates and shows that CT sediments with coefficients similar to those for the monomeric enzyme in aqueous solution. The conclusions are also valid for octane solutions of lysozyme and albumin. Hence, it is highly probable that in nonpolar solvents the enzymes exist in the non-associated state or as *molecular solutions*. However, the conclusion needs additional justification.

Absorption- and fluorescence spectra of CT in octane are quite similar to those in water. This is an indication of the compact structure of CT in the solutions.

As evidenced from electrophoresis in polyacrylamide gels, the molecular weight of CT of ca. 25 kDa remains unaltered after tritium labelling, lyophylization, dissolution in octane or toluene and final isolation after evaporation of the solvent. Hence, in organic solutions, CT retains its integrity.

3.3. *Catalytical properties of CT in the organic solutions*

CT catalyzes *trans*-esterification of CbzTNE by PrOH and other alcohols in the systems. The following observations support this, as well as the specificity of the reaction with respect to the enzyme.

(*i*) Both reaction products, *N*-carbobenzoxy-L-tyrosine propyl ester and *p*-nitrophenol, were identified by HPLC. The latter is isolated in a crystalline state and characterized. The reaction proceeds with more than 50% conversion and hydrolysis does not take place since the hydrolytic product, *N*-carbobenzoxy-L-tyrosine, is not detected. The same is true also for *trans*-esterification by PrOH of *N*-acetyl-L-tyrosine ethyl ester.

(*ii*) The reaction rate is proportional to a concentration of CT in octane. The dependence of the rate on the concentration of both substrates, CbzTNE and PrOH, obeys the Michaelis equation. The corresponding values of K_m are 0.66 mM and 0.15 M at 59°C.

(*iii*) Both CT, irreversibly inhibited by acylation of the active center with phenylmethanesulfonylfluoride, and a nonenzymic protein, bovine pancreatic trypsin inhibitor, do not catalyze *trans*-esterification in the organic solutions. Catalytic activity of CT is also specifically inhibited by addition of pancreatic trypsin inhibitor dissolved in octane. The K_i evaluated from the kinetic experiments in terms of the competitive inhibi-

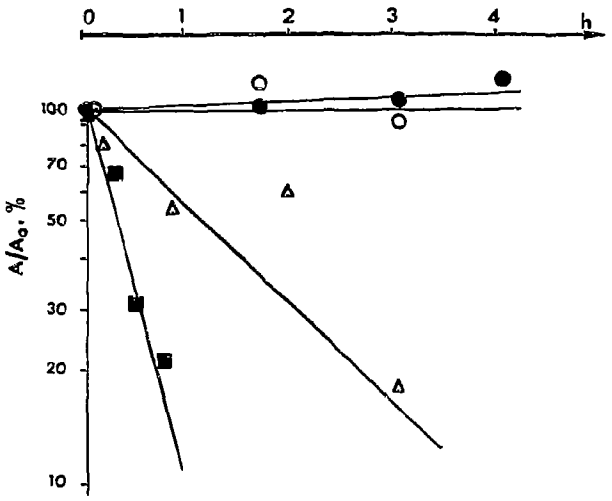

Fig. 2. Dependence of the residual catalytic activity, A (% of the activity before inactivation, A_0, in logarithmic scale) of CT on time of boiling (h) in solutions of octane at 126°C. (●), cyclohexane at 81°C (○), octane + PrOH, 7% (v/v) (△) and octane + PrOH, 7% (v/v) + water, 0.07% (v/v) (■).

tion is ca. 5 μM, several orders of magnitude higher than in water [12]. In any case, a great deal of specificity of protein– protein interaction is conserved that can be used in various analytical systems.

(*iv*) The initial maximal rate (V) strongly depends on the method of preparation of enzyme solutions, namely on the addition of effectors of CT and the pH of the bulk water before lyophilization. For example, for CT lyophilized without any effector or at pHs several units away from the optimum, the enzyme is practically inactive in octane solution. Hence, for CT solutions, we come across the same phenomenon as Zaks and Klibanov while working with powder suspensions in organic solvents, i.e. 'pH- and ligand-induced enzyme memory' [6]. The molecular reasons may be the same, i.e. high conformational rigidity of a protein molecule in waterless media [6].

3.4. *Thermostability of CT in organic solutions*

Another manifestation of rigidity of CT in organic solutions is the ability to retain high catalytic activity at elevated temperatures. The linear increase in activity ($\log V - T^{-1}$) is observed between 20 and 85°C showing the absence of denaturation. On the other hand, in aqueous solution CT has maximal activity at 40–45°C and loses it at higher temperatures [13].

In organic solutions, CT is highly stable against irreversible thermo-inactivation. Fig. 2 shows that the enzyme withstands boiling in octane (126°C) and cyclohexane (81°C) without inactivation for at least several h. For comparison, in aqueous solution CT is completely inactivated at 50°C in less than 1 h [13]. However, the inactivation becomes noticeable when a polar solvent (PrOH) is added and even more pronounced on addition of small amounts of water (in both cases, without a change of homogeneity of solutions). Similar correlation of thermostability with solvent polarity has been observed by Volkin et al. [14] with suspension of an enzyme powder in organic solvents. Unlike the heterogeneous system where protein–protein interactions may stabilize the enzyme, in our case, CT is in a purely homogeneous (probably molecular) solution.

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