

# Relationship between surface hydrophilicity of a protein and its stability against denaturation by organic solvents

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The stability of  $\alpha$ -chymotrypsin covalently modified with a strongly hydrophilic modifier, pyromellitic dianhydride, against solvent-induced denaturation in water–organic solvent binary mixtures has been studied. It was found that the hydrophilization results in a strong stabilization of the enzyme against denaturation by organic solvents. The stabilizing effect is explained in terms of the enhanced ability of the hydrophilized enzyme to keep its hydration shell, which is indispensable for supporting the native protein conformation, from denaturing stripping by organic solvents.

Chymotrypsin; Organic solvent; Enzyme denaturation; Enzyme modification

## 1. INTRODUCTION

Protein molecules in solution are surrounded by a hydration shell which is composed of water molecules attached to the protein surface mainly by hydrogen bonds and is indispensable for supporting the native protein conformation. If an organic solvent is present in solution, its molecules tend to displace water from the hydration shell thus distorting the finely balanced interactions responsible for maintaining the native conformation of the protein molecule. According to a generally accepted notion, the destruction of the hydration shell is one of the main reasons of protein denaturation by organic solvents [1,2].

An obvious way to protect proteins from such a denaturation consists of eliminating of the organic solvent from the immediate vicinity of the protein surface by entrapment of the protein molecule in a strongly hydrophilic matrix. In this case the matrix bearing tightly bound water molecules will create a favorable aqueous microenvironment around the entrapped protein and severely restrict the access of the organic solvent to the protein surface. The literature gives numerous examples of successful practical implementation of this approach. As a matrix, reversed surfactant micelles [3], hydrophilic solid supports [4] and hydrophilic high-molecular-weight polymers attached to the protein surface by covalent [5–8] or electrostatic [9] bonding have been used. In these systems the denaturation is prevented, in fact, due to the spatial

separation of the protein from the unfavorable contact with the organic solvent by the matrix-bound aqueous microphase (for a detailed discussion, see the recent review [10]).

An alternative, though closely related approach could consist of a covalent modification of the protein surface with strongly hydrophilic low-molecular-weight groups. Such a modification could help the protein to hold its hydration shell more tightly and thus to resist efficiently the denaturing stripping of water molecules from its surface by organic solvents without embedding the whole protein molecule into a segregated water-rich matrix-supported microphase.

The aim of the present work was to demonstrate the feasibility of this approach for protein stabilization against denaturation by organic solvents. As a model, use was made of  $\alpha$ -chymotrypsin modified at surface amino groups with pyromellitic dianhydride.

## 2. MATERIALS AND METHODS

$\alpha$ -Chymotrypsin from bovine pancreas (type II) was obtained from Sigma. Hexamethylphosphoramide (Koch-Light), 1,3-butanediol and 2,3-butanediol (Ferak) were used as received. Other solvents were of reagent grade and were purified immediately before use according to established procedures [11]. Surface amino groups of  $\alpha$ -chymotrypsin were modified with pyromellitic dianhydride [12] or by reductive alkylation with acetic aldehyde in the presence of sodium cyanoborohydride [13]. Spectrophotometric titration with trinitrobenzenesulfonic acid (Sigma) according to the procedure described in [14] showed that after modification no free (unmodified) amino groups were present at the protein surface. The catalytic activity of modified  $\alpha$ -chymotrypsin was measured spectrophotometrically on a Beckman E 25 spectrophotometer at 25°C using *N*-benzoyl-L-tyrosine *p*-nitroanilide (Sigma) as a substrate [15]. Maximal velocities ( $V_m$ ) of enzymatic reactions were determined from Lineweaver–Burke plots.

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Regression analysis of experimental data was performed using the Statgrafics computer program.

### 3. RESULTS AND DISCUSSION

#### 3.1. Protein denaturation by organic solvents: general features

The addition of increasing concentrations of organic solvents into aqueous solutions of proteins eventually always results in protein denaturation which manifests itself in a decrease of enzymatic activity and/or an abrupt change of protein spectra [15,16]. The decrease of activity shows a very well-defined threshold behavior as illustrated in Fig. 1. The threshold denaturation of proteins by organic solvents represents a general phenomenon operative regardless of the nature of specific solvents or protein used [16]. The concentration of the organic solvent at which half-inactivation occurs is called the threshold concentration,  $C_{50}$  (shown in Fig. 1 by arrows).

Recently, we have developed a thermodynamic model of protein denaturation by organic solvents [16] which quantitatively describes the denaturation process in terms of a competitive displacement of water molecules from the protein hydration shell by molecules of the organic solvent. According to the model, at increasing concentrations of the organic solvent in aqueous solution more and more water molecules are stripped off the protein surface, until a certain critical amount of removed water molecules is reached. After this point the threshold reversible denaturation takes place. The threshold concentration  $C_{50}$  is related to physical properties of the organic solvent according to the following equation [16]:

$$\log \frac{W_{50}}{C_{50}^n} = B_0 + B_1 n + B_2 E_T(30) + B_3 n \log P \quad (1)$$

where  $W_{50}$  is the concentration of water in the system at which half-denaturation of the protein occurs,  $n$  is the ratio of molecular surface area of water to that of the organic solvent,  $E_T(30)$  is the empirical solvent polarity parameter [17],  $P$  is the partition coefficient of the organic solvent in water-octanol biphasic system [18] ( $P$  is the measure of solvent hydrophobicity), and coefficients  $B_i$  are constant for a given protein at constant temperature. Concentrations standing in Eqn 1 are expressed in  $\text{mol} \cdot \text{l}^{-1}$ .

#### 3.2. Effect of surface hydrophilization on enzyme stability against denaturation by organic solvents

The modification of  $\alpha$ -chymotrypsin with pyromellitic dianhydride proceeds according to the following scheme [12]:

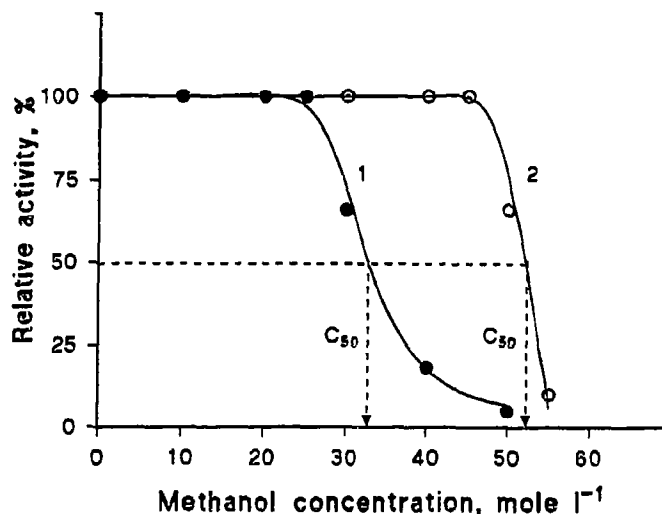
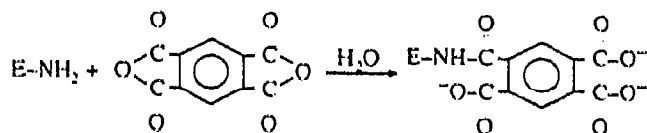


Fig. 1. Dependence of the relative catalytic activity (expressed in terms of the maximal velocity,  $V_m$ ) of  $\alpha$ -chymotrypsin on methanol concentration in water-methanol binary mixtures. 1, native  $\alpha$ -chymotrypsin; 2,  $\alpha$ -chymotrypsin modified with pyromellitic dianhydride. Vertical arrows indicate corresponding threshold concentrations of the organic solvent,  $C_{50}$ .

As a result, three charged carboxylic groups are introduced at the protein surface in place of each of the 15 accessible surface amino groups. Such a modification results in a pronounced increase of hydrophilicity of the  $\alpha$ -chymotrypsin molecule [12].

We found that hydrophilized  $\alpha$ -chymotrypsin could endure much higher concentrations of organic solvents without loss of catalytic activity as compared to the native enzyme. As an example, Fig. 1 shows that in the case of methanol as an organic co-solvent the hydrophilization of  $\alpha$ -chymotrypsin resulted in a shift of the  $C_{50}$  value towards higher methanol concentrations by approximately 20 vol.%. Similar shifts were observed also for a number of other organic solvents tested (Table I). These results clearly indicate that hydrophilization of the protein surface brings about a significant increase in protein stability against denaturation by organic solvents.

The occurrence of the hydrophilization-induced stabilization effect can be readily explained in terms of a quite reasonable assumption that the hydrophilization of the protein considerably enhances its ability to retain its hydration water. Such a reinforcement of the protein hydration shell makes it much more resistant to the stripping by organic solvents, and hence, according to the concept formulated in the preceding section, increases the protein stability against solvent-induced denaturation.

It has to be stressed that the modification of the equivalent number of surface amino groups of  $\alpha$ -chymotrypsin with ethyl radicals via reductive alkylation by acetic aldehyde did not result in the enzyme stabilization against denaturation by organic solvents,

Table I

Threshold concentrations of different organic solvents determined for native and hydrophilized  $\alpha$ -chymotrypsin, and physicochemical parameters of the solvents<sup>a</sup>

Solvent	$C_{50}$ (vol.%) for $\alpha$ -chymotrypsin		$n$	$E_T$ (30) (kcal·mol <sup>-1</sup> )	log <i>P</i>
	native	hydrophilized			
Acetone	23	41	0.254	42.2	-0.24
Ethanol	36	54	0.301	51.9	-0.32
Dioxane	7	27	0.240	36.0	-0.27
Glycerol	73	97	0.194	57.0	-2.50
Hexamethylphosphoramide	18	39	0.099	40.9	0.28
Methanol	32	54	0.414	55.5	-0.74
1,3-Butanediol	56	70	0.178	52.8	-1.02
2,3-Butanediol	39	67	0.179	51.8	-0.92

<sup>a</sup>The data for native  $\alpha$ -chymotrypsin and values of solvent parameters are taken from [16].

i.e. no changes in  $C_{50}$  values were observed (data not shown). This means that it is the hydrophilization of the protein surface, and not the modification of amino groups per se, that is responsible for the observed stabilization effect.

In full agreement with a thermodynamic model of protein denaturation by organic solvents [16], the behavior of hydrophilized  $\alpha$ -chymotrypsin can be quantitatively described by Eqn 1 using corresponding  $C_{50}$  values and the solvent parameters given in Table I. The computer regression analysis gave the following values for coefficients of Eqn 1:  $B_0$ , 1.65;  $B_1$ , 0.92;  $B_2$ , -0.007;  $B_3$ , 2.57 (for the unmodified protein  $B_0$ , 2.58;  $B_1$ , 0.65;  $B_2$ , -0.02;  $B_3$ , -0.207). The value of the adjusted coefficient of determination,  $R$ -squared [19], was computed to be as high as 0.90 (for the unmodified protein 0.91) indicating a good fit of the experimental data to the model.

In conclusion, we have demonstrated that hydrophilization of the protein surface greatly increases its stability against denaturation by organic solvents due to the reinforcement of the protein hydration shell. Previously, we found [12,20] that the hydrophilization resulted also in a very considerable increase in protein thermostability. From these findings we conclude that hydrophilization of the protein surface could represent a universal approach to enhance protein stability against unfavorable environmental conditions. Further investigations in this direction are in progress.

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