Enzyme–Polyelectrolyte Complexes in Water–Ethanol Mixtures: Negatively Charged Groups Artificially Introduced into α-Chymotrypsin Provide Additional Activation and Stabilization Effects

Elena V. Kudryashova,[1] Alexander K. Gladilin,[1] Alexander V. Vakurov,[1] Frederic Heitz,[2] Andrey V. Levashov,[1] Vadim V. Mozhaev[1]*

[1]Chemistry Department, Moscow State University, 119899 Moscow, Russia
[2]CRBM-CNRS (ERS 0155) and INSERM U 249, Route de Mende, BP 5051, 34033 Montpelier Cedex, France

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Abstract: Formation of noncovalent complexes between α-chymotrypsin (CT) and a polyelectrolyte, polybrene (PB), has been shown to produce two major effects on enzymatic reactions in binary mixtures of polar organic cosolvents with water. (i) At moderate concentrations of organic cosolvents (10% to 30% v/v), enzymatic activity of CT is higher than in aqueous solutions, and this activation effect is more significant for CT in complex with PB (5- to 7-fold) than for free enzyme (1.5- to 2.5-fold). (ii) The range of cosolvent concentrations that the enzyme tolerates without complete loss of catalytic activity is much broader. For enhancement of enzyme stability in the complex with the polycation, the number of negatively charged groups in the protein has been artificially increased by using chemical modification with pyromellitic and succinic anhydrides. Additional activation effect at moderate concentrations of ethanol and enhanced resistance of the enzyme toward inactivation at high concentrations of the organic solvent have been observed for the modified preparations of CT in the complex with PB as compared with an analogous complex of the native enzyme. Structural changes behind alterations in enzyme activity in water–ethanol mixtures have been studied by the method of circular dichroism (CD). Protein conformation of all CT preparations has not changed significantly up to 30% v/v of ethanol where activation effects in enzymatic catalysis were most pronounced. At higher concentrations of ethanol, structural changes in the protein have been observed for different forms of CT that were well correlated with a decrease in enzymatic activity. *Biotechnol Bioeng* **55**: 267–277, 1997.
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* *Present address:* Department of Chemical and Biochemical Engineering, 127 B Chemistry Building, University of Iowa, Iowa City, IA 52242; telephone: 1-319-353-2356; fax: 1-319-335-1415; e-mail: vmojaev@icaen.uiowa.edu
Correspondence to: V. Mozhaev
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INTRODUCTION

Biocatalytic systems in homogeneous water–cosolvent binary mixtures have been the subject of numerous investigations aimed at solving both fundamental and applied problems, because these media provide a number of obvious merits (Douzou, 1977; Khmelnitsky et al., 1991; Singer, 1962). However, one of the serious disadvantages of these systems is that enzymes are often inactivated at comparatively low cosolvent concentrations (10% to 40% v/v, depending on enzyme and cosolvent) because of denaturation (Khmelnitsky et al., 1991; Mozhaev et al., 1989; Tomiuchi et al., 1993). A number of approaches directed toward better adaptation of enzymes to water–cosolvent mixtures have been put forward (Dordick, 1992; Khmelnitsky et al., 1988; Mozhaev et al., 1990; Wong, 1992). Formation of noncovalent enzyme–polyelectrolyte complexes has been suggested as a new approach to obtain biocatalysts with enhanced resistance toward inactivation by organic solvents (Gladilin et al., 1995).

Protein–polycation complexes are spontaneously formed in aqueous solutions (Izumrudov et al., 1984), mainly due to multiple electrostatic interactions between positive charges in polycation and negatively charged carboxylic groups in the protein molecule. These interactions may lead to an even stronger electrostatic binding of a protein to a polyelectrolyte in organic solvents due to the lower dielectric constant of the medium. We assume that this binding may be responsible for a higher stability of the enzyme in the complex with the polyelectrolyte against denaturation by organic solvents (Gladilin et al., 1995).

The method of covalent modification enables us to introduce additional charged groups into the protein with retention of the majority of catalytic activity and substrate specificity of the enzyme, thus increasing the number of potential sites for electrostatic binding to a

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polyelectrolyte. In fact, polybrene (PB), used by our group as a polycation for obtaining complexes with enzymes, has 35 positively charged quaternary amino groups in its molecule (this value was calculated from the structural formula of PB and the molecular mass reported by Sigma). During formation of the complex, the overall concentration of positive charges in PB molecules is nearly 15-fold higher than the content of negatively charged residues in the molecules of α-chymotrypsin (CT) (Birktoft and Blow, 1972). It seems reasonable to assume that at least some of the negatively charged carboxylic groups introduced additionally by covalent modification into CT molecules are able to interact electrostatically with extra positive charges in the molecule of PB. These multipoint electrostatic interactions should increase conformational rigidity of the protein molecule. Therefore, supplementary stabilization of the enzyme can be expected against denaturation by organic solvents, in comparison with the complex of PB with nonmodified CT.

In this work, we studied the effect of negatively charged groups artificially introduced by covalent modification of an enzyme on its catalytic and structural properties in the complex with polyaction in homogeneous water–cosolvent mixtures. We have selected CT as a model enzyme for which behavior in water–cosolvent systems has been described in detail (Khmelnitsky et al., 1991; Kijima et al., 1996; Mozhaev et al., 1989; Tomiuchi et al., 1993; West et al., 1990). As a polycation, we have used PB, for which the existence of noncovalent complexes with CT in water–cosolvent mixtures has previously been shown (Gladilin et al. 1995). In such complexes, the enzyme has proved to be more active than free CT over a wide concentration range of several organic solvents (Gladilin et al., 1995).

MATERIALS AND METHODS

Covalent Modification of α-Chymotrypsin

α-Chymotrypsin (CT; Sigma, EC 3.4.21.1), was acylated with anhydrides of succinic (Reanal) and pyromellitic (Aldrich) acids (Mozhaev et al., 1988). Solution of an anhydride (10 to 500 molar excess, with respect to the enzyme) in dimethylsulfoxide (Sigma) was added at 4°C by drops during 1 min to 40 μ*M* CT (10 mL) in 0.1 *M* phosphate buffer (pH 8.0), containing 0.05 *M N*-acetyl-L-tyrosine (Sigma), which was added to protect the active site of CT from modification. The pH of the reaction mixture was kept constant by adding, dropwise, small portions of 1 *M* KOH. The reaction proceeded for 2 h, and then the enzyme was separated from low-molecular-mass compounds by dialysis and freeze-dried.

Reductive alkylation with glyceraldehyde (Reanal) was carried out as described by Mozhaev et al. (1992). The aldehyde and sodium cyanoborohydride (Sigma) (100 molar excess of both with respect to the enzyme) were added by small portions during 1 min to 40 μ*M* solution of CT (10 mL) in 0.1 *M* phosphate buffer, containing 0.05 *M N*-acetyl-L-tyrosine (pH 8.4). The solution was incubated at room temperature for 30 min and, after dialysis, the enzyme was freeze-dried.

Determination of the Degree of Modification

The number of modified amino groups was determined by following the decrease in free (nonmodified) amino groups by titration with picrylsulfonic acid (Sigma) on a Beckman 25 spectrophotometer (Mozhaev et al., 1992).

Titration of Active Sites in Preparations of α-Chymotrypsin

Concentration of active sites in all samples of CT in aqueous solutions was determined on a spectrophotometer with *N*-trans-cinnamoylimidazole (Sigma) as titration reagent at pH 5.05 and 25°C (Schonbaum et al., 1961).

Preparation of Enzyme–Polyelectrolyte Complexes

We studied enzyme–polycation complexes using polybrene (PB) (hexadimethrinebromide, trademark name of 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; Sigma) with the molecular mass of 6.5 kDa as a polycation. The complexes of CT with PB were prepared by mixing solutions of the enzyme and the polycation, both dissolved in 5 m*M* aqueous buffer of 3-[*N*-morpholino]-propanesulfonic acid (MOPS), pH 7.45 (Gladilin et al., 1995). For obtaining soluble complexes, the CT/PB ratio was always kept equal to 1:2.8 g/g (or 1:10.75 mol/mol) and enzyme concentration was 0.2 m*M*. After mixing the components, pH was adjusted to 7.45 on a PHM-82 pH-meter (Radiometer) with 1 *M* KOH.

Determination of Enzymatic Activity in Binary Water–Ethanol Mixtures

An aliquot of stock aqueous solution of free CT (0.2 m*M*) in 1 m*M* HCl or its complex with PB was added to a binary mixture of water with ethanol (Reakhim) containing 5 m*M* MOPS (pH 7.45) for a final enzyme concentration of 1.0 μ*M*. In the water–ethanol solution, pH was adjusted to 7.45 by 1 *M* KOH; see West et al. (1990) for a discussion of the applicability of this procedure. The enzymatic reaction was started by adding an aliquot of concentrated (40 m*M*) stock solution of *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTNA; Sigma) in 1,4-dioxane (VEB Laborchemie Apolda) to obtain a final substrate concentration of 0.04 to 1.0 m*M*. The initial rate of hydrolysis was measured by following the

formation of the product, *p*-nitroaniline, on a spectrophotometer at 390 nm; the molar extinction coefficient was detected for each concentration of ethanol. The values of the maximal rate of the reaction (V_{max}) were determined from depenencies of the initial reaction rate on the substrate concentration by using direct nonlinear regression analysis; the rate of spontaneous substrate hydrolysis was always negligible in comparison with the enzymatic rate.

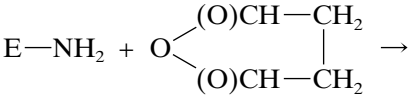
Spectroscopy of Circular Dichroism

Circular dichroism experiments were carried out with a Jobin Yvon Mark V spectrometer at 25°C with quartz cells of path length 1 mm in the far-ultraviolet region (200 to 260 nm) or 1 to 5 mm in the near-ultraviolet region (250 to 320 nm). The protein concentrations were 0.1 to 0.2 mg/mL and 0.5 to 3.0 mg/mL, respectively. The content of the secondary structures was calculated according to Greenfield and Fasman (1969).

RESULTS

Covalent Modification of α-Chymotrypsin: Properties of Modified Enzymes

For modification of CT, we used pyromellitic and succinic anhydrides as acylating reagents and glyceraldehyde as alkylating reagent. The reaction of amino groups of CT with succinic anhydride proceeded as follows:

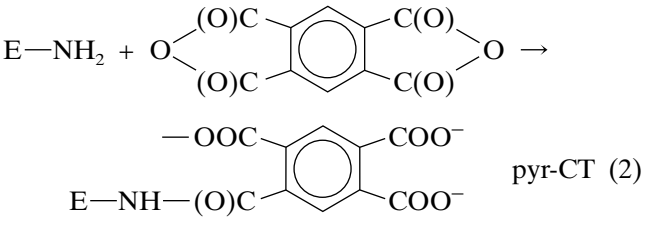


$$E{-}NH_2 + O\begin{cases}(O)CH{-}CH_2\\ \quad\quad\quad\quad\quad\quad|\\ (O)CH{-}CH_2\end{cases} \rightarrow$$

$$E{-}NH(O)CH{-}CH_2{-}CH_2{-}CH(O)OH \quad \text{suc-CT} \quad (1)$$

where E is the enzyme. By this reaction, one negatively charged carboxylic group is introduced into the protein while the positive charge of each amino group modified is eliminated. Acylation of hydroxyl groups of serine, threonine, and tyrosine residues cannot be excluded. However, at the dialysis step, during purification of the modified enzymes, the reverse reaction of deacylation by water leads to complete restoration of these OH-containing amino acid residues (Shiao et al., 1972).

Modification of CT with pyromellitic anhydride proceeds as follows:



$$E{-}NH_2 + O\begin{cases}(O)C\\ \quad\quad\quad\quad C(O)\\ \quad\quad\quad\quad\quad\quad O\\ \quad\quad\quad\quad C(O)\\ (O)C\end{cases} \rightarrow$$

$$\begin{matrix} & {-}OOC & & COO^- \\ E{-}NH{-}(O)C & & & COO^- \end{matrix} \quad \text{pyr-CT} \quad (2)$$

As a result, the positive charge of each modified amino group is eliminated and three negatively charged carboxylic groups are introduced. By using pyromellitic anhydride, the molecule of CT may be enriched by several dozens of additional carboxylate anions. Under the reaction conditions used by us, acylation of CT with both pyromellitic and succinic anhydrides does not produce either intermolecular or intramolecular protein cross-linking (Mozhaev et al., 1988).

Reductive alkylation of amino groups of CT with glyceraldehyde proceeds according to the following scheme:

$$E{-}NH_2 + O{=}CH{-}CH(OH){-}CH_2(OH) \xrightarrow{NaCNBH_3}$$

$$E{-}NH{-}CH_2{-}CH(OH){-}CH_2(OH) \quad \text{glyc-CT} \quad (3)$$

Amino groups are the only functional groups in proteins modified by this reagent (Means and Feeney, 1995). Modification with glyceraldehyde does not eliminate positive charges of amino groups: pK_a values of primary amino groups in the native enzyme and of secondary amino groups in the modified enzyme do not differ by more than 1 pH unit (Means and Feeney, 1995).

Table I summarizes some of the properties of the modified derivatives of CT. The modification reaction led to partial inactivation of the enzyme: the content of active sites in pyr-CT, suc-CT, and glyc-CT decreased to 40% to 60% as compared with the value of 80% for the native enzyme. Thus, less than half of the active enzyme was lost, which is a good result for the modification reactions. The values of k_{cat} and K_m for CT did not change significantly after the modifications.

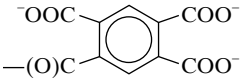
The structure of pyr-CT and glyc-CT was studied by several physical methods. Modification had only marginal effects on the circular dichroism (CD) spectrum of CT in aqueous solution in both far- and near-UV regions (see below). Also, the fluorescence spectra and differential absorption spectra of the modified CT did not differ from those of the native enzyme (data not shown). Thus, we concluded that the overall structure of the enzyme was not changed by the modifications. The catalytic behavior of covalently modified derivatives of CT in water–cosolvent mixtures is now under study (Kudryashova et al., unpublished data)—in the present study the data on modified CT are used for comparison of free enzymes with enzymes in complexes with polyelectrolytes.

Catalytic Activity of Native and Modified Enzymes in Complexes with Polybrene

Aqueous Buffer

Complex formation changes the microenvironment of CT molecules toward a more basic one: amino groups of PB increase the value of pH in the enzyme vicinity due to preferential sorption of hydroxyl ions. As pre-

Table I. Characteristics of covalently modified derivatives of α-chymotrypsin.

Modifying reagent	Introduced fragment	Abbreviation	Degree of modification	Concentration of active sites (%)[a]
Native enzyme		CT	—	80 ± 5
Pyromellitic anhydride		pyr-CT	8 11	50 ± 10 40 ± 10
Glyceraldehyde	—CH_2—CH(OH)—CH_2(OH)	glyc-CT	10	50 ± 5
Succinic anhydride	—C(O)—CH_2—CH_2—COO^-	suc-CT	5	60 ± 5

[a]Concentration of active sites was determined by using *N*-trans-cinnamoylimidazole as a titration reagent (Schonbaum et al., 1961).

dicted by Goldstein (1972), binding to polycations would shift the pH profile of enzymatic activity to lower pH values and this was the case with complexes of CT with PB. However, the maximal rate of the reaction (V_{max}) for the CT–PB complex determined at the optimal pH of 6.8 did not differ significantly from V_{max} for native CT at pH 8.0 corresponding to its pH optimum. This result agrees well with the data on other polyion-bound derivatives of CT (Goldstein, 1972; Suh et al., 1992). In all further experiments, enzymatic activity of CT, pyr-CT, and their complexes with PB was measured at a fixed pH of 7.4. This value was a compromise between pH 8.0 and 6.8, corresponding to the maximal activity of native CT and CT–PB, respectively: for each enzyme sample, the activity at pH 7.4 was equal to 60% to 70% of the maximal value.

Both CT and pyr-CT showed a five- to ten-fold higher affinity toward BTNA in complexes with PB in comparison with the free enzyme. To the best of our knowledge, such a large decrease in the value of the apparent Michaelis constant, $K_{m,app}$, has not been observed for polymer-bound forms of CT; for example, the value of $K_{m,app}$, with an anilide substrate for CT immobilized on polyionic supports, was exactly equal to the value for native CT (Goldstein, 1972). This decrease in $K_{m,app}$ might be explained by a partly negative character of a nitro group of BTNA—this group can hydrogen bond with positively charged groups in the polycationic matrix of PB.

Water–Cosolvent Mixtures

The dependencies of V_{max}, for hydrolysis of BTNA on the concentration of ethanol, are shown in Figure 1. Addition of 20% to 25% v/v of ethanol to aqueous solution led to a twofold increase in the activity of native CT (curve 1). The absence of an activation effect in some of our previous studies (Khmelnitsky et al., 1991; Mozhaev et al., 1989) can be explained by different experimental conditions, such as the buffer used, the value of pH, and the method of pH adjustment. Further addition of ethanol resulted in a decrease of activity and, at higher than 40% v/v of ethanol, native CT was fully inactive and formed visible precipitate. For pyr-CT, the activation effect at 20% to 25% v/v of ethanol was 1.5-fold higher than for native enzyme (curve 2 in Fig. 1). An abrupt decrease in the activity of pyr-CT was seen at concentrations of ethanol higher by 15% to 20% v/v than those for native CT; in addition, at 60% to 90% v/v of ethanol, pyr-CT was soluble and still retained high enzymatic activity (20% to 25% of the "aqueous" level).

Formation of noncovalent complex with PB produced two major effects (curve 3 in Fig. 1): the increase in enzymatic activity at moderate concentrations of ethanol was more pronounced than for free CT; and the range of cosolvent concentrations that the enzyme could tolerate without complete inactivation was much broader for CT–PB. The same two effects were obtained by us in binary mixtures of water with *N*,*N*-dimethylformamide and dioxane (Gladilin et al., 1995). At higher

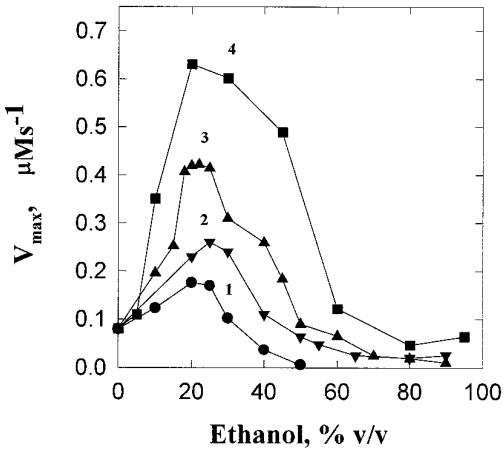


Figure 1. Dependence of the maximal rate of hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide, V_{max}, catalyzed by α-chymotrypsin preparations in binary mixtures with water on the concentration of ethanol: 1—CT; 2—pyr-CT (degree of modification = to 11); 3—CT–PB; 4—pyr-CT–PB.

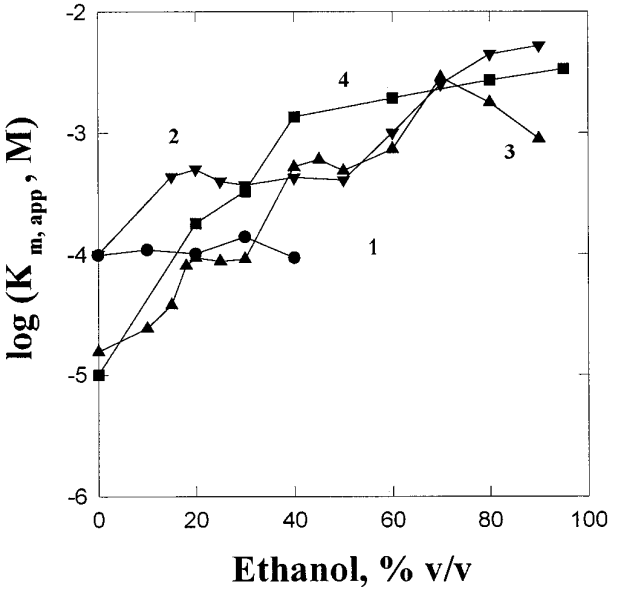


Figure 2. Dependence of the apparent value of K_m ($K_{m,app}$) for hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide catalyzed by α-chymotrypsin preparations in binary mixtures with water on the concentration of ethanol: 1—CT; 2—pyr-CT (degree of modification = 11); 3—CT–PB; 4—py-CT–PB.

than 90% v/v of ethanol, CT in the complex with PB lost the activityand a precipitate was similtaneously formed.

Enrichment of CT with negatively charged carboxylic groups by modification with pyromellitic anhydride additionally amplified both activation and stabilization effects in enzyme–polyelectrolyte complexes. At 20% v/v of ethanol, the complex between pyr-CT and PB showed a significant activation (more than sevenfold higher than the "aqueous" activity of the complex), that is, curve 4 in Figure 1. Also, this activity was much higher than the activity shown by pyr-CT and CT–PB. At higher than 20% v/v of ethanol, the complex of pyr-CT with PB was also more active than all other CT preparations. The difference in activity between pyr-CT–PB and CT–PB became maximal in the solution with 50% v/v of ethanol (a threefold effect). At 90% to 95% v/v of ethanol, where CT–PB is precipitated, pyr-CT–PB was still soluble and very active—three fourths of the activity of the complex in aqueous solution. Thus, an increase in the number of negative charges in the molecule of CT led to a remarkable additional stabilization in water–ethanol mixtures of the enzyme entrapped in complexes with PB. It is most likely that this stabilization effect was achieved due to multiple electrostatic interactions between the protein molecule and the polyelectrolyte.

The influence of ethanol on $K_{m,app}$ for hydrolysis of BTNA by different forms of CT is described by a more complex picture. For native CT, $K_{m,app}$ was nearly constant at all concentrations of ethanol (curve 1, Fig. 2) and all other CT derivatives showed an increase in $K_{m,app}$ on addition of ethanol (curves 2 to 4, Fig. 2). The increase in $K_{m,app}$ in water–cosolvent mixtures is rather typical for the substrates whose affinity to the enzyme active site is based on hydrophobic interactions, as is the case with CT (Maurel, 1978; Mozhaev et al., 1989). As a conclusion from Figure 2, the sensitivity of $K_{m,app}$ to medium effects was especially strong for the enzyme that had a microenvironment enriched with charged groups, due either to chemical modification (pyr-CT) or complex formation with the polycation (CT–PB and pyr-CT–PB).

Another explanation for the stabilization effect in Figure 1 may be that covalent modification of CT led to changes in some property of the protein molecule (e.g., solvation ability of its amino acid residues) and these changes were amplified by formation of the complex with PB. In this case, one could assume that the stabilization is not directly related to electrostatic interactions and can be equally achieved by modification with a noncharged reagent. To study this possibility, amino groups of CT were alkylated with glyceraldehyde, a hydrophilic reagent having two hydroxyl groups [Eq. (3)]. This modification did not change the total number of charges in CT, because the pK_a of the secondary amino groups formed is close to the pK_a of primary amino groups (Means and Feeney, 1995). Glyc-CT had a modification degree of 10, which was close to the value of 11 for py-CT. Modification with glyceraldehyde did not greatly change the enzyme resistance in the complex with PB against inactivation by ethanol as compared with the complex of native CT with PB (curves 2 and 3 in Fig. 3). Also, activities of glyc-CT in free and complex-bound forms differed less significantly at all concentrations of ethanol (curves 1 and 3 in Fig. 3) than in pyr-CT and pyr-CT–PB (curves 2 and 4 in Fig. 1). This may

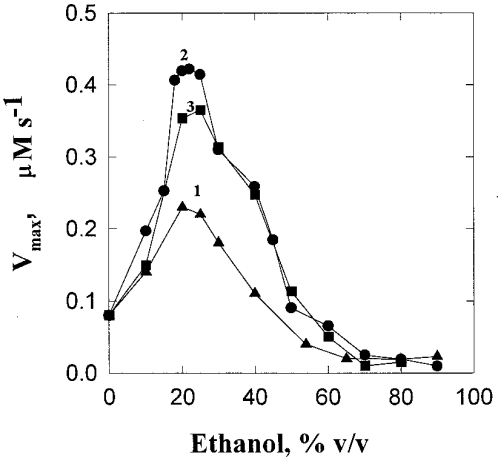
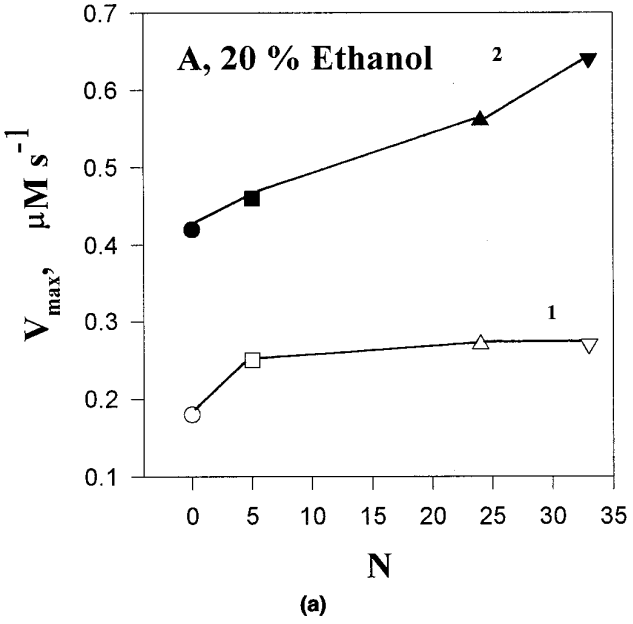


Figure 3. Dependence of the maximal rate of hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide, V_{max}, catalyzed by α-chymotrypsin preparations in binary mixtures with water on the concentration of ethanol: 1—glyc-CT (degree of modification = 10); 2—CT–PB; 3—glyc-CT–PB.



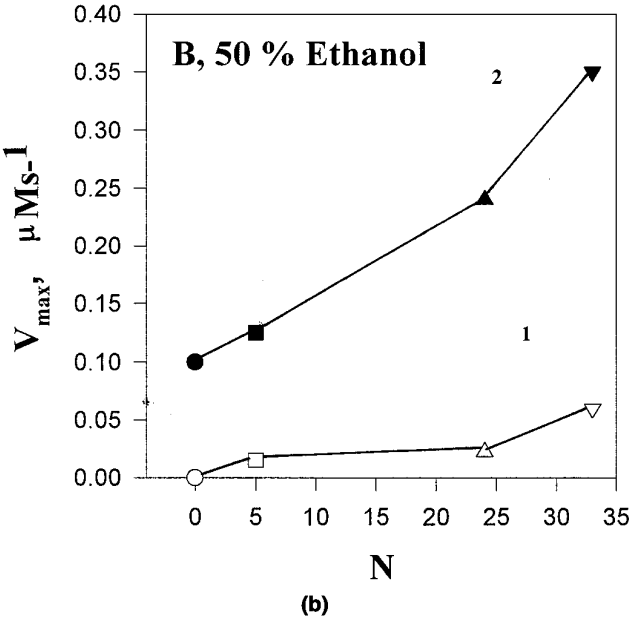


Figure 4. Dependence of the maximal rate of hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide, V_{max}, catalyzed by α-chymotrypsin preparations in water–ethanol mixtures on the number of carboxylic groups, *N*, introduced into α-chymotrypsin by covalent modification with: succinic anhydride, degree of modification = 5 (□); pyromellitic anhydride, degree of modification = 8 (△); pyromellitic anhydride, degree of modification = 11 (▽); and nonmodified CT is shown for coparison (○). Curves 1—free (nonbound) derivatives of CT; curves 2—derivatives of CT in complexes with PB. (A) 20% v/v of ethanol; (B) 50% v/v of ethanol.

mean that additional activation and stabilization of CT modified with pyromellitic anhydride in the complex with PB were caused mainly by supplementary charges interacting with the polyeletrolyte matrix rather than by some changes in the protein molecule induced by the modification.

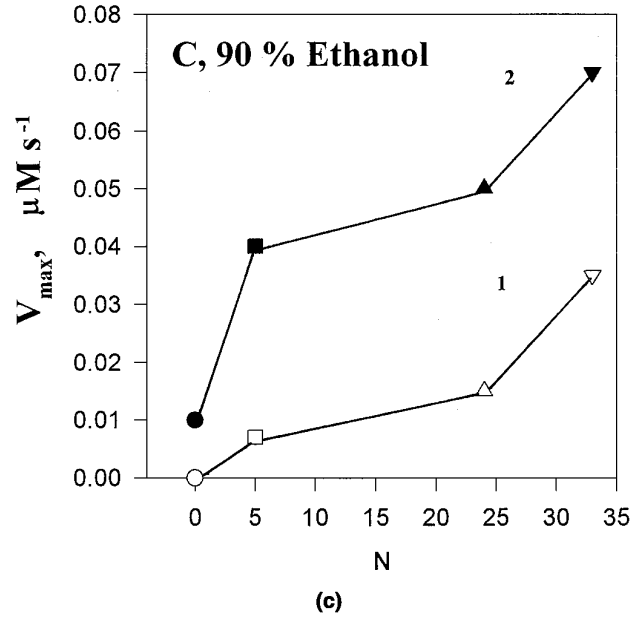


Figure 4. (*continued*) (C) 90% v/v of ethanol.

If electrostatic interactions are important for the stability of CT in complexes with PB in water–ethanol mixtures, the enzymatic activity should depend on the number of carboxylic groups introduced into the protein by chemical modification. This value was varied by using another modification reagent (succinic anhydride) and by changing the degree of modification of CT. Catalytic activities of different modified derivatives of CT in free form (curves 1) and in complexes with PB (curves 2) are compared in Figure 4 at three concentrations of ethanol. At 20% v/v, where CT had the maximal activity, additional carboxylic groups produced up to a 1.5-fold activation effect on both free and PB-bound CT (Fig. 4A)—at 20% v/v of ethanol, each CT derivative (either modified or not) was 2- to 2.5-fold more active in the complex with PB than in the free form. The differences in catalytic activity for various modified forms of both free and complex-bound CT were the highest at 50% v/v of ethanol (Fig. 4B). Whereas the native enzyme was inactived due to precipitation, modified CT retained well-detectable actvity, probably because additional negative charges on the protein surface retarded aggregation—such a deceleration of polymolecular interactions has been established for modified CT in aqueous solutions (Mozhaev et al., 1988). In complexes with PB, these additional charges increased the activity of CT up to 3.5-fold, and there was good correlation between the enzymatic activity and the number of these charges (curve 2 in Fig. 4B). On average, each modified derivative of CT was sevenfold more active in complexes with PB than in free form. At 90% v/v of ethanol, the activity of all free or complex-bound forms of CT was tenfold lower compared with their corresponding maximum lev-

els at 20% v/v of ethanol, and was smaller than their "aqueous" activities (Fig. 4C). As a conclusion from Figure 4A–C, the stablization effect of PB in water–ethanol mixtures was much amplified by an increase in the number of negative charges in CT due to the better ability of the modified protein to interact with the polycation.

To unravel molecular reasons underlying the kinetic effects in water–ethanol mixtures, we studied structural changes in CT derivatives by CD spectroscopy.

Circular Dichroism

Aqueous Solution

In the far-ultraviolet region, the spectrum of CT–PB and pyr-CT–PB showed a negative maximum at 233 nm, a shoulder at 222 nm, and a negative band at 208 nm (curve 1 in Fig. 5A and B). These features coincided with the spectrum of native CT which, in turn, agreed well with the literature data (Gorbunoff, 1971). From the secondary structure analysis, we determined ca. 10% of α-helices, 20% of β-sheets, and 70% of random coil contents (Gorbunoff, 1971; Greenfield and Fasman, 1969). In the near-ultraviolet region, the enzymes showed spectra (curve 1 in Fig. 6A and B) with maxima at 289 and 296 nm, which are best assigned to tryptophan residues. Thus, in aqueous solution, both CT–PB and pyr-CT–PB had nativelike secondary and tertiary structures.

Water–Ethanol Solutions

Addition of less than 30% v/v of ethanol changed far- and near-UV CD spectra of free CT (not shown), CT–PB, and pyr-CT–PB (curve 2 in Figs. 5 and 6) only slightly, and thus had only a marginal effect on the overall structure of proteins. Further addition of ethanol up to 40% v/v generated strong modification of the CD spectra (curve 3 in Figs. 5 and 6). In the near-UV region, we saw a loss in intensity accompanied by "destructurization" of the spectrum (curves 4 and 5 in Fig. 6) and at 70% v/v of ethanol, precipitation occurred due to the high protein concentration (0.5 to 3.0 mg/mL) used in registration of the spectra. In the far-UV region, the 233-nm band disappeared, whereas a shoulder at 218 nm appeared at 40% v/v of ethanol (curve 3 in Fig. 5). At 50% to 60% v/v of ethanol, a single minimum at 218 nm was found for both CT–PB and pyr-CT–PB (curves 4 and 5 in Fig. 6). At 70% v/v of ethanol, CT–PB precipitated, whereas pyr-CT–PB remained soluble (concentration of 0.1 to 0.2 mg/mL) and the spectrum showed a further increase in the ellipticity at 218 nm (not shown).

All these variations indicate large structural changes at high concentrations of ethanol. The loss of near-UV spectral bands suggests strong modifications in the tertiary structure and the fracture of the far-UV spectrum indicates that ethanol induced transition into a basically β-sheet structure.

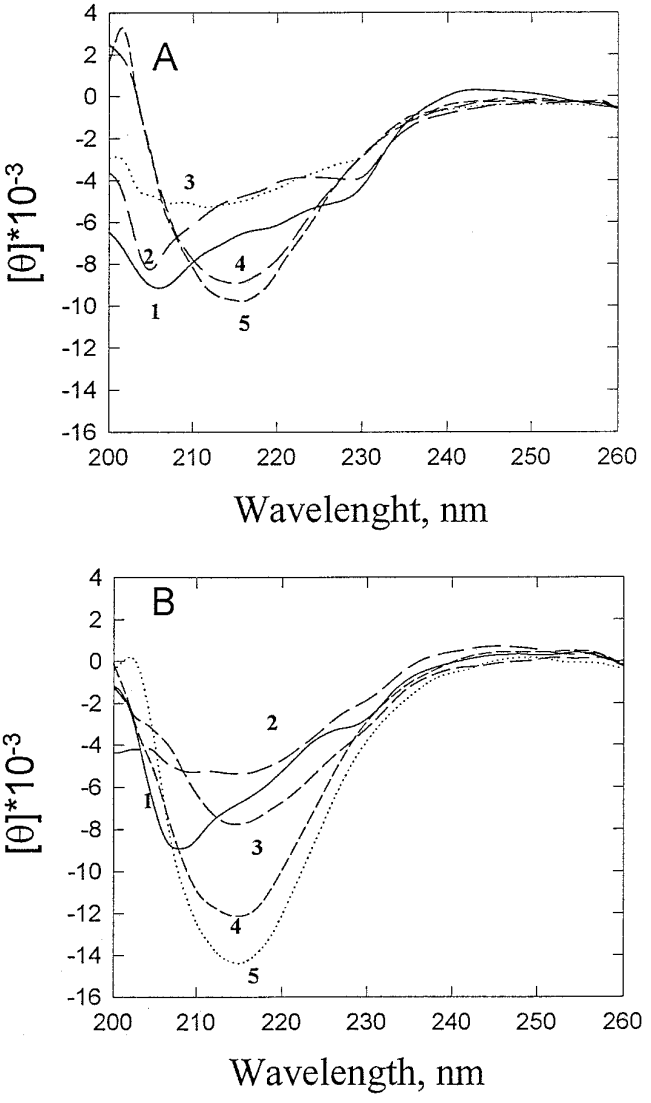


Figure 5. The effect of ethanol on the circular dichroism spectra of α-chymotrypsin preparations in the far-UV region: (A) CT–PB; (B) pyr-CT–PB. 1—Aqueous solution; 2—20% v/v ethanol; 3—40% v/v ethanol; 4—50% v/v ethanol; 5—60% v/v ethanol.

DISCUSSION

In this work we have studied CT samples over a wide range of ethanol concentrations—from purely aqueous solutions and up to low water media (5% to 10% v/v of water). In the literature there are few works in which catalytic activity and structure of enzymes were studied over the whole concentration range of a cosolvent, the results from Kise's group being most relevant to our study (Kijima et al., 1996; Tomiuchi et al., 1992, 1993). Activity of free CT dissolved in binary water–polar cosolvent mixtures started to decrease and reached zero (for ethanol at 50% v/v), and this inactivation was ac-

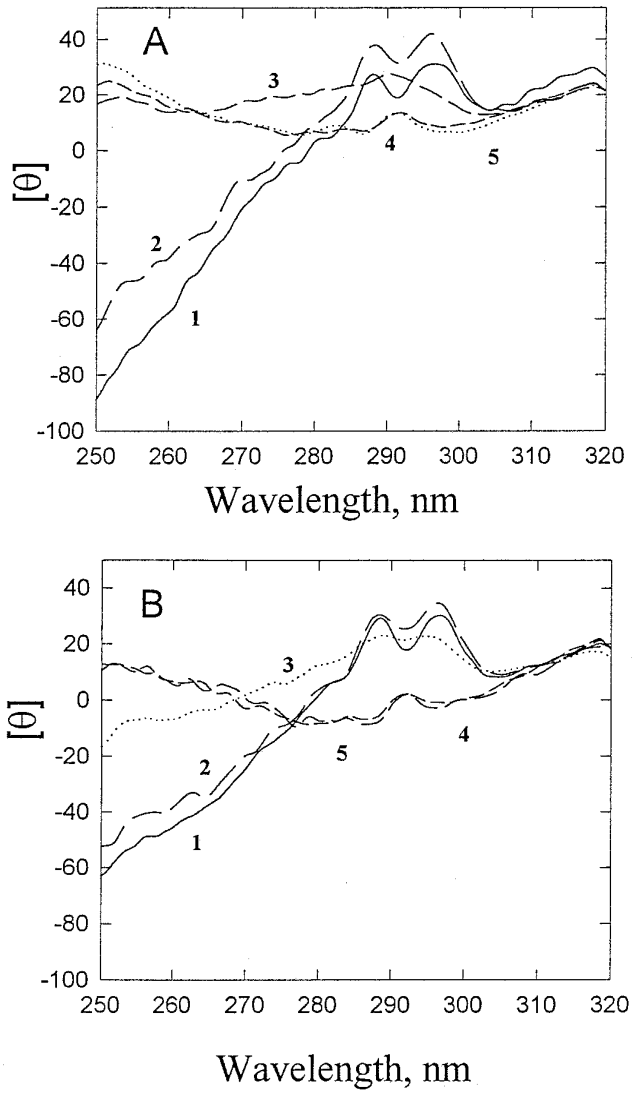


Figure 6. The effect of ethanol on the circular dichroism spectra of α-chymotrypsin preparations in the near-UV region: (A) CT–PB; (B) pyr-CT–PB. 1—Aqueous solution; 2—20% v/v ethanol; 3—40% v/v ethanol; 4—50% v/v ethanol; 5—60% v/v ethanol.

companied by precipitation. At higher solvent concentrations (70% to 80% v/v of ethanol), the precipitate showed activity of about 10% of the aqueous level. At a still higher cosolvent concentration, the activity in the precipitate again diminished, reaching zero in low water systems. Thus, a typical picture of enzymatic activity in water–cosolvent mixtures showed two concentration intervals where CT was active (either soluble or precipitated) separated by a region where the enzyme was inactive (Tomiuchi et al., 1993).

Covalent modification of CT as well as formation of complexes with PB, led to a significantly different picture. First, all CT derivatives, except native CT, remained soluble at micromolar enzyme concentrations up to 90% v/v of ethanol. Thus, in our systems, changes in catalytic activity took place in the absence of phase transitions (without formation of visible aggregates). Second, modified and complex-bound CT showed dependencies on ethanol concentration without any gap in catalytic activity (Figs. 1 and 3). Several characteristic zones are seen in these dependencies. At relatively small concentrations of ethanol, activity of all forms of CT increased severalfold. At higher cosolvent concentrations, the activity decreased to some finite level, in contrast to free CT, which was totally inactive. At still higher concentrations of ethanol, the activity remained constant until precipitation started (at 90% v/v for pyr-CT and CT–PB), or even increased slightly (at 95% v/v for pyr-CT–PB).

Activation

Activation of enzymes by moderate concentrations of organic solvents has been reported by many investigators, and this effect may be as high as several dozen times (Dreyfus et al., 1978; Ruwart and Suelter, 1971; Selman-Reimer et al., 1981; Singh and Wang, 1979; Tan and Lovrien, 1972). Summarizing these studies, we sort out the following molecular mechanisms behind these activation effects:

1. Cosolvent molecules can *bind at* specific or nonspecific *activation sites* in the protein molecule. This binding induces small perturbation; for example, it promotes more favorable orientation of a substrate in the enzyme active site (Inward and Jencks, 1965). Kinetically, this promotion is equivalent to the phenomenon of "activation by a substrate" when binding of the second substrate molecule to a low-affinity site increases the reaction rate. Competition in enzyme binding and activation was observed between the molecules of substrate and organic cosolvent (Barke and Jencks, 1969; Inward and Jencks, 1965). For oligomeric enzymes, cosolvents compete with activators in binding at allosteric sites (Dreyfus et al., 1978) and in stabilization of a more relaxed activated enzyme conformation which has more solvent-exposed nonpolar groups (Dreyfus et al., 1984).
2. Some extraneous inhibitors may remain bound to an enzyme after purification or the products of enzymatic reaction act as inhibitors. Organic solvents may induce *dissociation of* these *inhibitor* molecules from the active site, thus leading to an increase in the apparent enzyme affinity toward substrates (Faller and Sturtevant, 1966; Inouye et al., 1995).
3. In catalysis by hydrolases, organic cosolvents can act as *additional* efficient *nucleophiles* competing with water molecules. For example, hydrolysis of esters catalyzed by CT was profoundly accelerated by addition of alcohols (Faller and Sturtevant, 1966; Inward and Jencks, 1965).
4. A *change in the structure* of high-molecular-mass *substrates* caused by cosolvent addition can be an im-

portant source of activation in enzymatic reactions. This was the case with enzymatic hydrolysis of cellulose swelling in organic solvents (Kumakura and Kaetsu, 1983); the 46-fold activation of glycogen phosphorylase kinase by methanol can be explained in part by structural changes in the protein substrate, phosphorylase b (Singh and Wang, 1979).

5. The affinity of an enzyme towards substrates is very sensitive to changes in medium—this *alteration in* the value of K_m (Fink, 1974; Maurel, 1978) changes the enzyme effectivity parameter when the activity is measured in the regime of V_{max}/K_m. Binding of specific substrates to CT is mainly determined by hydrophobic interactions and the value of $K_{m,app}$ increases in water–alcohol mixtures (Faller and Sturtevant, 1966; Maurel, 1978) due to a smaller preference of substrate extraction from solution to the enzyme active site. By contrast, organic solvents decrease the value of $K_{m,app}$ in enzyme–substrate pairs supported by coulombic forces (Maurel, 1978; Ruwart and Suelter, 1971) which are strengthened in media with a low dielectric constant.
6. Addition of cosolvents may change *intersubunit interactions in oligomeric enzymes* (Ahmed and Miles, 1994) up to their dissociation (Ohama et al., 1977), due to weakening of hydrophobic interactions often responsible for stability of inter-subunit contacts.
7. Activation may be accompanied by *changes in protein conformation* (Bradbury and Jacoby, 1972; Singh and Wang, 1979). However, structural transitions cannot be regarded as commonplace in enzyme activation by organic solvents because often they are not seen by physical methods.
8. Finally, activation may be a consequence of a general effect of the medium *on catalytic steps* of enzymatic reactions—sometimes the activation effect correlates with hydrophobicity and the dielectric constant of organic solvents (Dreyfus et al., 1984; Lozano et al., 1995; Selman-Reimer et al., 1981).

The activation of CT derivatives in the presence of 10% to 30% v/v of ethanol may be a result of the simultaneous action of several of the above mechanisms. Still, hydrolysis of nitroanilides by CT does not show ''activation by a substrate''; thus, it is unlikely that the activity increase results from a direct binding of ethanol molecules to an activator site in CT. The rate of hydrolysis of anilides by CT is determined by the acylation step in both aqueous solution and water–solvent mixtures (West et al., 1990); thus, participation of ethanol as a competing nucleophile is hardly probable. In this work, we studied the influence of ethanol on the value of V_{max} after separating its effect on $K_{m,app}$. So, the activation cannot be explained by a higher enzyme affinity to BTNA—actually, ethanol increased the value of $K_{m,app}$ (Fig. 2). It is not likely that the activation is a result of conformational changes in the enzyme—by using two methods, fluorescence and CD spectroscopy, we did not see any structural changes in CT at 10% to 30% v/v of ethanol. Most likely, the activation is due to the influence of the medium on the catalytic steps of the reaction. This assumption will be studied in future experiments by thorough investigations of the effect of organic cosolvents on the catalytic mechanism of CT.

Inactivation: Structure of Denatured CT

At concentrations of ethanol higher than 25% to 30% v/v, the enzymatic activity of all CT derivatives started to diminish (Figs. 1 and 3). This activity decrease came in parallel with perturbations in CD spectra of CT (Figs. 5 and 6) pinpointing to simultaneous changes in the enzyme secondary and tertiary structures. Such a simultaneous disruption of secondary and tertiary structures by organic solvents was observed by other investigators (Fan et al., 1993). At 50% to 60% v/v of ethanol, near-UV CD spectra of CT were absolutely unstructured indicating a disordered microenvironment of aromatic amino acid residues. At the same ethanol concentrations, we observed an increase in negative ellipticity at 218 nm and ascribed this to formation of additional β-sheets. This might be unexpected because usually the percent of α-helices in proteins increases in water–cosolvent mixtures at the expense of β-sheets and random coils (Acharya, 1992; Buck et al., 1993; Fan et al., 1993; Singer, 1962). On the other hand, proteins with high β-sheet content showed a very low ability to change their conformations into α-helical ones (Wang et al., 1992). CT is an example of mainly β-protein (Birktoft and Blow, 1972) and β-sheet-to-α-helix transitions may be prohibited for it as well.

An alcohol-induced increase in β-pleated sheets in CT may be a consequence of intramolecular α-helix-to-β-structure transition as was the case with poly(L-lysine) in alcohols (Shibata et al., 1992). Another explanation may be that formation of β-sheets in CT in ethanol results from intermolecular processes leading to protein aggregation (Jackson and Mantsch, 1992). All our kinetic and structural studies were made with optically transparent solutions. However, formation of small ''soluble'' (not visible by eye) protein associations cannot be totally excluded. Augmentation of β-sheet content for CT and pyr-CT in complexes with PB at 50% to 60% v/v of ethanol might be regarded as a beginning of accumulation of soluble associates. It may be that at these cosolvent concentrations, protein denaturation, and/or aggregation of CT was stopped at an intermediate step due to interactions with the polycation, thus preventing formation of insoluble and inactive aggregates. These mechanisms of denaturation versus aggregation in organic solvents will be further studied by using different physical techniques.

It has been hypothesized (Buck et al., 1993; Fan et al., 1993) that proteins denatured by organic solvents

resembled molten globule states. At intermediate concentrations of ethanol, CT and pyr-CT in complexes with PB shared some common structural features with molten globules, such as existence of a significant secondary structure and loss of a major tertiary structure. This molten globulelike structure of CT was probably "frozen" due to multiple interactions with the polycationic matrix of PB which, in this respect, acted as a chaperone. In cells, chaperones bind with partly folded intermediates and thus inhibit their nonspecific aggregation and misfolding (Hartl et al., 1994). Due to the importance of molten globules as possible intermediates in protein folding in vivo, they have been extensively studied in the last decade (Ptitsyn 1995). A question still to be answered concerns the catalytic activity of enzymes in the molten globule state. In view of the experimental evidence that several derivatives of CT are catalytically active in solutions with a high content of ethanol (Figs. 1 and 3), this question does not seem unwise.

Stabilization

In this work, we have found that formation of noncovalent coplexes between an enzyme and a polycation significantly increased the enzyme stability against denaturation by organic solvents. This stabilization led to a pronounced extension of the concentration interval for ethanol (up to 90% to 95% v/v) where the enzyme showed high catalytic activity. This result may be of practical importance because the systems with high concentrations of polar cosolvents seem to be very promising for enzymatic synthesis of peptides (Wong, 1992). Simple methods developed in this study for construction of biocatalysts working under such hazardous conditions may be a reasonable alternative to attempts at engineering enzyme stability by mutagenesis (Chen and Arnold, 1993; Dordick, 1992; Wong, 1992).

Stabilization of CT in complexes with polycation can be explained by several theories. Adlercreutz (1991) suggested that a polymeric support can compete with the bound enzyme in binding with molecules of water and organic solvents. This competition may lead to a shift in the catalytic activity versus solvent content profiles. It may also be that PB inhibited the polymolecular processes, resulting in enzyme aggregation and inactivation by organic solvents. We believe that multiple noncovalent interactions of CT with PB make the major contribution to enzyme stabilization in water–solvent mixtures. Complexes of proteins with polyelectrolytes, and CT–PB in particular, are stabilized by coulombic forces (Izumrudov et al., 1984). CT has 16 negatively charged carboxylic groups on the surface (Birktoft and Blow, 1972) that could maintain multiple contacts with excess positively charged amino groups in the polycation. Due to covalent attachment to a support, CT has previously been stabilized in water–ethanol mixtures and a correlation was found between the number of enzyme–polymer bonds and the shift in the activity profile versus ethanol concentration (Mozhaev et al., 1990). In that system (CT crosslinked to polyacrylamide), an increase in the denaturation threshold concentration by 20% v/v of ethanol was achieved due to formation of 12 covalent bonds. Stabilization effects of the same magnitude and even higher were obtained in the present study. Thus, it is reasonable to assume that the number of noncovalent electrostatic bonds in the CT–PB system is actually high. Furthermore, it also seems reasonable that the artificial increase in the number of such interactions (by introduction of new negative charges by chemical modification) led to additional stabilization of CT. This stablization cannot solely be explained by the influence of chemical modification on the enzyme, although modified CT is more stable than the native enzyme. Formation of complementary electrostatic bonds between the newly introduced negative charges in the CT molecule and noncompensated positive charges in PB is in a great part responsible for this additional stabilization. This conclusion is supported by the correlations shown in Figure 4 between the catalytic activity of CT in the complex with PB and the number of supplementary carboxylic groups in the molecule of CT. It was the additional electrostatic interactions that made the enzyme structure in the complex with polyelectrolyte more rigid and, this amplified the enzymatic activity in the water–cosolvent mixtures.

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