

Effects of crown ethers and small amounts of cosolvent on the activity and enantioselectivity of α -chymotrypsin in organic solvents

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Abstract: Addition of crown ethers to α -chymotrypsin, subtilisin, and other proteases considerably enhances the activity of these enzymes in transesterification reactions of *N*-acetyl-alanine and -phenylalanine esters in organic solvents. Even much higher enhancements of activity (up to 640 \times) are obtained by prior lyophilization of the enzymes in the presence of 18-crown-6. Several possibilities for the origin of the crown ether activation are discussed. Isosteric variation of the leaving ability of the alcoholate group revealed that for more reactive esters the rate of the acylation process becomes determined by a relatively slow physical process, most likely a conformational change of the enzyme and/or displacement of water molecules from the active site. This rate limiting process has important consequences for the enantioselectivity of the enzyme and enables the variation of the enantioselectivity by addition of small amounts of different cosolvents to the organic reaction medium.

Introduction

The high catalytic efficiency and selectivity of enzymes in the transformation of organic compounds have been exploited for decades in industrial processes and research laboratories (1). However, the long held belief that enzymes are only active in aqueous solution has limited the application of enzymes in reactions with water-sensitive or water-insoluble substrates. Nowadays it is well established that enzymes can be catalytically active in organic solvents. Compared to aqueous solutions, the use of an organic reaction medium can have some interesting advantages, like enhanced thermal stability of the enzyme, easy separation of the suspended enzyme from the reaction medium, increased solubility of substrates, favorable equilibrium shift to synthesis over hydrolysis, suppression of water-dependent side reactions, and possibly new (stereo-)selectivity properties of the enzyme (2).

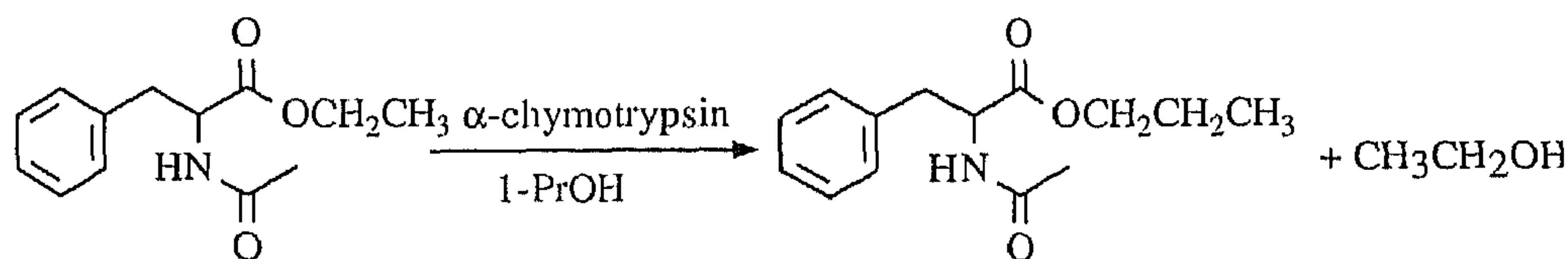
An important drawback of the use of organic solvents for enzyme reactions is that the activity of the enzyme is generally several orders of magnitude lower than in aqueous solution. Prior lyophilization of the enzyme from an aqueous solution which is buffered at the pH of optimal aqueous enzyme activity, if necessary in the presence of an inhibitor, improves the activity in organic solvent. It is proposed that both the optimal pH and the inhibitor contribute to the fixation of the enzyme in a catalytically active conformation during lyophilization (3). Another parameter which influences the activity of the enzyme is the amount of water present in the organic solvent. An essential amount of water on the enzyme surface, often less than a monolayer, is necessary to retain the catalytic activity. The necessity of water has been explained in terms of introducing flexibility to the protein backbone and the screening of charged groups at the enzyme surface. This was confirmed by the finding that the enzyme activity in organic solvents does not depend on the total concentration of water in the reaction system, but on the number of water molecules at the enzyme surface (4). Increment of the water content of a given organic solvent can result in an up to three orders of magnitude higher enzyme activity. However, high levels of water in the solvent result in the loss of the advantages typical for non-aqueous enzymology.

Several strategies have been developed to enhance the enzyme activity in organic solvents, like protein engineering (5), chemical modification of the enzymes (6), immobilization (7), and the addition of "water mimicking" compounds like formamide, glycol, or DMF (4b,8).

During recent years we have investigated the effects of crown ethers on enzyme reactions in organic solvents. Depending on their ring size and structure, crown ethers can form complexes with metal ions, ammonium groups, guanidinium groups, and water, species that are all common in enzymatic reactions. Moreover, the strongest interactions of crown ethers with these guest species occur in organic solvent. In this paper we discuss the effect of crown ethers and the presence of small amounts of cosolvents in the organic reaction medium on enzyme activity and enantioselectivity of serine proteases (in particular α -chymotrypsin) in the transesterification of *N*-acetylalanine and -phenylalanine esters in organic media.

Effect of macrocycles on the activity of α -chymotrypsin

A suitable reaction to study the effect of addition of macrocycles is the α -chymotrypsin-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol in organic solvent (Scheme 1).



Scheme 1

The catalytic mechanism of α -chymotrypsin in water is one of the best understood mechanisms of enzyme catalysis and most mechanistic information available in the literature about enzymes suspended in organic solvents deals with the transesterification of *N*-acetyl amino acid alkyl esters by α -chymotrypsin and other serine proteases (4b,9). However, the activity of the enzyme in organic solvent is much lower than that of the enzyme dissolved in water. For example, in octane, one of the organic solvents in which the enzyme shows its highest activity, the rate of acylation by *N*-acetyl-L-phenylalanine ethyl ester is 10^4 - 10^5 times slower than in water (3). α -Chymotrypsin has been proposed as a useful catalyst in (peptide) synthesis (1), but the advantages of the use of this enzyme under non-aqueous conditions are only attractive if the enzyme shows a reasonable activity. We have investigated whether enhancement of the activity by addition of crown ethers can offer a possibility to reach this goal (10).

Table 1 shows the effect of various crown ethers, a cryptand, and pentaglyme on the activity of α -chymotrypsin suspended in toluene in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol. All macrocycles increase the activity of α -chymotrypsin, but the most significant effect is found for 18-crown-6 (18-C-6).

TABLE 1. Influence of various macrocycles on the transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol, catalyzed by α -chymotrypsin in toluene.^a

Entry	Crown ether	$V_0(\text{crown ether}) / V_0$
1	18-C-6	19.6
2	monoaza-18-C-6	11.3
3	dibenzo-18-C-6	6.0
4	decyl-18-C-6	1.9
5	dicyclohexyl-18-C-6	4.0
6	dibenzo-24-C-8	1.8
7	15-C-5	1.8
8	2.2 didecyl Kryptofix	2.6
9	pentaglyme	1.4

^aConditions: 2.5 mM ester, 1 M PrOH, 2 mM crown ether, 0.5 mg enzyme.mL⁻¹ (pretreated with potassium phosphate buffer), 25°C.

Pentaglyme (CH₃-O-[-CH₂-CH₂-O]₅-CH₃, the open chain analog of 18-C-6) shows only a minor effect on the rate of the reaction. This implies that the hydrogen-bond accepting properties of polyethers cannot account for the rate-enhancing effect alone. Therefore, it can be concluded that a *macrocyclic effect is responsible for the acceleration of the reaction*.

Since the largest effect is obtained with 18-C-6 addition, species which prefer complexation with 18-C-6 must play a role in the enzyme activation mechanism. Such species are alkali metal ions, especially potassium, alkylammonium groups, and water.

Effect of macrocycle concentration

The relation of the enzyme activation with the concentration of 18-C-6 in the solution was studied by varying the concentration of 18-C-6 in the range 0 to 4 mM for the chymotrypsin-catalyzed transesterification in toluene (Fig. 1).

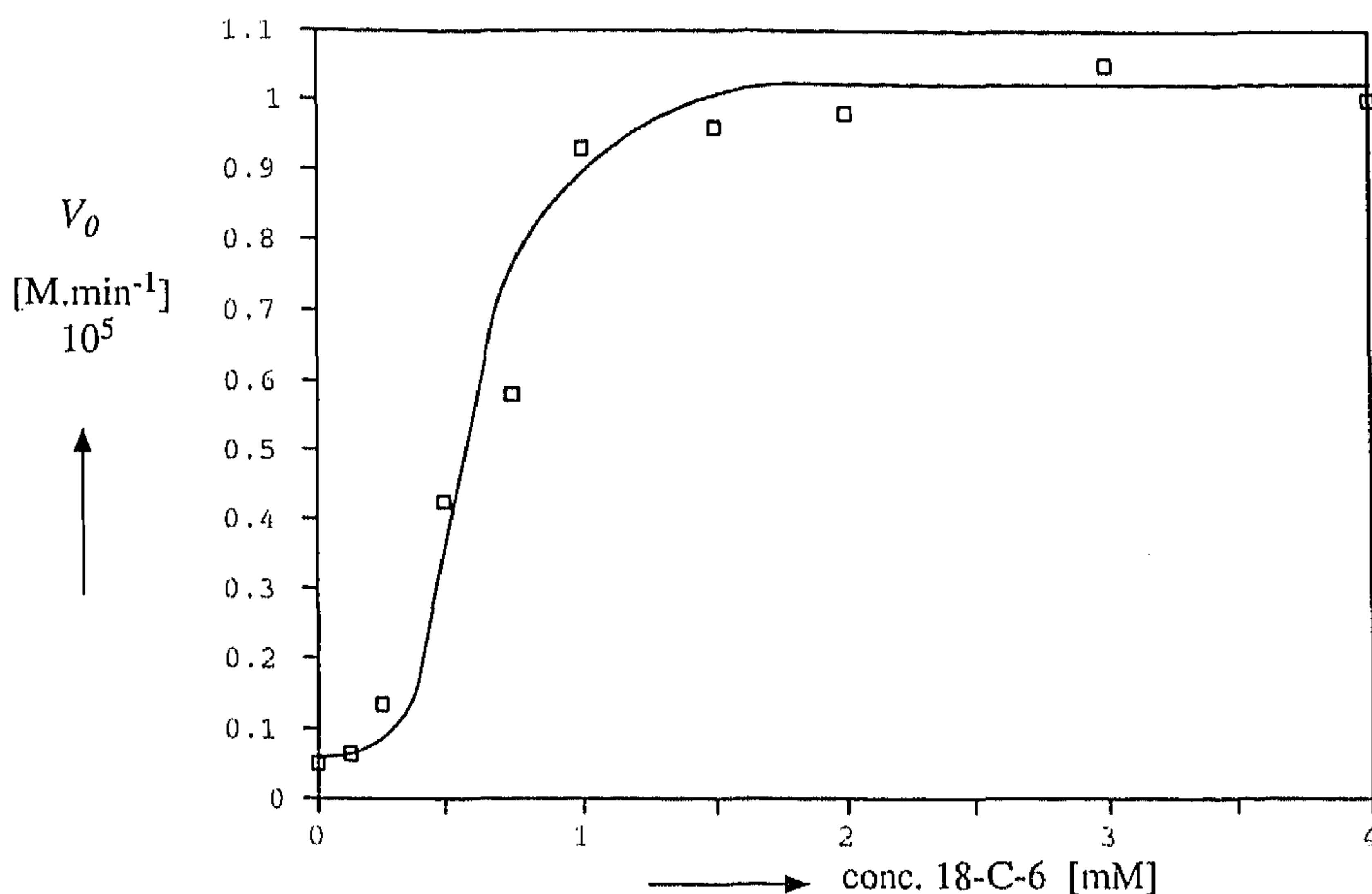


FIG. 1. Influence of the 18-C-6 concentration on the activity of α -chymotrypsin in the transesterification of N-acetyl-L-phenylalanine ethyl ester with 1-propanol. Conditions: 2.5 mM ester, 1 M 1-PrOH in toluene,) 0.5 mg enzyme. mL^{-1} (pretreated with potassium phosphate buffer), 25 °C.

From this fig. it can be seen that after initial addition with relatively small effects, a sharp rate increase in enzyme activity is obtained in the range of 0.5-1 mM 18-C-6. Further addition above 1 mM leads to a plateau value. This concentration profile suggests that after that initial amounts of 18-C-6 are apparently bound in an inactive form, subsequent amounts become catalytically active in an equilibrium process which goes to saturation.

Also the influence of the solvent on the crown ether effect was investigated. In aqueous solution no effect of addition of 18-C-6 on the activity of α -chymotrypsin was found. In organic solvents the magnitude of enzyme activation by 18-C-6 is dependent on the solvent used.

Table 2 gives the initial rates (V_0) of the α -chymotrypsin-catalyzed transesterification both in the absence and the presence of 2 mM 18-C-6 in different solvents, ranked in decreasing hydrophobicity (decreasing log P values). The data in the absence of 18-C-6 show that the enzyme activity is very sensitive for the solvent used, which is in accordance with results reported earlier in the literature (3).

Addition of 2 mM 18-C-6 has a pronounced effect on the rate of the reaction in all the solvents, especially in the more hydrophobic solvents where the complexation properties of 18-C-6 are probably most dominant.

A very remarkable increase of the enzyme activity was found by pretreatment of the enzyme by prior lyophilization in the presence of 18-C-6 (11). Lyophilization in the presence of 250 equiv. of 18-C-6 increases the activity of α -chymotrypsin 640 times, resulting in a k_{cat}/K_M value of the suspended enzyme towards N-Ac-L-PheOEt of $770 \text{ M}^{-1} \cdot \text{s}^{-1}$. This enzyme activity is only 50 times lower than that of α -chymotrypsin in aqueous solution (Table 3).

Several complexation processes in which crown ethers are involved can contribute to the enzyme activation: complexation of buffer cations present in the enzyme preparation, complexation of charged residues on the enzyme surface, and/or complexation of water at the surface and/or in the active site of the enzyme. It can be expected that 18-C-6 forms complexes with potassium ions which are present in the enzyme preparation as a result of the pretreatment of the enzyme with potassium phosphate buffer. This

TABLE 2. Influence of 2 mM 18-C-6 on the transesterification of N-acetyl(L)phenylalanine ethyl ester with 1-propanol, catalyzed by α -chymotrypsin in various solvents.^a

Solvent	V_0 (no 18-C-6)	V_0 (2 mM 18-C-6)	$\frac{V_0(18-C-6)}{V_0}$
octane	54	1545	29
cyclohexane	42	805	19
dibutyl ether	1.7	53	31
toluene	5.0	98	20
<i>t</i> -amyl alcohol	0.20	0.40	2
THF + 1% H ₂ O	1.8	14.5	8

^aConditions: 2.5 mM ester, 1 M 1-PrOH, 0.5 mg enzyme.mL⁻¹ (pretreated with 0.1 M potassium phosphate buffer pH 7.8), 25 °C, V_0 in 10⁷ M.min⁻¹, $\pm 5\%$.

TABLE 3. Effect of additives, present during freeze-drying, on the activity of α -chymotrypsin in the transesterification of N-acetyl-L-phenylalanine ethyl ester in cyclohexane/1 M 1-PrOH.^[a]

Additive	$\frac{\text{Mole additive}}{\text{Mole } \alpha\text{-chymotrypsin}}$	$V_0(+\text{additive})$ [10 ⁻⁷ M.min ⁻¹] ^[b]	$\frac{V_0(+\text{additive})}{V_0}$
18-C-6	0	8.0	1
	50	330	41
	250	5100	640
	500	4700	585
	1000	620	77
pentaglyme	500	660	82

^[a]Conditions: 2.5 mM substrate, 1 mg.mL⁻¹ enzyme powder, 25 °C.

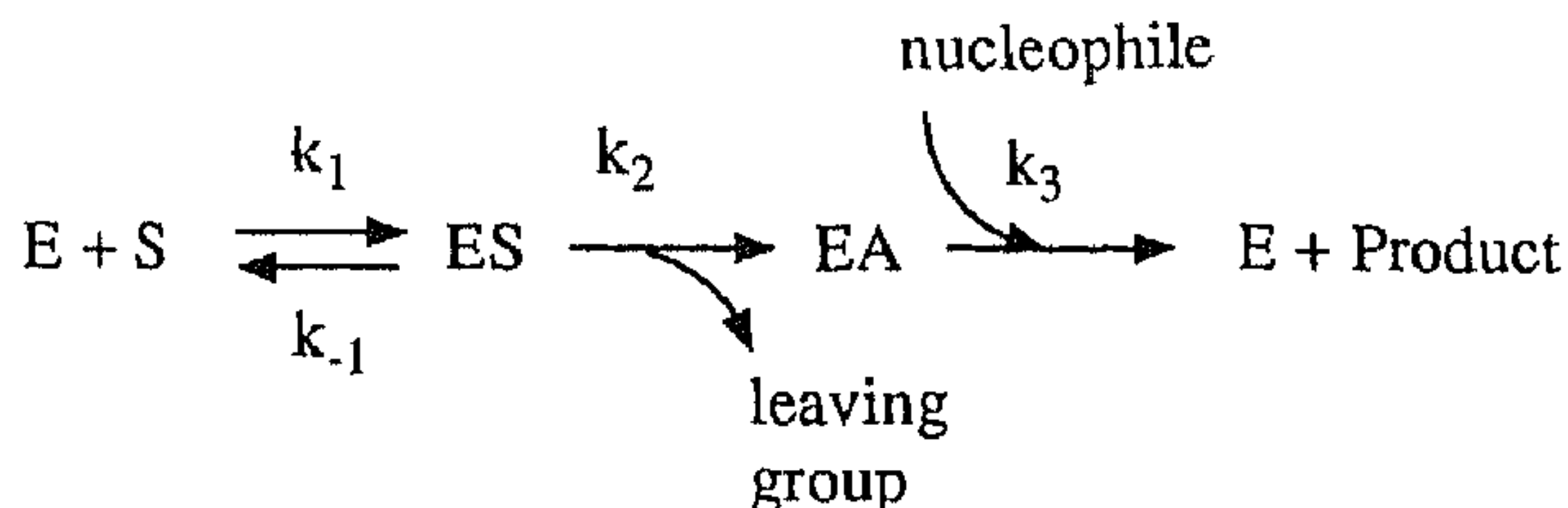
^[b]Initial rate (V_0), expressed in mg of protein per mL.

complexation could then remove potassium ions from the active site, resulting in a better accessibility of the active site for the substrate. However, active site titrations both in the absence and in the presence of 18-C-6 showed that the percentage of accessible active sites is not increased by 18-C-6 (12).

An important property of the crown ethers could also be that these form complexes with one or more of the fourteen ammonium groups on the α -chymotrypsin surface. Important support for this possibility is the observation that 18-C-6 gives no increase of activity of *acetyl*trypsin. In this enzyme all accessible amino groups are acetylated and complexation with crown ether is therefore no longer possible. This indicates that interaction of 18-C-6 with the ammonium groups of α -chymotrypsin may indeed contribute to the enhancement of the enzyme activity. However, 18-C-6 does not increase the enzyme flexibility of α -chymotrypsin on the nanosecond timescale as was concluded from time resolved fluorescence depolarization experiments with α -chymotrypsin labeled at the active site with an anthranoyl group (13).

The initial rate (V_0) of the transesterification reaction can be represented by the second order rate equation, $V_0 = k_2/K_S [E_0] [S]$, where $K_S (= k_{-1}/k_1)$ is the dissociation constant of the ES complex and k_2 involves all chemical and physical processes related to the acylation of the enzyme (scheme 2).

For enzyme reactions in water, frequently a dissection can be made between binding step and catalytic turn-over step by measurement at increasing substrate concentrations until saturation kinetics is observed. However, saturation kinetics could not be observed in the solubility range of the substrate (1-12 mM in octane, 1-70 mM in toluene) and therefore it was not possible to establish whether the increase of enzyme activity by 18-C-6 addition is due to changes in k_2 and/or K_S . This absence of saturation kinetics for enzymes in organic solvents is rather common and is most likely due to the much higher K_S values compared to water as the solvent (9b).



Scheme 2

Effect of leaving group activation

In a further approach to obtain more information about the effect of crown ethers on enzyme activity, we have investigated the activity of α -chymotrypsin (and subtilisin Carlsberg) towards a series of *N*-acetyl-D,L-alanine and *N*-acetyl-D,L-phenylalanine esters with ethoxy, 2-fluoroethoxy, 2,2-difluoroethoxy, and 2,2,2-trifluoroethoxy leaving groups in the transesterification of these substrates by 1-propanol in cyclohexane (12). Due to the comparable size of hydrogen and fluoro in the leaving groups and their similar polarities (14), the binding constant K_S may be assumed to remain almost constant within the series of isosteric amino acid esters. Therefore, changes in V_0 , which are proportional to k_2/K_S , give information about the sensitivity of the k_2 step for substrate activation.

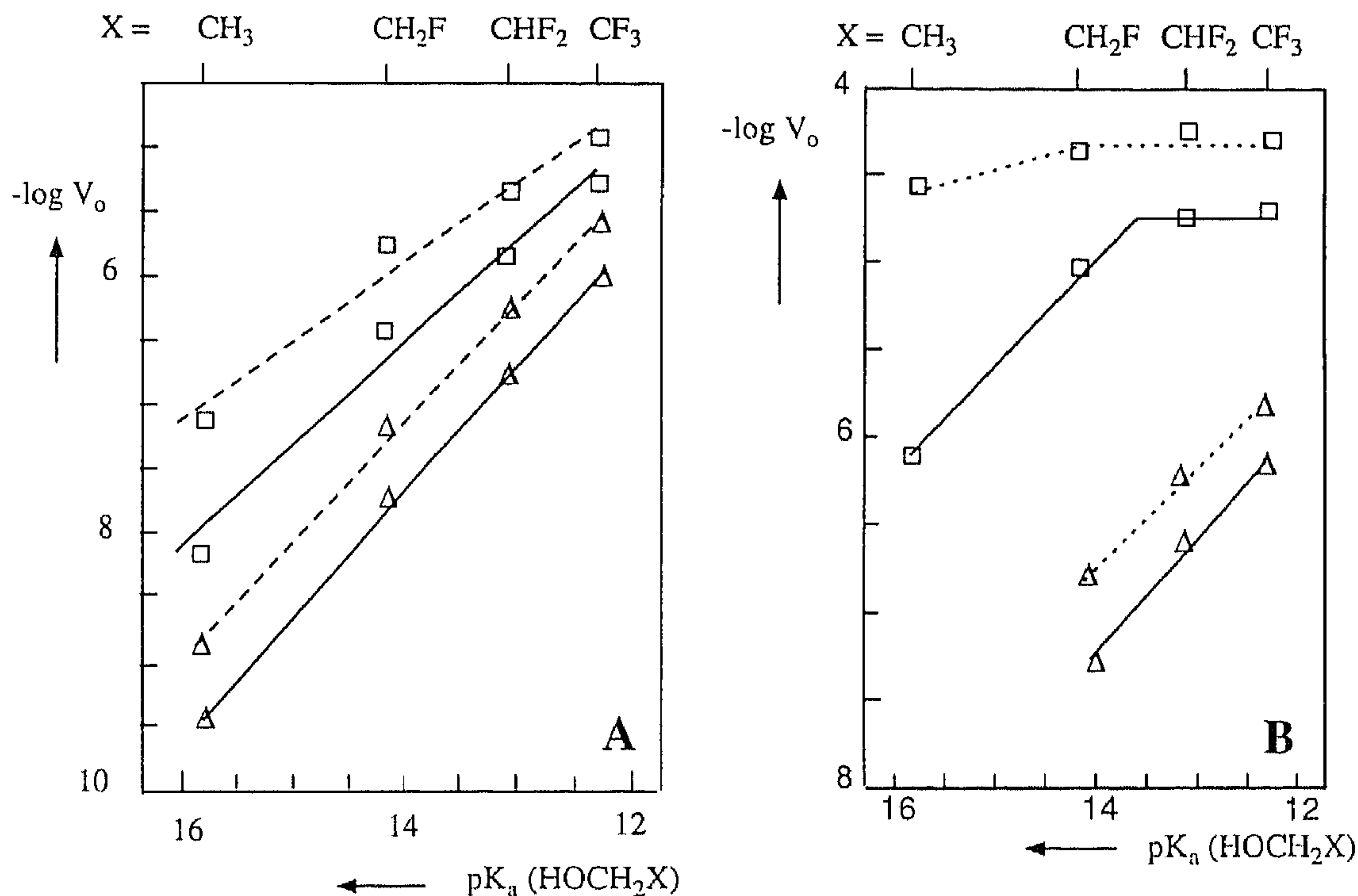


FIG. 2. Effect of the base strength of the leaving group of *N*-acetyl-D,L-alanine esters (A) and *N*-acetyl-D,L-phenylalanine esters (B) on the activity of α -chymotrypsin in the transesterification with 1-propanol in cyclohexane. (\square , L-enantiomer; Δ , D-enantiomer). The solid lines give the initial rate in absence of 18-C-6, the broken lines give the initial rate in the presence of 2 mM 18-C-6. V_0 in $\text{mol}\cdot\text{min}^{-1}(\text{mg enzyme}\cdot\text{mL}^{-1})^{-1}$.

In Figs. 2A and 2B the initial rates ($\log V_0$) are given as a function of the leaving ability of the alcoholate groups (expressed in the pK_a values of the conjugate alcohols, which are 15.83 (ethanol), 14.20 (2-fluoroethanol), 13.11 (2,2-difluoroethanol), and 12.32 (2,2,2-trifluoroethanol)). The α -chymotrypsin-catalyzed transesterification of the D,L-alanine esters (Fig. 2A) increases linearly upon increase of the leaving ability of the alcoholate group, but for the more reactive D,L-phenylalanine esters (Fig. 2B) a distinctly different response on increase of the leaving group is observed. Activation of the leaving group leads to a plateau value in the transesterification rate, indicating that for these relatively reactive substrates the chemical acylation (leaving group expulsion) no longer determines the rate of reaction. As under these conditions the reaction is still first order in substrate concentration, deacylation of the enzyme is not the rate limiting step. Therefore, the occurrence of the plateau in reaction rate must be due to a rate-limiting physical step, probably a slow dehydration of the active site upon productive binding of the substrate, and/or a slow conformational change of the enzyme necessary for acylation.

Addition of 18-C-6 to the organic reaction medium enhances the reaction rate for all esters, and the effect is larger for the L-enantiomers than for the D-enantiomers. This can be reconciled with facilitated water transport from the active site by 18-C-6, improving substrate binding. Promoted water transport is more advantageous for the tighter fitting and more reactive L-enantiomers than for the more loosely bound and less reactive D-enantiomers. This difference in sensitivity for water transport from the active site has important consequences for the enantioselectivity of the enzymes. In Figures 2A and 2B it is shown that the enantioselectivity decreases when the leaving ability of the leaving group increases. Similar effects were observed for these reactions catalyzed by subtilisin Carlsberg. This is shown in Figure 3. Whereas essentially absolute L-selectivity is observed for subtilisin Carlsberg in reaction with N-acetylphenylalanine methyl ester in aqueous solution, in cyclohexane (containing 0.33 M 2-methyl-2-butanol and 5 mM 1-propanol) the enantioselectivity of this enzyme towards a racemic mixture of N-acetylphenylalanine ethyl ester (15) is only 18 times in favor of the L-enantiomer (Fig. 3). The preference of the enzyme for the L-enantiomer becomes further reduced when the leaving ability of the alcoholate group in the ester is (isosterically) increased. Activation of the ester by using 2,2-difluoroethoxy and 2,2,2-trifluoroethoxy leaving groups even results in an inversion of the enzyme enantioselectivity.

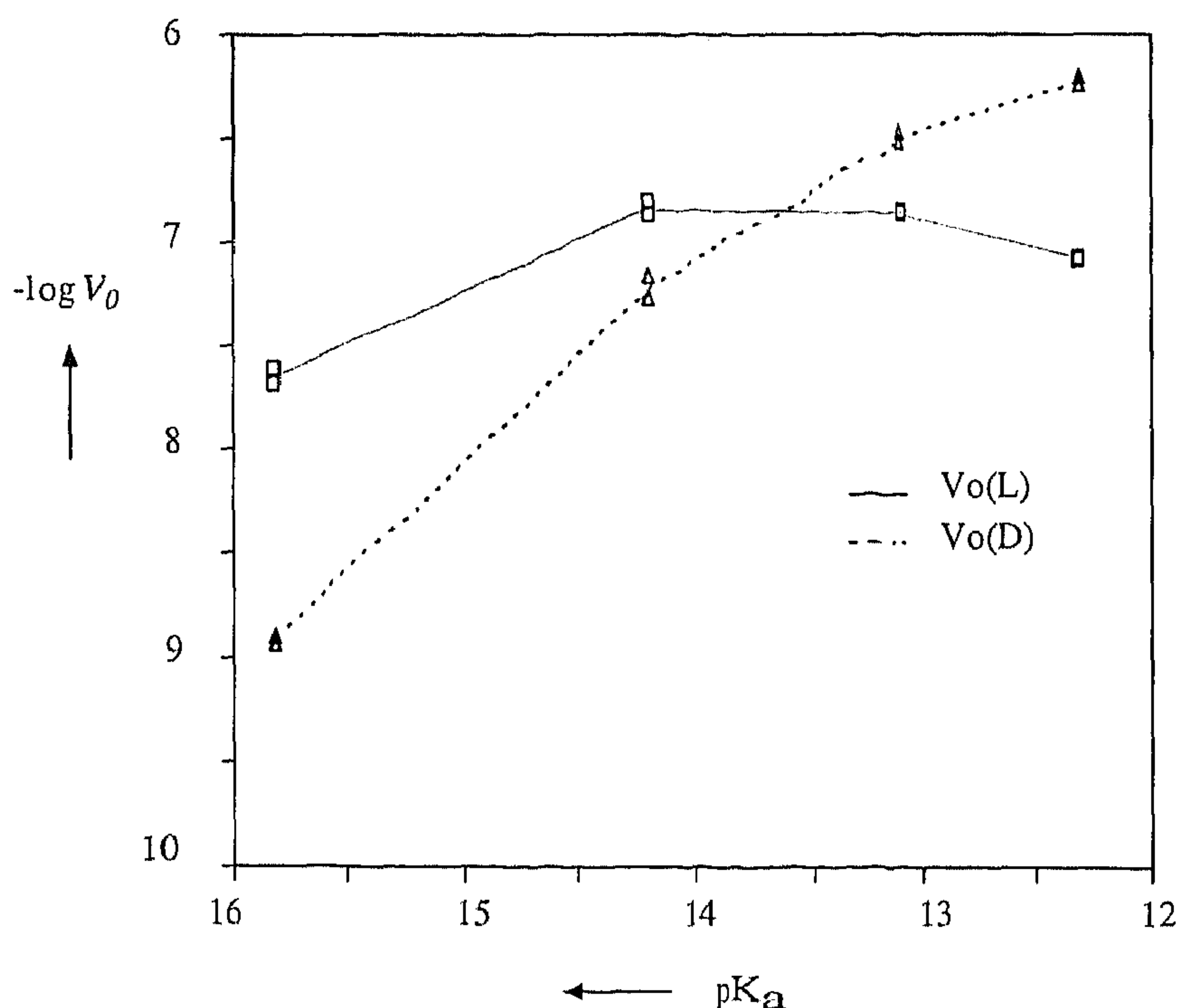


FIG. 3. The effect of the pK_a of the leaving group of N-acetyl-D,L-phenylalanine esters on the activity and enantioselectivity of subtilisin Carlsberg in the transesterification reaction with 1-propanol (5mM) in cyclohexane containing 0.33 M 2-methyl-2-butanol.

The sensitivity of the enzyme for the nature of the leaving group offers a unique possibility to manipulate the enantioselectivity of enzymes in organic media (15). This is illustrated in Figure 4 where the effect of the addition of low amounts (0.33 M) of organic cosolvents to cyclohexane is shown on the transesterification of N-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester catalyzed by subtilisin Carlsberg.

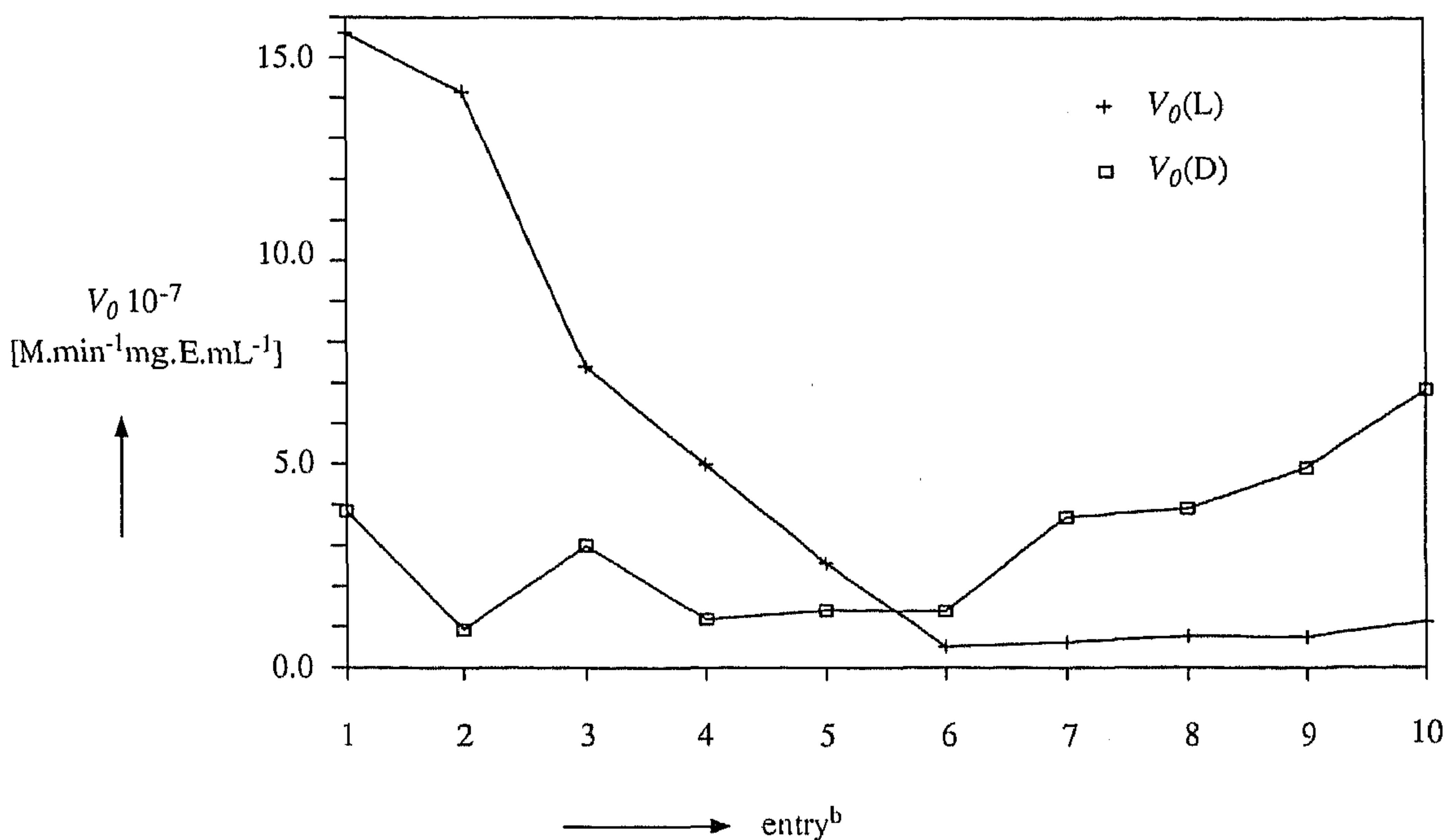


FIG. 4. Effect of various additives on the enantioselectivity of subtilisin Carlsberg in the transesterification of N-acetyl-D,L-phenylalanine trifluoroethyl ester.^a

^aConditions: 1 mM ester, 5mM 1-PrOH, 0.33 M additive in cyclohexane, 25 °C. ^bAdditive: 1, ethanol; 2, acetonitrile; 3, 1-propanol; 4, propionitrile; 5, butyronitrile; 6, no additive; 7, tert-butanol; 8, 2-methyl-2-pentanol; 9, 3-methyl-3-pentanol; 10, 2-methyl-2-butanol.

Figure 4 shows that the enantioselectivity is remarkably dependent on the structure of the cosolvent. Addition of bulky alcohols (which do not react as nucleophiles in this reaction) increases the enzyme activity towards the D-enantiomer, whereas the activity towards the L-enantiomer remains essentially unchanged. In contrast, addition of small alcohols and nitriles increases the activity towards the L-enantiomer, leaving the activity towards the D-enantiomer almost unaffected. These effects are not confined to reactions of subtilisin only; also for α -chymotrypsin, elastase, and *Aspergillus oryzae* protease similar responses were obtained under these conditions. A rationalization for the observed difference in cosolvent effect may be that only small cosolvents have access to the active site of the enzyme where they can promote the transport of water molecules necessary for substrate binding and acylation. Moreover, these cosolvents promote the enzyme flexibility by hydrogen bonding. Removal of water from the active site is more important for the binding of the tightly fitting L-enantiomer than for the loosely bound D-enantiomer, which explains then the increase of activity towards the L-enantiomer. The more bulky cosolvents cannot fulfil this role, but, like the smaller cosolvents and water, these hydrogen-bonding species can increase the flexibility of the enzyme backbone by hydrogen-bond interactions. This increased enzyme flexibility will be primarily reflected in a higher rate of the D-enantiomer because of the higher binding constant of this enantiomer to the active site.

In conclusion, these results show that pretreatment of enzymes by lyophilization with crown ethers or by simply adding 18-C-6 to the organic solution, can enhance the enzyme activity to a level which makes them suitable for practical applications. Moreover, it is shown that for relatively reactive substrates the enantioselectivity of proteases in organic solvent is very sensitive for small changes in solvent composition. This offers the possibility to tune the enantioselectivity and to apply these enzymes as catalysts for conversions of both the L- and the D-enantiomers.

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