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Studies on the mechanism of crown-ether-induced activation of enzymes in non-aqueous media.

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Abstract

Studies on the mechanism of crown-ether-induced activation are described in this paper. Michaelis Menten kinetics of α-chymotrypsin in toluene in the presence and absence of 18-crown-6 showed that only V_{max} is increased upon crown ether treatment. Parallel Lineweaver–Burk plots indicate that crown ethers do not activate the enzyme by specific interactions in the active site, such as transition state stabilization or facilitated transport of water molecules. Increased V_{max} values of crown-ether-treated enzyme most probably originate from conformational changes, which alter k_{cat} as well as the amount of catalytically active enzyme.

Keywords: α-Chymotrypsin; Crown ethers; Organic solvents

1. Introduction

Non-aqueous enzymology is one of the major fields in biotechnological research [1]. The advantages of using enzymes in organic solvents are well recognized, e.g. synthesis is favored over hydrolysis, enhanced enzyme stability, tunable enzyme selectivity via medium engineering, and obviously increased substrate solubility. Although these new and exciting properties are promising with respect to industrial applications, practical applications are rare. The reason for this is that most enzymes in organic solvents are far less active compared to the situation in water. After extensive research in various laboratories, most part of the molecular origin for this drop in activity is understood nowadays [2]. Among these factors are a reduced conformational mobility, unfavorable substrate desolvation energetics, transition state destabilization, and conformational changes. The latter are induced during the inevitable dehydration procedures [3], such as lyophilization, and upon suspension of the biocatalysts in an organic solvent [4]. The current view is that the enzymes are kinetically locked in various conformations in dry nonpolar organic solvents. Only a few of these will be catalytically active and contribute to the catalysis. Several remedies to encounter the activity reduction are currently available. Most widely applied are the use of additives during lyophilization, such as inorganic salts [5],

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sugars [6], and crown ethers [11], as well as immobilization procedures [1]. Also organic solvent soluble amphiphile–enzyme complexes are known to induce activation in organic solvents [7]. Most of these activation procedures stabilize and optimize the enzyme conformation prior to suspension into the organic solvent.

Crown ethers, discovered in the 60s by Pedersen [8], are cyclic polyethers composed of ethylene-oxy units. They have an extraordinary ability to solvate alkali metal ions by sequestering the metal in the center of the polyether cavity. 18-Crown-6 can also complex very efficiently (alkylated) ammonium ions, and even water molecules. Weak interactions between crown ethers and proteins are reported in methanol [9]. It has been shown that the simple addition of crown ether to the organic solvent does enhance the enzymatic activity significantly [10]. A few years later we reported that addition of crown ether prior to lyophilization enhances the activity up to a level only two orders of magnitude lower compared to water [11]. This suggests that crown ethers are protecting the enzymes against conformational changes during dehydration, so called lyoprotection. However, although a part of the activation can be explained by lyoprotection it cannot explain why enzymes are also activated upon crown ether addition to the reaction solvent. The mechanism of this specific crown-ether-induced activation is not understood in detail, although a few possibilities have been suggested previously [12–14]. These are prevention of salt bridge formation by complexation of lysine residues and facilitated transport of water molecules from the active site into the bulk organic solvent. This paper describes our ongoing studies to unravel the molecular origin of crown ether activation.

2. Results and discussion

As mentioned in the introduction, we have discussed previously a number of possibilities for the molecular origin of the specific crown ether activation of enzymes in organic solvents [12–14]. These are based on the combination of experimental observations and knowledge of likely crown ether interactions.

The first hypothesis for the activation induced by crown ethers is the facilitated transport of water molecules from the active site into the bulk organic solvent. Removal of water molecules from the active site is necessary in order to accommodate the substrate and enable specific substrate–enzyme interactions in the transition state. In water the removal of water molecules from the active site is energetically favorable due to an increase in translational and rotational entropy. In organic solvents, however, the situation is dramatically changed because the release of water molecules is thermodynamically unfavorable in this case [16]. Determination of Michaelis Menten constants in organic solvents in the presence and absence of crown ether can provide information on substrate-active site interactions. Generally, K_m values in organic solvents are so high that no saturation kinetics can be obtained [17]. However, for certain enzyme–substrate combinations saturation kinetics can be observed. An example is the protease-catalyzed transesterification of *N*-CBZ-L-Ala-ONp with 1- butanol in toluene [18]. Fig. 1 shows indeed Michaelis Menten behavior for α-chymotrypsin that is lyophilized in the presence and absence of 18-crown-6. The enzymatic activity in the presence of 18-crown-6 is significantly enhanced. In principle, both parameters K_m and/or V_{max} can be responsible

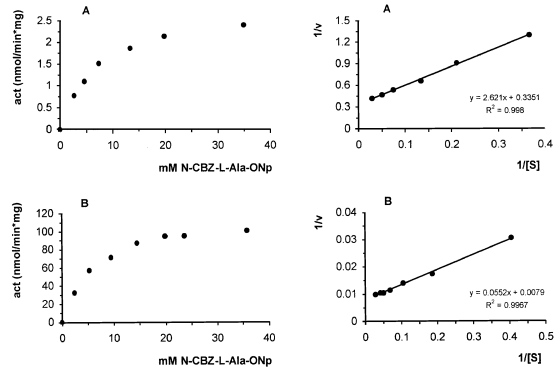

Figure 1. Saturation kinetics for the α-chymotrypsine-catalyzed transesterification of *N*-CBZ-L-Ala-ONp with 1-BuOH in toluene. (A) Enzyme lyophilized without 18-crown-6, (B) enzyme lyophilized with 250 eq. 18-crown-6). Conditions: 2 mg/ml of lyophilized α-chymotrypsine powder, 1 M 1-BuOH, $a_w = 0.113$, 30°C.

for the enhanced activity. Increases in V_{max} will obviously result in increased enzymatic activity. Under subsaturation conditions, which is often the case in non-aqueous enzymology, a decreased K_m value, i.e. an enhanced binding of the substrate by the enzyme active site, will also result in higher enzymatic activity. The experimental results (Fig. 1) point out that the presence of crown ether during the lyophilization process does not significantly alter the Michaelis Menten constant K_m for *N*-CBZ-L-Ala-ONp. K_m values in the presence and absence of 18-crown-6 of 7.0 and 7.8 mM, respectively, can be calculated from the Michaelis Menten equation. However, in organic solvents the evaluation of K_m values is not straightforward [18]. For enzyme–substrate binding the substrate molecule has to become desolvated and the partitioning of the substrate between the bulk organic solvent and the enzyme active site is different for different organic solvents as desolvation energetics will vary from solvent to solvent. Therefore in organic solvents only apparent Michaelis Menten constants are determined. Reimann et al. [18] proposed that these Michaelis Menten constants should be corrected for the differences in thermodynamic activity (solubility) of the substrate in the different solvents. An additional aspect that has to be taken into consideration is that the presence of crown ether in the reaction system may alter the thermodynamic properties and hence influence the apparent binding properties of the enzyme. This possibility is ruled out for two different reasons. Determination of the solubility *N*-CBZ-L-Ala-ONp in toluene + 1 M 1-BuOH in the presence and absence of 18-crown-6 as well as calculations of thermodynamic activity coefficients with UNIFAC showed that 18-crown-6 does not alter the thermodynamic properties of the substrate in the reaction system. Hence it can be concluded that the desolvation energetics are not altered by the presence of 18-crown-6 in the organic reaction medium and that the experimental K_m values will also in their solvent-corrected form, be practically equal in the presence and absence of 18-crown-6.

From these results it is apparent that V_{max} is the only kinetic parameter altered. The V_{max} value increases from 3 to 126 nmol min^{-1} mg^{-1} upon addition of 18-crown-6 prior to the lyophilization process. Since V_{max} is determined by the product of k_{cat} and the concentration of free, catalytically active enzyme, one or both of these parameters might be influenced. Active site titrations in the absence and presence of crown ether show that crown ethers do not influence the percentage of active sites [19]. The percentage of active sites is 11% in cyclohexane/1 M 1-PrOH both in the presence and in the absence of 18-crown-6. These data are based on experiments with crown ether addition to the reaction solvent. Data of active site percentages after lyophilization in the presence of 18-crown-6 are currently not available due to practical limitations. Obviously, a 500-times increased enzymatic activity in the presence of 18-crown-6 in the case of both the α-chymotrypsin-catalyzed transesterification and the peptide bond formation reactions [11,13] can, however, not be explained by an increased percentage of active sites. In general, we should be cautious with the interpretation of active site data. Most active site determination methods are based on a pre-acylated enzyme, which is deacylated upon suspending in an organic solvent. A titratable active site only means that deacylation of an active site is possible but does not reveal information about the whole conversion. Active site titrations may therefore overestimate the available active sites. The only remaining parameter that might contribute to the crown ether activation is k_{cat}. In serine proteases charge separation in the charge relay system is the key catalytic step. The formation of an anionic intermediate and the related transfer of a proton are the key processes involved. Approximately 30% of the transition state is solvent exposed [2]. Obviously, charge separation in nonpolar organic solvents is an overall unfavorable process. In organic solvents the formation of the anionic tetrahedral intermediate during the catalysis is generally accepted as the rate-limiting step in the catalysis, and one of the main contributions to the decrease in activity upon the transfer of enzymes from water to non-aqueous organic solvents. This tetrahedral intermediate is formed twice during the conversion, viz. during the formation of the acyl–enzyme intermediate and during the nucleophilic attack of the substrate, in this case 1-butanol. Depending on the leaving group ability of the original amino acid ester either the first or the latter of these two intermediate conversions is rate limiting. Transition state stabilization by the addition of water to the reaction solvent

is a method to enhance catalytic turnover. Furthermore, the solvent can be tuned to afford favorable interactions with the transition state [2]. It may be envisioned that the polar crown ether is assisting in the stabilization of the transition state by either bringing in complexed, hydrogen bond donating water molecules, or complexed sodium cations which stabilize by electrostatic interactions. However, for this process the complexed crown ether has to penetrate into the active site and will most likely compete with the substrate. In such a case an enhanced K_m value should be observed. The observed Michaelis Menten curves clearly demonstrate noncompetitive interactions as K_m is slightly decreased. On the basis of this observation it can be concluded that active site interactions, such as transition state stabilization and/or crown-ether-facilitated transport of active site water molecules, are not likely to occur.

The second hypothesis to explain the crown ether activation was based on the fact that crown ethers, particularly 18-crown-6, are very efficient complexing agents for (alkylated) ammonium groups. In organic solvents with a low dielectric constant ε-ammonium groups of lysine residues in proteins are likely to form salt bridges with anionic residues, like aspartic and glutamic acid. Such an intra- and intermolecular salt bridge formation may result in conformational changes and/or blockage of the enzymes active site. Complexation of the ammonium residues by 18-crown-6 might prevent salt bridge formation with anionic amino acid residues, resulting in enhanced levels of active enzyme. This hypothesis would be supported by kinetic information of crown ether effects on enzymes which lack these solvent accessible ammonium residues. An example is acetylated trypsin in which all 14 lysine and four to seven out of 10 tyrosine residues are acetylated [15]. Table 1 shows the influence of crown ether addition to the reaction solvent as well as prior to lyophilization in case of normal trypsin and acetylated trypsin.

It is obvious that crown ether addition prior to lyophilization is much more effective compared to addition to the reaction solvent. On the basis of these results we propose a model for the crown ether effects of enzymes in organic solvents. The activation may be divided into two parts. The first is the specific crown ether effect that is obtained by addition of 18-crown-6 to the reaction solvent. This activation is limited to 18-crown-6 derivatives since smaller or larger crown ethers hardly have any effect [10]. Furthermore, the activation is caused by a macrocyclic effect as pentaglyme, the linear chain analog of 18-crown-6, does not show any activation. The second contribution to the activation is observed in the case of addition during lyophilization. This activation is less specific and most probably caused by lyoprotection. This model is in line with the experimental results in Table 1 as almost no crown ether effect is found upon addition to the reaction solvent in the case of acetylated trypsin. However,

Table 1
Crown ether (pre)treatment of trypsin and acetylated trypsin

Treatment	Enzyme	[18-crown-6]	Activity (nmol/min mg)	Activation
Addition solvent	Trypsin	–	0.071	–
		5 mM	0.31	4.4
	Acetylated trypsin	–	0.013	–
		5 mM	0.018	1.4
Addition lyophilisation	Trypsin	–	0.071	–
		50 eq.	4.6	65
		250 eq.	3.9	55
		1000 eq.	0.57	8
	Acetylated trypsin	–	0.013	–
		50 eq.	0.57	43
		250 eq.	0.17	13
		1000 eq.	n.d.	–

Conditions: 5 mg/ml enzyme powder, 5 mM *N*-Ac-L-Phe-OEt, 1 M 1-PrOH, cyclohexane, $a_w = 0.113$, 30°C. n.d. nondetectable.

lyoprotection is a general effect and gives significant activation even in the case of acetylated trypsin. The total activation, caused by the specific crown ether effect and by the lyoprotection, is therefore lower in the case of acetylated trypsin. The specific crown ether activation of enzymes in organic solvents is most probably caused by complexation to ammonium groups of lysine and/or hydrogen bonding to tyrosine which limits salt bridge formation and thus stabilizes the enzyme conformation in organic solvents. Complexation of 18-crown-6 with ammonium groups of lysine residues on the enzyme exterior was reported to solubilized the enzymes in polar organic media, like methanol [9]. This may minimize diffusional limitations for the substrate and hence lead to an enhanced enzymatic activity. However, solubilization of the enzymes was not observed in our experiments, which were performed in the apolar solvent cyclohexane.

In an earlier paper our group reported that acetylated trypsin was not activated by lyophilization in the presence of 18-crown-6, while trypsin was activated effectively [11]. These experiments were performed with enzyme preparations lyophilized in the presence of 500 molar equivalents of 18-crown-6 with respect to the enzyme. This amount of added crown ether was based on the fact that 500 equivalents was the optimal amount for the α-chymotrypsin-catalyzed transesterification of *N*-Ac-L-Phe-OEt with 1-PrOH in cyclohexane. Table 1 clearly demonstrates that there is no activation in case of acetylated trypsin with large amounts of 18-crown-6. No activity could be detected in the case of acetylated trypsin that was lyophilized in the presence of 1000 equivalents of 18-crown-6. Recent studies revealed that the activity of enzymes lyophilized in the presence of larger amounts of 18-crown-6 dramatically drops, when they are incubated for 15 min in the reaction solvent prior to substrate addition. This is not observed with significantly smaller amounts of 18-crown-6. Due to this phenomenon the optimum is shifted towards 50 equivalents of 18-crown-6. Most probably, reequilibration of the water is the origin for this behavior, despite the fact that the crown-ether-pretreated enzyme preparation and solvent are preequilibrated at a thermodynamic water activity of 0.113. 18-Crown-6 will be slowly released from the enzyme powder into the apolar cyclohexane. Due to an increased solvent polarity the solvent becomes more "water-demanding" at this thermodynamic water activity. Water will therefore slowly be redistributed between the crown ether containing apolar solvent and enzyme. The enzyme becomes dehydrated and will consequently become less active. In a more polar solvent, like acetonitrile, this reequilibration is fast, due to fast dissolution of the crown ether and water into the organic solvent. Therefore 50 equivalents of 18-crown-6 was found to be optimal in the case of α-chymotrypsin-catalyzed dipeptide formation in acetonitrile [13]. In order to correct for the water redistribution in the case of *crown-ether-pretreated* enzyme samples these measurements were now performed in an alternative way. The thermodynamic water activity equilibrated enzyme is suspended in an organic solvent with the same thermodynamic water activity. The substrate is added after 30 min of reequilibration.

The present studies reveal that crown ethers do not activate enzymes in organic solvents by active site interactions, as was hypothesized with the facilitated transport of water molecules from the active site in the bulk organic solvent. Crown ethers rather act by enhancement of the enzymes turnover frequency and/or percentage of catalytically active enzyme. Both of these can be explained by crown ether-induced conformational stabilization in organic media. Part of this conformational stabilization is likely to be caused by specific crown ether interactions with ammonium residues of lysine residues and/or hydrogen bonding to tyrosine residues as is shown by our investigations on trypsin and acetylated trypsin. Work, which focuses on the possibility of crown-ether-induced conformational stabilization during lyophilization as well as addition to the reaction solvent, is currently under investigation.

3. Experimental section

3.1. Pretreatment of the enzymes

The enzymes (Sigma, highest available purity), trypsin, acetylated trypsin, and α-chymotrypsin, were lyophilized (5 mg/ml) from 20 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7.8 containing the indicated

amounts of 18-crown-6. The corresponding enzyme preparations are stored at −20°C after lyophilization.

3.2. Transesterification of N-Ac-L-Phe-OEt

The enzyme preparations and cyclohexane (Merck; p.a.) with 1 M 1-PrOH (Merck; p.a.) are equilibrated at a thermodynamic water activity of 0.113 (saturated LiCl solution). The enzyme preparations are suspended in the solvent, followed by stirring for 30 min. Subsequently 5 μmol of *N*-Ac-L-Phe-OEt (Sigma) in 20 μl 1-PrOH is added per milliliter of reaction mixture. The formation of propyl ester is followed with GC.

3.3. Michaelis Menten studies [18]

The enzyme preparations and toluene (Aldrich; p.a.) with 1 M 1-BuOH (Aldrich; p.a.) are equilibrated at a thermodynamic water activity of 0.113 (saturated LiCl solution). The indicated concentration of *N*-CBZ-L-Ala-ONp is dissolved in the solvent. Subsequently the enzyme preparations are suspended. Samples of 100 μl are taken at selected time intervals and quenched with 500 ml 1 M Na_2CO_3. After 1 min of vigorous mixing the aqueous layer is analyzed for *p*-nitrophenolate with UV–VIS spectroscopy at 405 nm.

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