Journal of Molecular Catalysis B: Enzymatic 64 (2010) 38–44

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Effect of prolonged exposure to organic solvents on the active site environment of subtilisin Carlsberg

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ARTICLE INFO

Article history:
Received 6 August 2009
Received in revised form 21 January 2010
Accepted 25 January 2010
Available online 2 February 2010

Keywords:
Enzyme catalysis in organic solvents
Enzyme storage stability in organic solvents
Active-site polarity
Subtilisin Carlsberg

ABSTRACT

The potential of enzyme catalysis as a tool for organic synthesis is nowadays indisputable, as is the fact that organic solvents affect an enzyme's activity, selectivity and stability. Moreover, it was recently realized that an enzyme's initial activity is substantially decreased after prolonged exposure to organic media, an effect that further hampers their potential as catalysts for organic synthesis. Regrettably, the mechanistic reasons for these effects are still debatable. In the present study we have made an attempt to explain the reasons behind the partial loss of enzyme activity on prolonged exposure to organic solvents. Fluorescence spectroscopic studies of the serine protease subtilisin Carlsberg chemically modified with polyethylene glycol (PEG-SC) and inhibited with a dansyl fluorophore, and dissolved in two organic solvents (acetonitrile and 1,4-dioxane) indicate that when the enzyme is initially introduced into these solvents, the active site environment is similar to that in water; however prolonged exposure to the organic medium causes this environment to resemble that of the solvent in which the enzyme is dissolved. Furthermore, kinetic studies show a reduction on both V_{max} and K_M as a result of prolonged exposure to the solvents. One interpretation of these results is that during this prolonged exposure to organic solvents the active-site fluorescent label inhibitor adopts a different binding conformation. Extrapolating this to an enzymatic reaction we argue that substrates bind in a less catalytically favorable conformation after the enzyme has been exposed to organic media for several hours.

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1. Introduction

Although during the last 20 years enzymes in organic solvents have been successfully used to catalyze a range of important reactions, there are still some drawbacks to overcome before their potential can be fully exploited. Among then is the fact that their initial activity decreases during prolonged exposure to organic solvents. This is particularly significant for lengthy reactions, for the construction of bioreactors, or for reusing the biocatalyst. Several studies have addressed issues such as shelf life, bioreactor stability and operational stability of a variety of enzymes suspended in organic solvents and in aqueous–organic solvent mixtures [1–3]. It is well documented that the dehydration step (usually lyophilization), typically required prior to suspension in organic media, causes minor structural perturbation [4], whereas subsequent exposure to organic media generally does not perturb the enzyme's structure [5,6], although there are some accounts in the literature that contradicts this assessment [7,8]. Nevertheless, the mechanism of enzyme partial inactivation in organic solvents is still unclear. Recent studies on the serine protease subtilisin Carlsberg (SC) have shown that this detrimental effect was observed in different solvents and under different experimental conditions, suggesting that this effect is independent of the physicochemical properties of the organic medium, its hydration state, and the reaction temperature. It was also determined that the enzyme remains structurally defined during prolonged exposure to organic solvents [9,10]. In a separate study electron paramagnetic resonance spectroscopy (EPR) was used to gain vital information about the effect of prolonged exposure to an organic solvent on the mobility of a spin label bound to the active site of SC (which in turn could be extrapolated to a similarly bound substrate in the active site). Although those results were inconclusive as to pinpointing the mechanistic reason for this observed decrease of initial enzyme activity, two different components were observed in the EPR spectra whose percentages changed during the incubation period, giving the first indication that perhaps the observed activity loss was due to an

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1381-1177/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.molcatb.2010.01.021

effect localized at the active site [9]. Furthermore, two recent studies shed additional light to this phenomenon: the first study looked at the enzyme dynamics by measuring the H/D exchange by NMR, and showed that globally, the enzyme becomes more rigid upon storage in acetonitrile (ACN) and 1,4-dioxane [11]. As previously observed the enantioselectivity remained constant, which is an indication that the active-site structure remained intact. More importantly however, was the fact that there was no correlation between enzyme flexibility and activity. The most active preparation (PEG-SC) was less flexible than the less active lyophilized preparation, and both preparations were more flexible in ACN than in 1,4-dioxane (the enzyme is more active in the second solvent) [11]. These results suggested to us that decreased global flexibility might not be the only cause of the observed loss of activity upon prolonged storage in organic media, and that other factors must be involved. The second study looked at the dynamics of the same enzyme in acetonitrile during 92 ns. This molecular dynamics approach showed that a key residue in the active site change its orientation, diminishing the ability of the oxyanion hole to stabilize the tetrahedral intermediates [12]. This study also showed that most internal water molecules are exchanged by the solvent used (ACN), thus changing the environment (the polarity) of the active site. Although the study represents a snapshot of the initial events after exposure to organic media, it is an indication that additional changes (such as water removal and reorientation of additional residues and side chains) might take place during prolonged exposure to organic media (Cruz et al., 2009). In an effort to shed more light to the mechanism of enzyme partial inactivation upon prolonged exposure to organic solvents, we decided to study the environment of the active site of SC during storage in ACN and 1,4-dioxane—solvents in which SC exhibits different catalytic properties, directly, by fluorescence spectroscopy. There is a common characteristic shared by most enzyme-in-organic solvents systems that limits the types of studies that can be carried out with then, and that is the fact that they are virtually insoluble in organic solvents. This is particularly relevant for the types of studies we are about to discuss since suspended enzyme particles would impede and obscure fluorescence experiments. To overcome this problem we chemically modified our enzyme with polyethylene glycol (PEG) of 5 kDa molecular weight, a technique which has been shown to increase the solubility of several enzymes in some organic solvents without affecting or altering their secondary and tertiary structure [10,13–15]. However, the degree of solubility of a particular PEG-enzyme system varies among solvents. In our case, we found the enzyme to be highly soluble in ACN, 1,4-dioxane, toluene and dichloromethane. The last one was found to be detrimental to enzyme structure (determined by ^{1}H NMR), and toluene turned out to be a bad choice for our spectroscopic experiments. Other solvents tested render a partially soluble enzyme while in others the PEG-enzyme precipitated during the long incubation period. This restricted our choices to 1,4-dioxane and ACN, which are actually good picks since previous studies found that SC is structurally stable in these solvents [9]. Additionally, in these two solvents SC exhibits vastly different activity and enantioselectivity (the enzyme is much more active and enantioselective in 1,4-dioxane than in ACN).

Fluorescence spectroscopy is a powerful tool to study discrete sites in proteins by looking at the emission of intrinsic or extrinsic fluorophores. For these studies we chose two very basic analyses based on steady-state fluorescence emission in organic solvents. Similar studies (but in aqueous systems) have previously been reported by Vaz and Schoellmann [16,17]. Variables such as quantum yields and molar extinction coefficients were calculated for the system with respect to the solvents being used. Our model enzyme was studied with respect to two fluorophores: Trp residue present at position 113 (Trp_{113}) as the intrinsic fluorophore (the enzyme's only intrinsic fluorophore) and a dansyl group (D), bound to the active site Ser_{221} (Ser_{221}-D) of the enzyme, as the extrinsic fluorophore. D binds exclusively at the active site serine residue of SC with a concomitant loss of enzymatic activity (indicative that it does bind to the active site) and hence serves as an effective probe for the active site studies [17]. According to Turner and Brand the emission maximum of adsorbed probes gives the most reliable estimate of the binding site polarity [18]. The high solvent sensitivity of both these fluorophores (Trp and D) makes them particularly useful for studying their microenvironment on the enzyme such as the polarity. We have thus used this system to study the effect of ACN, 1,4-dioxane and aqueous medium (sodium phosphate buffer) on the active-site polarity of the enzyme. The fluorescence and kinetic studies presented here and those previously published suggests that over time, substrates prefer a binding orientation that is not catalytically favorable.

2. Materials and methods

2.1. Materials

Subtilisin Carlsberg (alkaline protease from *Bacillus licheniformis*, EC 3.4.21.14) was purchased as lyophilized powder, the solvents were all purchased in the anhydrous form (Aldrich Sure/Seal bottles—water content below 0.005%) and used without further drying, and sec-phenethyl alcohol were purchased from Sigma–Aldrich (St. Louis, MO). Vinyl butyrate and 5-(dimethylamino)-1-naphthalenesulfonyl fluoride (dansyl fluoride, DF) were purchased from TCI America (Portland, OR). N-Succinimidyl PEG (5 kDa) was ordered from NeKtar Therapeutics (Huntsville, AL). Sephadex PD-10 desalting columns were purchased from Amersham Biosciences.

2.2. Methods

2.2.1. Chemical modification of the enzyme with polyethylene glycol (PEG)

Subtilisin Carlsberg was PEGylated with 10-fold molar excess of 5 kDa PEG in sodium borate buffer (pH 9.2). The PEGylation was allowed to proceed with constant stirring for 15 min at 20 °C and then 3 h at 4 °C. After this the pH of the reaction mixture was reduced to 5.5 with dilute HCl to stop the reaction and the solution was dialyzed for 24 h in 25 kDa cutoff dialysis bag. The dialyzed solution was finally lyophilized for 48 h.

2.3. Preparation of dansyl methoxide (D-OMe) solution

Fifty milligrams of (0.198 mmol) of dansyl fluoride were dissolved in 1 mL of methanol in a vial. 0.350 mL of a 5.6 M solution of sodium methoxide was added and the solution let under stirring for 15 min at room temperature. The suspension was centrifuged at 5000 rpm for 5 min. The supernatant was separated and used for further analysis.

2.4. Preparation of dansyl hydroxide (D-OH) solution

Fifty milligrams (0.198 mmol) of dansyl fluoride were dissolved in 1 mL of isopropyl alcohol. 0.02 mL of the solution was added to a 1 mL of 1 M solution of sodium hydroxide in water and the solution let under stirring at room temperature for 15 min.

2.5. Fluorescence spectroscopy

The enzyme was labeled with DF according to the method of Vaz and Schoellmann [16]. Active site titration (using N-trans cinnamoyl imidazole as a substrate) to monitor the labeling effectiveness revealed 100% inhibition of enzyme activity, thus

indicating 100% labeling with the dansyl group (D). Note that fluorine is released during the reaction.

2.6. Steady-state fluorescence emission spectra

Corrected steady-state emission spectra of the enzyme with and without dansyl were recorded with a Horiba Jobin Yvon Fluoromax-3 spectrofluorometer equipped with a xenon arc lamp. Trp fluorescence emission was collected from 310 to 600 nm by exciting directly the Trp_{113} in the non-labeled as well as dansyl-labeled SC (Ser_{221}-D) at 295 nm. Ser_{221}-D fluorescence emission was collected from 370 to 700 nm by exciting directly the dansyl at 360 nm. The excitation and emission slit widths were 2 nm for all spectra. Background fluorescence from the solvents was recorded and subtracted from the protein spectra. Steady-state measurements were performed in a 3.0 ml square fluorescence cuvette with 10 mm excitation/emission path lengths (emission collected through a perpendicular face). The absorbance of the samples was lower than 0.05 at λ_{ex} = 295 nm to avoid inner-filter effects. All measurements were performed at 20 °C. The effect of long-term exposure of the enzyme to the organic solvents was studied by collecting the emission spectra at regular intervals of 24 h for 4 days. The molar extinction coefficients at 360 nm were determined for free DF dissolved in the respective solvents.

2.7. Data analysis

The corrected emission spectra from the D-free and D-labeled SC were used to determine the emission maxima of Trp/D in the enzyme in different solvents. The emission maxima of dansyl bound to the active site of SC were further used to calculate the active-site polarity on the basis of data published by Vaz and Schoellmann and Kosower [17,19]. A linear correlation ($y = -113.03x + 288.24$) was obtained from a plot between the wave number (ν) and empirical polarities (Z) listed by Vaz and Schoellmann (1976) for dansyl-ethyl ester as a reference compound for SC [16], which was used to convert the emission maxima obtained for Ser_{221}-D in different solvents into the microenvironment polarities on the Z-scale. Absorption spectra were obtained using a Hewlett Packard 8453 UV–vis spectrophotometer.

2.8. Enzyme preparation and kinetic measurements

PEGylated SC powder was prepared by lyophilization from a solution of 5 mg SC per mL of a 20 mM potassium phosphate buffer, pH 7.8, for 24 h. The transesterification reaction between sec-phenethyl alcohol and vinyl butyrate was used in all of the initial activity and enantioselectivity measurements, and the reactions were carried out at 25 °C under anhydrous conditions (except in the case of the controlled water activity experiments). These reactions were done as previously described [20]. The retention times and instrument calibration of the "R" and "S" products were obtained using samples of the pure enantiomers synthesized from the corresponding alcohol enantiomers, and the activity was determined from the sum of both enantiomers of the product [21]. V_{max} and K_M were measured following the reaction of phenyl alcohol and vinyl butyrate and the data analyzed as previously described by us [9].

2.9. Degree of PEGylation

The degree of PEGylation in terms of number of PEG molecules per molecule of SC was found to be approximately 4 as determined by the method of Habeeb et al. [22].

2.10. Protein concentration

Protein concentrations were assayed by the bicinchoninic acid (BCA) method using bovine serum albumin as standard [23].

2.11. Water activity control

Some of the activity determinations and fluorescence experiments were performed under controlled water activity by adding salt hydrate pairs to the samples (sodium acetate 3/0, water activity = 0.28) [24]. Equal weights (50 mg each) of each of the two differently hydrated sodium acetate salts were added to 750 μl of the solvent and allowed to equilibrate for 30 min and then added 13.0 mg of protein to the solvent–salt mixture and allowed to equilibrate for another 60 min. Fluorescence spectra/activity were determined thereafter.

3. Results

3.1. Enzyme activity in organic solvents

The transesterification activity of PEGylated SC was determined in dioxane and in ACN during incubation at 25 °C under controlled and uncontrolled water activity conditions (Fig. 1). As previously reported, the enzyme activity decreased approximately 10-fold during a 4-day incubation performed in neat organic solvents [10]. Under controlled water activity conditions (using saturated salt solutions), the initial activity was low and it decreased further by 1.6-fold during incubation. A similar effect was observed in the case of ACN. However, the activity in 1,4-dioxane was higher than that in ACN (Fig. 1). We proceeded henceforth to explore the polarity

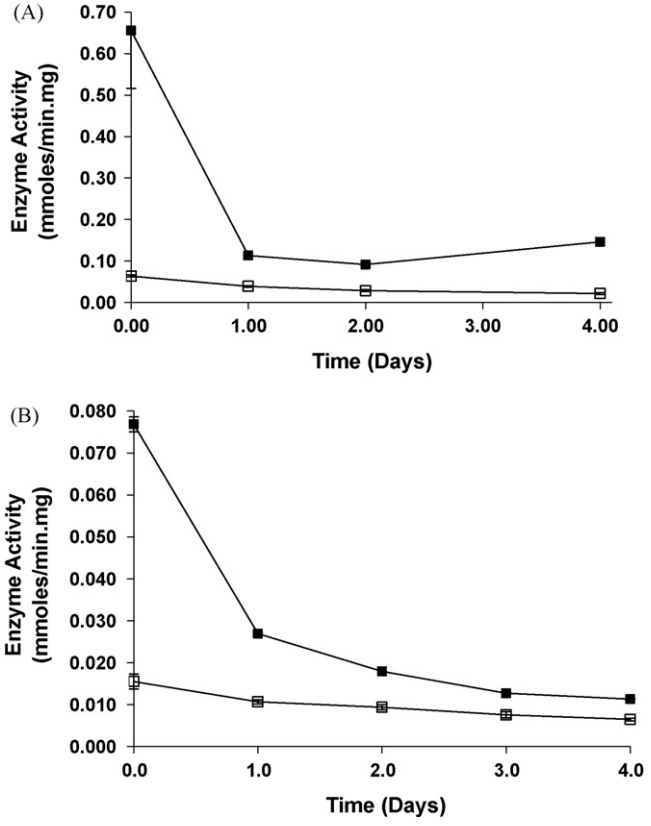

Fig. 1. Initial rate of PEGylated subtilisin Carlsberg in organic solvents during incubation at 25 °C (measured before 10% product conversion). PEGylated enzyme concentration: 1.0 mg/mL. Close squares: no water activity control; open squares: with water activity control (sodium acetate): (A) 1,4-dioxane; (B) acetonitrile.

Table 1
Emission maxima of all fluorophores used in the study (all measurements are within a maximum error of ±0.91%).

	$\lambda_{excitation}$ (nm)	Emission maxima (nm)[a]				
Days of storage in the respective solvents		0	1	2	3	4
Native SC Trp_{113}_buffer[b]	295	360				
PEG-SC Trp_{113}_buffer		356				
Free Trp_{113}_ACN		337	339	341	341	341
Free Trp_{113}_1,4-dioxane		333	334	330	333	330
PEG Ser_{221}-D_ACN	360	524/**445**[c]	515/**445**	515/**445**	515/**445**	515/**445**
PEG Ser_{221}-D_1,4-dioxane		510/**450**[c]	502/**450**	502/**450**	502/**450**	502/**450**
PEG Ser_{221}-D_buffer		510				510

The emission maxima written in bold letters refer to wavelength at which emission intensities increase during storage.
[a] Approximately ±2 nm.
[b] Not PEGylated enzyme.
[c] Shoulder: blank spaces stand for no measurement/storage.

of the active site (by fluorescence spectroscopy) and the binding kinetics of the substrate to gain a deeper understanding of the reasons that led to the observed partial loss of activity after prolonged exposure to organic solvents. We proceed to describe each study in detail.

3.2. Fluorescence spectroscopy

SC was labeled at the active site Ser_{221} with dansyl fluoride (Ser_{221}-D). A 100% inhibition of enzyme activity was achieved under optimal reaction conditions with a labeling ratio of one (see Section 2). The fluorescence studies in buffer were performed using two different preparations of the enzyme: the native SC powder and PEGylated (5 kDa) SC. However, for the studies in ACN and 1,4-dioxane only PEGylated SC was used for the solubility reasons discussed in Section 1.

3.3. Polarity studies

According to Stryer, the polarity of the binding site of a fluorophore can be evaluated in terms of two parameters: the emission maximum and the quantum yield of fluorescence of bound dye. Emission maximum is indicative of the overall dipolar character of the solvent shell, while the quantum yield is related to more localized deactivating processes [25]. We examined the polarity at the enzyme surface and the active site by following the fluorescence properties of Trp_{113} and Ser_{221}-D, respectively. Shifts in emission maxima and changes in quantum yields of both Trp_{113} and Ser_{221}-D were seen in H_2O, ACN and 1,4-dioxane (Table 1).

3.4. Polarity on the surface of the enzyme

Since the enzyme's only Trp residue (Trp_{113}) is on the surface and therefore its emission maximum will be determined by its interaction with solvent molecules and neighboring residues' side chains [26,27], we were able to study the polarity at the surface of the enzyme in the different solvents here used and the effect of prolonged exposure to these solvents. The emission maximum of Trp_{113} in the enzyme (without the dansyl moiety) showed a clear red shift in going from the organic to aqueous solvent (330 nm in 1,4-dioxane to 360 nm in water, Table 1, Fig. 2). No differences were seen between the emission maxima of the Trp_{113} and free Trp (unbound Trp) in any of the solvents indicating that the Trp_{113} emission is determined only by its interaction with the solvent (Table 1). Additionally, no substantial shift in emission maximum of Trp_{113} was seen during the 5-day incubation period in organic solvents, which indicates that the surface of the protein arrives at a fast equilibrium with the bulk of the solvent system (Table 2).

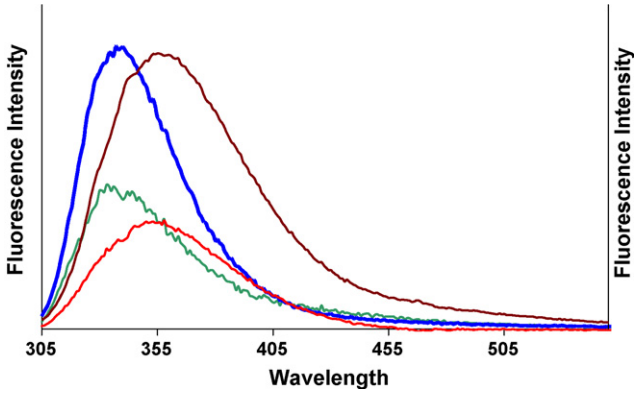

Fig. 2. Emission spectra of PEGylated and native subtilisin Carlsberg in different solvents (excitation at 295 nm). Green: 1,4-dioxane; red: buffer; blue: acetonitrile; brown: native (not PEGylated) in buffer.

3.5. Active-site polarity

The fluorescence emission of the active site bound dansyl fluorophore (Ser_{221}-D) was monitored to estimate the active-site polarity of SC in different solvents after different periods of incubation at 25 °C (Table 1). The emission maxima of Ser_{221}-D did no show a considerable red shift on initial exposure to the solvents studied here, as compared to our controls D-OMe and D-OH (in the absence of the enzyme, Table 2). The emission maximum of Ser_{221}-D was more red-shifted in ACN (525 nm) than in buffer (510 nm), while in dioxane it was similar to the one in buffer (Table 1). This means that the polarity experienced by Ser_{221}-D in dioxane is similar to that experienced by Ser_{221}-D in buffer. This

Table 2
Fluorophore properties for tryptophan and modified dansyl moiety.

	$\lambda_{excitation}$ (nm)	Emission maxima (nm)
Free Trp_buffer	295	360
Free Trp_ACN		335
Free Trp_1,4-dioxane		330
FreD-F_Buffer	360	524
FreD-F_ACN		586
FreD-F_1,4-dioxane		540
FreD-OH_Buffer	360	502
FreD-OH_ACN		460
FreD-OH_1,4-dioxane		450
FreD-OCH_3_Buffer	360	500
FreD-OCH_3_ACN		450
FreD-OCH_3_1,4-dioxane		450

Same color emission maxima are for comparison purposes only.

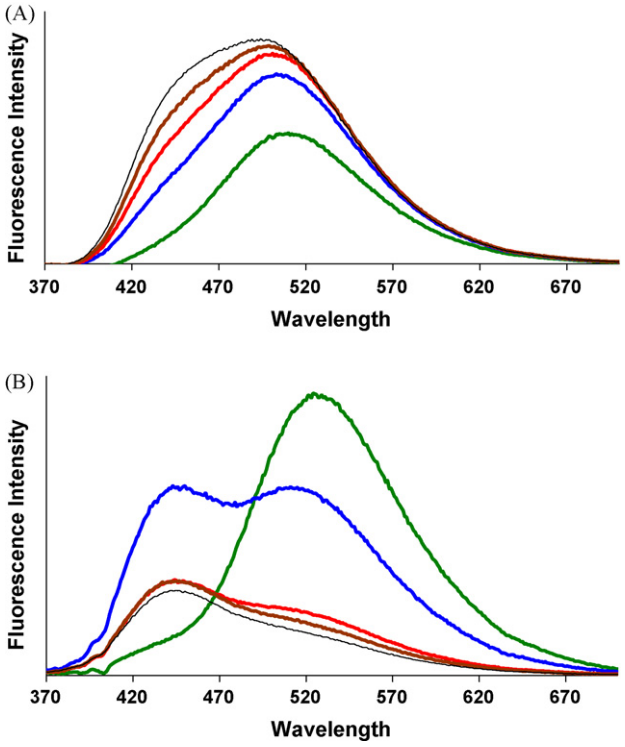

Fig. 3. Fluorescence emission spectra of dansyl-PEG-subtilisin Carlsberg in (A) 1,4-dioxane and (B) in acetonitrile. Day 0; day 1; day 2; day 3; day 4 (excitation at 360 nm).

finding can account for the fact the PEG-enzyme shows a higher activity in dioxane than in most other organic solvents [9,10,15]. D-labeled PEGylated and native SC, on being excited at 360 nm in buffer showed a clear peak of D emission at 510 nm (Table 1). Also, no shift in emission maximum occurred when the labeled enzyme was stored in buffer for 5 days. However, a distinctly different and complex behavior was seen during incubation in the organic solvents (Table 1). The Ser_{221}-D emission spectrum in dioxane on day 0 showed an almost well defined peak centered at 510 nm as a result of excitation at 360 nm (Fig. 3A). After nearly 24 h, a shoulder started to appear in the emission spectrum at 450 nm. The shoulder at 450 nm became increasingly pronounced during subsequent days of incubation and by day 4 the emission spectrum showed a broad peak spanning from 450 to 500 nm (Fig. 3, Table 1). The quantum yield of Ser_{221}-D also increased during the incubation in dioxane as is noticed from the increase in D fluorescence intensity during this period. These results would indicate a decrease in the polarity of the environment around the fluorophore during incubation in dioxane. A similar effect was observed in ACN, where again the D-labeled PEGylated subtilisin, on being excited at 360 nm, showed a clear peak at 524 nm on day 0 (Fig. 3B, Table 3). After 24 h, another peak appeared at 445 nm, and on continued incubation in ACN, the longer wavelength peak was reduced to a mere shoulder while the peak at 445 nm became the major peak. These changes in the emission spectra for Ser_{221}-D in organic solvents could also be due to a transition between two different states or subpopulations, the midpoint being approximately at 470 nm for both dioxane and ACN. It can be hypothesized that there is a gradual reduction of the fraction of the first subpopulation (λ_{em} = 510/524 nm) and a corresponding increase in the size of the second subpopulation (λ_{em} = 450/445 nm) during incubation in dioxane and ACN. It has been previously suggested, for a similar system (SC-Ser_{221}-D) in THF, that the second emerging peak resulted from free dansyl in the solution due to its leaching from

Table 3
Active-site polarity of subtilisin Carlsberg based on fluorescence emission maxima of dansyl fluoride bound to the active site Ser_{221}.

Solvent	ν_F, μ^{-1} (peak 1/2)	Estimated "Z" (Peak 1/2)
Buffer	1.96	66.61
ACN_Day 0	1.91	72.53
Day 1	1.94/**2.25**	72.53/**34.24**
Day 2	1.94/**2.25**	72.53/**33.67**
Day 3	1.94/**2.26**	72.53/**33.43**
Day 4	1.94/**2.26**	72.73/**33.67**
Dioxane_Day 0	1.96	66.61
Day 1	1.99/**2.22**	66.61/**37.06**
Day 2	2.00/**2.23**	66.61/**36.50**
Day 3	2.00/**2.23**	66.61/**36.50**
Day 4	2.03/**2.23**	66.61/**36.50**

The values in bold are derived from emission peaks that increase in intensity during the incubation.

the active site [28]. However, hydrolysis of the dansyl group is ruled out since parallel studies showed Förster Resonance Energy Transfer (FRET) (obtained by activating Trp_{113}, located about 20 Å away from the active site) throughout the incubation period (data not shown). If the dansyl acceptor was being hydrolyzed and released from the enzyme, the FRET effect would be drastically reduced or even disappear as huge concentrations of the acceptor are required for FRET to occur when the donor and acceptor are not on the same molecule, or are free in solution [29]. Furthermore, if the dansyl group is hydrolyzed and released from the active site, the new fluorophore (dansyl hydroxide) should give rise to a new peak in the absorption spectrum at 320 nm (Table 2), while we only observe a small shift to 335 nm (from 343 nm) in the absorption spectrum of the sample during incubation in 1,4-dioxane (Fig. 4). This small shift in λ_{max} could also be the result of changes of the environment felt by the Ser_{221}-D. Table 2 also shows the emission maxima of free dansyl hydroxide (D-OH), dansyl fluoride (DF) and dansyl methoxide (D-OCH_3) in buffer, 1,4-dioxane and ACN for comparison purposes. A possible explanation is that these different populations (which are responsible for these two emission bands) arise from local conformational changes due to the penetration of solvent molecules into the active site, forcing the dansyl group to reorient itself towards the solvent. It is also possible that water from the hydration shell quenches the fluorescence towards the beginning of the incubation period and the gradual displacement of this water by the organic solvent results in an increase in fluorescence [30]. Quantitative estimation of the active-site polarity on the basis of emission maxima of active site bound fluorophores was proposed by Vaz and Schoellmann using the "empirical polarity" scales established by Kosower [16,19]. They reported that, for the evaluation of microenvironment polarity using fluorescent probes,

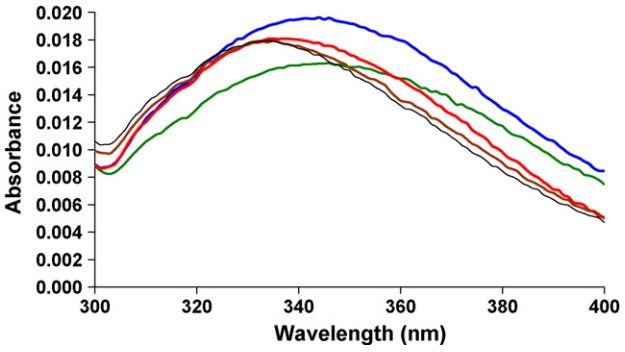

Fig. 4. Absorption spectra of dansyl-PEG-subtilisin C. during incubation in 1,4-dioxane. Day 0 (green); day 1 (blue); day 2 (red); day 3 (brown); day 4 (black).

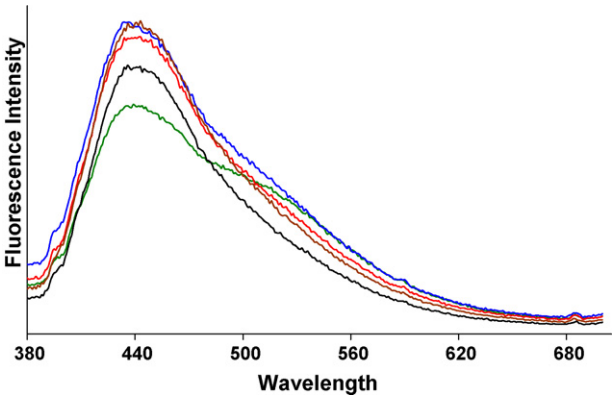

Fig. 5. Emission spectra of dansyl-labeled subtilisin Carlsberg during incubation in 1,4-dioxane under controlled water activity. Day 0; day 1; day 2; day 3; day 4. Excitation at 360 nm.

the use of the "empirical polarity" scales is more appropriate than the use of polarity scales based upon dielectric constant. We have deduced the 'empirical polarities' for the microenvironments of the dansyl group in the active site of SC in different solvents at different times of incubation on the basis of the *Z*-values obtained by Vaz and Schoellmann using the model compound dansyl-ethyl ester as a reference [16]. The results obtained are listed in Table 3. The *Z*-values reflect upon the microenvironment experienced by the fluorophore and such low *Z*-values as seen here can be accounted for by the presence of non-polar side chains of some amino acid residues in the active site as suggested by Vaz and Schoellmann [16]. Initially, for at least one subpopulation of the Ser_{221}-D fluorophore the active site environment in ACN seems to be more polar than in buffer, while in dioxane it is similar to that in buffer on day 0 and becomes less polar during the subsequent incubation (Table 3). These results clearly demonstrate a decrease in active-site polarity during incubation in organic solvents. After initial exposure of the enzyme to organic solvents, the polarity of the active site is similar to that in water, but during incubation it becomes similar to the polarity of the respective organic solvent, which can be explained by penetration of organic solvent into the enzyme active site.

3.6. Water activity control experiments

To rule out the effect of water these fluorescence studies were performed under controlled water environment by adding a hydrated salt mixture of sodium acetate to the sample. The spectra obtained in 1,4-dioxane are shown in Fig. 5. Unlike what was previously observed, the Ser_{221}-D emission peak obtained after initial exposure to this solvent was centered at 445 nm with a shoulder at 510 nm (without controlling the water activity we observed a peak centered at 510 nm and a shoulder at 445 nm which increased during incubation in this solvent, Fig. 3). During the incubation period the peak at 445 nm showed an increase in intensity as compared to the shoulder (at 510 nm). Results obtained from a similar water activity control experiment in ACN were analogous to those in the case of 1,4-dioxane (spectra not shown). It seems that the effect of incubation seen in samples with water activity controlled was accelerated. This in turn suggests that when the system is not pre-equilibrated with respect to water the effect seen during incubation could actually be the consequence of the equilibrium that enzyme and solvent molecules are trying to reach, while in case of pre-equilibration (with saturated salt solution), the emission spectra at time zero reflect a system already at equilibrium. However, it is important to point out that a pre-equilibrated system also shows the blue shift in Ser_{221}-D emission during incubation in both solvents.

3.7. Effect of incubation of V_{max} and K_M

In addition, both V_{max} (min) and K_M (mM) decrease during 4-day incubation in these two organic solvents. In ACN at day 0, $V_{max} = 5 \pm 0.6$, and $K_M = 322 \pm 87$. After 4 days of incubation, $V_{max} = 0.63 \pm 0.09$, and $K_M = 126 \pm 28$. In 1,4-dioxane at day 0, $V_{max} = 15 \pm 3$, and $K_M = 440 \pm 120$. After 4 days of incubation in this last solvent, $V_{max} = 1.7 \pm 0.4$, and $K_M = 192 \pm 65$. This clearly indicates that changes are occurring at the enzyme's active site during this period. The drastic decrease in V_{max} suggests that the efficiency of the enzyme is jeopardized, and although a decrease in K_M could be due several reasons such as to minor and local (at the active site) structural changes, or to a tighter binding of the substrate or poorer stabilization of the tetrahedral intermediate, it clearly shows that the catalytic machinery is been affected. As it will be discussed in the following section, results obtained here and recently reported will support two possible mechanisms.

4. Discussion

When PEG-subtilisin C. was initially exposed to 1,4-dioxane, the emission maximum of the dansyl group bound to its active site was similar to that in buffer (510 nm), whereas the emission maximum in ACN shifted 14 nm to a higher wave number (to 524 nm). However, during prolonged exposure to these two organic solvents a new, shorter wavelength peak, indicative of a drastic decrease in the polarity felt by the fluorophore begins to emerge. Both V_{max} and K_M decreased during prolonged exposure to these two solvents, also indicative of major changes taking place in the active site, which could be a diminished ability to stabilize the transition states, or minor structural distortions that might force substrates to adopt a less catalytically active conformation. Previous H/D exchange results obtained from this laboratory show that the enzyme becomes more rigid during prolonged exposure (96 h) to these two solvents [11], and a theoretical study obtained after 92 ns in ACN suggest that SC exchange its internal water molecules by the organic solvent, while a critical residue in the oxyanion hole changes its orientation [12]. The implications of theses findings is that while the enzyme becomes more rigid by the removal of its internal water molecules over a 96-h period, the active-site polarity should decrease (as suggested by our fluorescence data) and disruption of the oxyanion hole might reduce the enzyme's ability to stabilize the tetrahedral intermediates, decreasing both V_{max} and K_M, as shown by the results here presented. Furthermore, a decrease in the polarity of the active site and disruption of the oxyanion hole could lead to reorientation of the bound substrate [12] or allow the substrate to bind in a less restricted fashion, letting it move more freely, as it has been shown on previous studies involving EPR spectroscopy [9,31]. In short, one interpretation that is emerging from these studies is that changes in active-site polarity during prolonged exposure to organic solvents are the major cause of the observed decrease in enzymatic activity.

It is important to mention that efforts have been made by other research groups to explain the solvent dependence of enzyme activity using similar techniques and systems than us. However, most studies were not geared to address the effect of prolonged exposure to organic solvents on an enzyme's activity, and therefore they concentrate on the initial effect of exposure to organic solvents. Nevertheless, it is important to discuss some of these results since the effects that are linked to enzyme activity are enzyme flexibility and active-site polarity, both related to our work. Eppler et al. ^{19}F NMR active-site polarity studies show that increasing the salt concentration (NaCl, NaF, KCl and KF) in hexane from 0 to 98% did not augment enzyme polarity (while enzyme activity was increased), concluding that changes in polarity do not affect enzyme activity [31]. On a similar and more recent study however, it was concluded

that active-site polarity and hydration do affect enzyme activity, but that this effect was also dependent on the solvent used [32]. Actually, our results show that initially (when first introduced to an organic solvent) the enzyme retains the polarity of the aqueous solution from which it was last dissolved in, and changes in enzyme polarity are a slow process which might involve penetration of the solvent onto the enzyme fold. In a system whose water activity is controlled, besides the equal hydration of enzyme and solvent, the 2 h incubation period (required for the experiment) would allow for some solvent molecules to enter the enzyme fold, and therefore change the active-site polarity as we observed.

We propose that prolonged exposure to organic solvents leads to a decrease in enzyme dynamics and active-site polarity, and a reorientation of the active site inhibitors. These affect the ionization state of the catalytic triad residues as well as the transition states and intermediates' stability, resulting in reduced enzyme activity, V_{max} and K_M.

5. Conclusion

Previous studies have shown that an enzyme's initial activity in organic solvents decreases after prolonged exposure to this medium. It was also reported that this effect was unrelated to structural perturbations of the enzymes under study, since their structures remained "unharmed". Considering those studies and the fluorescence spectroscopic results presented here, we suggest a mechanism that involves two possible orientations/conformations of the active site bound fluorophore after prolonged exposure of PEG-SC to ACN and 1,4-dioxane. The dominant conformation of the inhibitor (or substrate), upon initial exposure to organic solvents is the one that interacts to a greater extent with the active side residues. During incubation there is a gradual transition of the active site bound fluorophore to a more flexible and mobile conformation that has less interaction with the active site environment. These changes seem to be brought about by water stripping effect and the movement of solvent molecules into the enzyme fold that brings about polarity changes in the active site. In the case of a bound substrate in the enzyme active site in an organic solvent, we believe that the first substrate conformation and active-site polarity (similar to that in aqueous solvent) favors catalysis more than the later conformation that results after prolonged exposure to the organic solvent. However, additional studies are needed to rule out other possible mechanisms and to unequivocally ascertain the contribution of enzyme dynamics, active-site polarity and substrate reorientation to the phenomenon of enzyme partial inactivation upon prolonged exposure to organic solvents.

Acknowledgements

The project described was supported by Grants P20 RR016470, S06 GM-08216, and GM-08102 from the National Center for Research Resources, and the National Institutes of Health (SCORE). The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Center for Research Resources or the National Institutes of Health. In addition, the authors would like to thank Dr. Carmelo Garcia and Dr. Roland Oyola from the University of Puerto Rico at Humacao for their assistance in the use of and for providing us access to the Fluorescence Spectrophotometer.

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