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Flexibility of Enzymes Suspended in Organic Solvents Probed by Time-Resolved Fluorescence Anisotropy. Evidence That Enzyme Activity and Enantioselectivity Are Directly Related to Enzyme Flexibility

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Abstract: A time-resolved fluorescence anisotropy study on the molecular flexibility of active-site labeled anthraniloyl- α -chymotrypsin, dansylsubtilisin Carlsberg, and native subtilisin Carlsberg, suspended in organic solvents, is described. The internal rotational mobility of the fluorophore in the nanosecond time range could be separated from rotation of enzyme aggregates and rapid energy transfer processes. The enzymes suspended in dry organic solvents are less flexible than when dissolved in water. The enzyme flexibility increased with increasing hydration level. The results confirm that the increase in enzyme activity observed upon addition of low amounts of extra water is related to an increase in enzyme flexibility. Differences in enantioselectivity of subtilisin Carlsberg in different organic solvents have been correlated with differences in enzyme flexibility. The relationship between the internal rotational mobility of the fluorophore and the enantioselectivity provides the first experimental evidence that enzyme flexibility and enzyme enantioselectivity are correlated.

Introduction

During the last decade, enzymatic catalysis under nonaqueous conditions has become a new important field in enzymology. In an organic reaction medium enzymes display several interesting properties like enhanced (thermal) stability and different substrate and stereospecificities.¹⁻³ In addition to their practical importance, these new reaction conditions offer the possibility

to study several mechanistic aspects of enzyme catalysis in more detail, like the role of the water shell surrounding the enzyme molecule. In organic solvents the enzyme is not completely hydrated.⁴ On the basis of studies with enzyme powders of different humidities that are equilibrated via the gas phase, less hydrated enzymes are expected to be more rigid.⁵⁻⁸ A decreased intramolecular mobility of enzymes in anhydrous organic solvents is often used to explain increased thermal stability and the preservation of conformations induced by high

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concentrations of ligands in aqueous solution (bioimprinting).⁹ Moreover, the lower activity of enzymes in anhydrous media might be the result of the restricted flexibility.¹⁰

Direct measurements of the flexibility of enzymes in organic solvents are rare and are based on ESR^{11–13} and solid state NMR¹⁴ studies. These studies suggest that enzymes suspended in dry organic solvents are more rigid than when dissolved in water.^{6–8} However, the relationship between higher enzyme activity and higher enzyme flexibility was observed in only one study.¹² In two other studies this relationship was absent.^{11,14} A correlation between different enzyme (enantio)selectivities, the reaction medium, and enzyme flexibility has not been reported.

In this paper, we show that time-resolved polarized fluorescence spectroscopy is a useful technique to study the flexibility of enzymes suspended in organic media. Both fluorescence technique and data analysis have now been developed to a level that important dynamic and structural information on enzymes can be obtained.¹⁵ In particular, time-resolved polarization anisotropy enables a detailed investigation of internal motional dynamics, with respect to overall protein tumbling on a (sub)-nanosecond time scale.¹⁶ The advantage of the polarized time-resolved fluorescence technique is its sensitivity combined with time-resolution in the picosecond–nanosecond time window. On this time scale the major dynamic processes in proteins take place, including conformational changes at room temperature. Time-resolved fluorescence anisotropy measurements have been used to obtain information on protein motion in homogeneous solution,¹⁷ associated with membranes,¹⁸ or in two-phase systems like reverse micelles.¹⁹ All these publications dealt with solution studies of proteins. The number of time-resolved polarized fluorescence studies with solid samples (powders, crystals, aggregates) is very scarce. There has been a report on α -chymotrypsin,²⁰ but in this study information was obtained from time-resolved tryptophan fluorescence only and not from fluorescence anisotropy.

In this investigation, the time-resolved fluorescence technique has been applied for the first time on enzymes *suspended* in organic solvents. In this complex system of protein aggregates,

the maximum entropy method (MEM) is the preferable approach in analyzing time-resolved fluorescence and anisotropy patterns.²¹

For the application of fluorescence spectroscopy on enzymes suspended in organic media, a suitable fluorophoric reporter group is needed. Besides probing intrinsic fluorophores like tryptophan or tyrosine, site-specific chemical modification of the enzyme with a fluorophore can be performed. In this way, the serine proteases α -chymotrypsin and subtilisin Carlsberg have been specifically labeled in the active site with anthranilic acid^{22,23} (**1**) and dansyl derivatives²⁴ (**2**), respectively. Both

reporter groups are highly fluorescent and show absorption and emission spectra distinct from the corresponding protease spectra. Therefore, these reporter groups can be specifically excited at a wavelength where no absorbance of protein residues (tryptophan and tyrosine) takes place. An additional advantage of using subtilisin Carlsberg is that this enzyme contains only one tryptophan residue which enables probing the enzyme dynamics at two distinctly different sites. Since α -chymotrypsin and subtilisin Carlsberg have been extensively studied in organic solvents, we have chosen these two enzymes to investigate the enzyme dynamics under anhydrous conditions.

A comparison of the correlation–time distribution spectra of the labeled enzymes recorded in aqueous and nonaqueous media will be presented. Subsequently, the effect of added extra water to the enzyme/organic solvent suspension on the enzyme flexibility will be discussed. The most important issue that we have addressed is the relation between the enantioselectivity of subtilisin Carlsberg and the organic solvent in relation to changes induced in enzyme flexibility.

Experimental Section

Subtilisin Carlsberg, dansyl fluoride, and *p*-nitrophenyl anthranilate were from Sigma. α -Chymotrypsin was from Boehringer. Enzymes (5 mg·mL⁻¹) were dissolved in 0.1 M KH₂PO₄ buffer pH 7.8 and lyophilized. α -Chymotrypsin and subtilisin Carlsberg were modified according to literature procedures.^{22,24} Unless stated otherwise, all experiments were performed with enzymes and organic solvents previously equilibrated separately above a saturated LiCl solution at 5 °C for at least 40 h.²⁵ THF was freshly distilled from sodium benzophenone ketyl. Measurements were performed in stoppered 1 cm quartz cuvetts (Hellma 111 QS) at 25 °C with suspensions of 1 mg·mL⁻¹ under continuous stirring. As a blank, native α -chymotrypsin and subtilisin Carlsberg were taken. In case of the tryptophan experiments, RNase (Type XII A, Sigma), an enzyme containing no tryptophan, was used as blank.

(21) In this non-a-priori approach the decay (either fluorescence or anisotropy) is fitted to an exponential series with evenly spaced decay components (either lifetimes τ or correlation times ϕ) on a log(τ or ϕ)-axis. By using the Shannon–Jaynes information entropy as a regularizing function in the MEM a solution is chosen with no more correlation than inherent in the experimental data (Livesey, A. K.; Brochon, J. C. *Biophys. J.* **1987**, 52, 693).

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Figure 1. Fluorescence decay (A) of dansylsubtilisin Carlsberg dissolved in aqueous buffer and the corresponding lifetime distribution pattern (B). Fluorescence anisotropy decay (C) and corresponding correlation-time distribution pattern (D).

The determination of the enantioselectivity of subtilisin Carlsberg ($1 \text{ mg} \cdot \text{mL}^{-1}$) toward N-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester (1 mM) in various solvents at 25°C was determined as described in detail elsewhere.²⁶ The enantioselectivities at low conversions were calculated using the formula given by Chen and Sih² after separation of the product enantiomers on a chiral GC column.

Polarized fluorescence decay curves were measured by the time-correlated single photon counting technique. The excitation source consisted of the frequency-doubled output of a cavity-dumped dye laser which was synchronously pumped by a mode-locked Antares 76-YLF laser (Coherent, Palo Alto, CA). The excitation pulse frequency was reduced to 951 kHz, the pulse duration was less than 4 ps, and the excitation light was vertically polarized. Further details are given by Pap et al.²⁷ For excitation at 295 nm (tryptophan in subtilisin Carlsberg), rhodamine 6G as laser dye was selected while for excitation at 345 nm (anthranoyl- α -chymotrypsin and dansylsubtilisin Carlsberg) DCM (Eastman Kodak) was chosen. Tryptophan emission was measured through a combination of a WG320 cutoff filter (Schott, Mainz, Germany) and a 349 nm interference filter (Schott). The instrumental response function, corresponding to the laser pulse convolution with the detection response, was determined by measuring the fluorescence decay of *p*-terphenyl (BDH, scintillation grade) in fluorescent grade ethanol (Merck) having a reference fluorescence lifetime of 1.05 ns.¹⁶ For anthranoyl- α -chymotrypsin fluorescence a combination of a KV380 cutoff filter (Schott) with a 402 nm interference filter (Baird Atomic) was selected while for dansylsubtilisin Carlsberg this combination consisted of KV380 cutoff and 498 nm (Balzers) interference filters. The reference compound was a solution of POPOP (Eastman Kodak) in fluorescent grade ethanol having a fluorescence lifetime of 1.35 ns.¹⁶ The fluorescence was acquired during 10 cycles of 10 s in each polarization direction. Background signal (a suspension of a similar amount of nonfluorescent protein, see above) was collected at one quarter of the sample acquisition time. One complete measurement consisted of measuring the polarized fluorescence decays of the reference compound, the sample, the background, and again the reference compound.

Data Analysis. The fluorescence anisotropy decay of fluorophores in proteins can be adequately described by a superposition of two

independent motions, namely a rapid internal motion of the fluorophore within the protein (characteristic time ϕ_{int}) and the much slower protein rotation (characteristic time ϕ_{prot}):

$$A(t) = [\beta_1 \exp(-t/\phi_{\text{int}}) + \beta_2] \exp(-t/\phi_{\text{prot}}) \quad (1)$$

Analysis of the total fluorescence decay and fluorescence anisotropy decay $A(t)$ was performed with the commercially available maximum entropy method (MEM, Maximum Entropy Data Consultants Ltd., Cambridge, U.K.). The principle of MEM has been described in the literature.^{21,28} For the analysis of the total fluorescence decay of the protein samples into a distribution of lifetimes τ with amplitudes α , 100 equally spaced values on a $\log(\tau)$ scale between 0.01 and 20 ns were used. The starting distribution model was chosen to be flat in $\log(\tau)$ space (all lifetimes have equal probability) since no *a priori* knowledge about the system was present. Similarly, in the (one-dimensional) anisotropy analysis a spectrum of amplitudes β against correlation times ϕ is obtained. In this case, the final image $\alpha(t)$ of the fluorescence decay was introduced as a fixed image in the analysis of the anisotropy decay. As a starting model, 100 equal spaced values on a $\log(\phi)$ scale (from 0.1 to 100 ns) were used in the analysis. Similar considerations regarding the starting model in the recovery of $\alpha(t)$ apply to the recovery of $\beta(\phi)$, i.e., in case no *a priori* knowledge of the distribution is present, one should start with a flat spectrum in the $\log(\phi)$ space. The integrated amplitude $\beta(\phi)$ corresponds to the initial anisotropy $A_{t=0}$.

In all cases studied the fitting parameter χ^2 was close to 1.0, and, in addition, the weighted residuals between experimental and calculated decay curves and the autocorrelation function of the residuals¹⁶ were randomly distributed around zero, indicating an optimal fit of the data. Because the MEM data analysis program does not provide a detailed error analysis we carried out in some cases a global analysis of the experimental data according to eq 1 in which the ϕ_{prot} was fixed to a very long correlation time ($1 \mu\text{s}$). Software from Global Unlimited (Urbana, IL) was used for this error analysis. The errors in the adjustable parameter ϕ_{int} were then determined at a 67% confidence interval according to a published procedure.²⁹ The quality of these fits were as good as for the MEM results.

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Results and Discussion

Time-Resolved Polarized Fluorescence of Subtilisin Carlsberg and α -Chymotrypsin in Water and Organic Media. **Subtilisin Carlsberg.** In Figure 1, the fluorescence decay and fluorescence anisotropy decay curves and the corresponding fluorescence-lifetime and correlation-time distribution patterns are shown of dansylsubtilisin dissolved in aqueous buffer (0.1 M KH_2PO_4 , pH 7.8).

Figure 1A shows the experimental total fluorescence decay of dansylsubtilisin Carlsberg in order to illustrate that the fluorescence intensity persists over at least 40 ns. Figure 1B is the representation in a distribution of lifetimes as observed by MEM. A major contribution centered at 13 ns and a minor component at 4 ns are evident. Figure 1C presents the initial part of the anisotropy decay, in which it can be immediately observed that a rapid decay component is superimposed on the slower decaying part of the anisotropy. The anisotropy (A) is constructed from the individual parallel- and perpendicular-polarized decay curves. The time axis is slightly shifted so that the onset of the anisotropy is seen. Figure 1D is the corresponding correlation-time distribution obtained by global analysis of the two polarized fluorescence intensity decays. The anisotropy (A), which is 0.24 at $t = 0$ ($A_{t=0}$), vanishes within 20 ns. In case of an isolated protein this decay of anisotropy is caused by two processes, viz. rotation of the whole labeled protein molecule (ϕ_{prot}) and internal rotation of the fluorophore relative to the enzyme molecule (ϕ_{int}). No contribution of a correlation-time at about 13 ns was observed in the corresponding correlation time distribution pattern (Figure 1D). This ϕ_{prot} value is expected for rotation of subtilisin Carlsberg dissolved in aqueous buffer according to the Stokes–Einstein relation. In contrast, the anisotropy decay is due to two contributions centered around 3.8 and <0.15 ns. The contribution at 3.8 ns must be due to mobility of a large protein segment carrying the fluorophore (ϕ_{int}) while the very short correlation time arises from rapid rotation of the dansyl group around its point of attachment.

Since an increase of the ratio of areas contributing to ϕ_{int} and ϕ_{prot} implies that the rotational space of the fluorophore becomes larger,³⁰ the absence of a ϕ_{prot} of 12–13 ns in Figure 1D indicates that the dansyl group possesses a large angle of displacement.^{30,31}

In Figure 2A the anisotropy decay curve is shown on an expanded scale of the same modified enzyme, but now suspended in cyclohexane. Compared with Figure 1C, the anisotropy, which is 0.23 at $t = 0$ ($A_{t=0}$), becomes almost constant after some initial decay.³² The corresponding rotational correlation-time distribution pattern, depicted in Figure 2B, shows two contributions responsible for the anisotropy decay, one centered around 8.8 ns of low amplitude and the other, major contribution with a ϕ of >30 ns. The long unresolved rotational correlation times are caused by slow rotation of the protein/buffer-salt aggregates being immobilized on the time scale of the experiment. Since ϕ increases linearly with the volume of

Figure 2. Fluorescence anisotropy decay of subtilisin Carlsberg suspended in cyclohexane (A) and the corresponding correlation-time distribution pattern (B).

the labeled protein aggregate, the long unresolved ϕ values shown in Figure 2B imply that large aggregates are present in the suspension. Of more interest in Figure 2B is the ϕ_{int} value centered around 8.8 ns. Compared with the situation in aqueous solution, ϕ_{int} is slower in cyclohexane. Moreover, due to the larger area of the signal of ϕ_{prot} at >30 ns than that of ϕ_{int} , it can be concluded that the rotational space of the dansyl group is significantly decreased when the enzyme is suspended in this organic solvent. Also, the rate of internal rotational diffusion is considerably smaller.

α -Chymotrypsin. The rotational correlation-time distribution pattern of anthraniloyl- α -chymotrypsin dissolved in aqueous solution showed only one signal at 12.6 ns, similar to the value reported by us earlier.³⁰ The virtual absence of an internal correlation time implies that the anthraniloyl group has no rotational mobility in the active site when the enzyme is dissolved in aqueous solution.³⁰ We observed anisotropy-decay curves and rotational correlation-time distribution patterns for anthraniloyl- α -chymotrypsin in organic solvents similar as for dansylsubtilisin Carlsberg (Figure 2).

The only discrepancy is that the initial anisotropy is lower ($A_{t=0} = 0.16$ – 0.22) than in aqueous solution ($A_{t=0} = 0.28$). This might be due to ultrarapid excitation energy transfer processes, in which the excitation energy of the anthraniloyl fluorophore is transferred to another anthraniloyl fluorophore.³³ Since the orientation of the transition dipole moment of this newly excited fluorophore is most likely different from that of its precursor, the light emitted by the second fluorophore has another, smaller polarization degree. Therefore, depolarization occurs which is not due to rotation of the fluorophore. High concentrations of fluorophores and consequently short distances increase the probability of this process, as might be the case in the enzyme aggregates studied. Since the anisotropy is already low at $t = 0$, this process must be very fast, in the range of a

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(32) Although the decrease in anisotropy is quite low, no fitting problems were encountered since a duplo experiment with a different batch of dansylsubtilisin Carlsberg resulted in exactly the same correlation-time distribution pattern as shown in Figure 2B.

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Figure 3. Internal rotational correlation-time (ϕ_{int}) of anthraniloyl- α -chymotrypsin, suspended in acetone/1 M 1-propanol, at various hydration levels.

few ps, and therefore independent of the depolarization process with the longer ϕ_{prot} and ϕ_{int} values.

In summary, the flexibility of labeled α -chymotrypsin and subtilisin Carlsberg in heterogeneous systems can be properly monitored by estimation of ϕ_{int} . Compared with a homogeneous solution, the results for the heterogeneous system are more difficult to interpret. Most likely, only labeled enzyme molecules at or located directly under the surface of the suspended aggregates are monitored. Light scattering, rapid depolarization due to energy transfer processes between fluorophores, or slow tumbling of the enzyme aggregates do not interfere with the ϕ_{int} contribution. In the following sections, changes in ϕ_{int} will be described when we change the reaction medium via the addition of extra water and by changing the organic solvent.

Effect of the Addition of Water on the Internal Mobility of the Fluorescent Probe. α -Chymotrypsin. We have investigated the relationship between the internal rotational diffusion correlation-time (ϕ_{int}) of anthraniloyl- α -chymotrypsin and the hydration level of the enzyme suspended in several organic solvents. Suspended in a mixture of acetone and 1 M 1-propanol, the ϕ_{int} decreases from 6.3 to 1.1 ns upon addition of 0.2–3% (v/v) of water (Figure 3).³⁴

The fluorescence lifetime distribution pattern, showing two main contributions centered around 5.3 and 8.4 ns when no extra water was present, did not markedly change upon the introduction of extra water.

When anthraniloyl- α -chymotrypsin was suspended in cyclohexane or in octane (1 M 1-propanol added), ϕ_{int} values of 4.0 and 4.8 ns were recorded. These values shifted to 3.3 and 3.7 ns, respectively, upon the addition of 0.1% (v/v) extra water.

From the results in acetone (Figure 3), cyclohexane, and octane it can be concluded that the ϕ_{int} of anthraniloyl- α -chymotrypsin is sensitive to changes in the reaction medium, like solvent structure and water content. A shorter ϕ_{int} implies that the internal rotational diffusion of the anthraniloyl moiety becomes less hindered by the active-site environment. This is due either to a higher flexibility or a rearrangement of the amino acid residues in the active site pocket. A higher mobility of

water molecules in the active site, which has been observed upon increasing the hydration level of protein powders,⁸ might also play a role. It is well documented that addition of small amounts of water to α -chymotrypsin suspended in dry organic solvents significantly increases the activity.⁴ This increase might be directly related to a higher enzyme flexibility and/or water mobility. Visser and co-workers³⁰ observed a comparable response of ϕ_{int} as shown in Figure 3 in this study on the effect of increased hydration of anthraniloyl- α -chymotrypsin encapsulated in reverse micelles. The highest value of ϕ_{int} observed (4 ns) gradually decreases to about 0.5 ns upon addition of extra water. Moreover, the pattern observed when the enzyme is suspended in organic solvents with the fluorescence lifetime distribution pattern of the enzyme in dry reverse micelles (one major contribution) shows great similarities. The higher ϕ_{int} and the more homogeneous lifetime distribution pattern compared to aqueous solution indicate that α -chymotrypsin is less flexible and exists in less different enzyme conformers as compared to the enzyme dissolved in aqueous solution.³⁵

Subtilisin Carlsberg. Compared with anthraniloyl- α -chymotrypsin,²² dansylsubtilisin is known to be more sensitive for deacylation of the fluorescent probe by water.³⁶ We observed upon addition of more than 0.5% (v/v) water to dansylsubtilisin Carlsberg suspended in dry THF an anisotropy pattern with a rapid decay followed by an increase of anisotropy with time. This complex pattern indicates that there are at least two populations of fluorophores.³⁷ In order to avoid this complex behavior, the mobility of the single tryptophan residue present in native subtilisin Carlsberg was used to monitor the changes in enzyme flexibility at various hydration levels. Up to 4.0% (v/v) of extra water was added to a suspension of native subtilisin Carlsberg in dry THF.

As shown in Figure 4, the anisotropy decay of the tryptophan is faster when the hydration level of the enzyme is increased. In addition, the anisotropy starts at a significantly lower level under more "dry" conditions. This again indicates a state of aggregation where an ultrarapid depolarization process like excitation energy transfer takes place. The addition of water causes two effects, viz. the initial anisotropy increases and the anisotropy decays faster. We can define a new parameter ΔA ($= A_{t=0} - A_{t=\infty}$) which accounts for these two effects. Both values ($A_{t=0}$ and $A_{t=\infty}$) are obtained from maximum entropy analysis of the fluorescence anisotropy decay (see Experimental Section).³⁸ The change in anisotropy decay ΔA plotted against the hydration level is given in Figure 5.

The net increase in ΔA implies that the enzyme becomes more flexible upon increase of the hydration level and also that the ultrarapid energy transfer between interprotein tryptophan residues diminishes.

As for α -chymotrypsin, the activity of subtilisin Carlsberg is known to increase upon introduction of small quantities of water to the organic solvent.^{4,12} Affleck et al.¹² estimated the activity of subtilisin Carlsberg in THF at various hydration levels. The published data show that the enzyme activity increases sharply upon the addition of 0.5% of water to dry THF/1 M 1-propanol

(34) The errors in the ϕ_{int} values are relatively large because the anisotropy decay from the internal motion is relatively small compared to the anisotropy due to immobilized protein aggregates, which remains constant (see, for example, Figure 2A, where the anisotropy decays from 0.23 to 0.20 and the latter values arises from immobilized protein). In the error analysis we have used the exhaustive search method (at 67% confidence level), described by Beecham et al. (ref 29), to recover the errors. It has been demonstrated by Beecham et al. that the errors obtained by this method are more realistic and larger than those obtained by linear approximation. It was also shown that the recovered error bars are asymmetric when the rotational correlation time becomes longer than the average fluorescence lifetime. This tendency is also illustrated in Figure 3: the positive confidence interval is very large while the negative confidence interval is still bounded.

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(38) The correlation-time distribution patterns were too complex to be useful, probably because of the complex photophysics of tryptophan.

Figure 4. Fluorescence anisotropy decay of subtilisin Carlsberg suspended in THF containing 0.1% (v/v) H₂O (A) and 4.1% H₂O (B).

Figure 5. Change in the initial fluorescence anisotropy minus the anisotropy at "infinite" time ΔA of the single tryptophan residue in subtilisin Carlsberg, suspended in THF, at various hydration levels.

but a further increase of the water content results in a lower enzyme activity. A hydration range of $>0.5\%$ corresponds with the region where we observe complex anisotropy patterns (vide supra). Local denaturation of the active site region resulting in a lower enzyme activity can be the origin for this observation because it is known that higher quantities of water in organic solvents destabilize the enzyme.^{12,39} However, the tryptophan data (Figures 4 and 5) show that the enzyme is not completely denatured at water concentrations $\geq 0.5\%$ (v/v) since in that case very fast depolarization of the fluorescence is expected. Interestingly, Affleck et al.¹² also found a sharp increase in mobility of an ESR probe, covalently linked in the active site of subtilisin Carlsberg, suspended in THF upon increase of the percentage of water. Both in this ESR study and in our study the ϕ_{int} becomes shorter upon an increase of the hydration level of the enzyme, an observation in line with previous studies on enzyme powders equilibrated via the gas phase at various hydration levels.⁶⁻⁸

Relationship between Flexibility and Enantioselectivity of Subtilisin Carlsberg Suspended in Various Organic Solvents.

An intriguing feature in nonaqueous enzymology is that the enzyme enantioselectivity depends on the type of organic solvent.⁴⁰ Although several hypotheses have been suggested in the literature, no general explanation for this phenomenon is available. For example, in one paper the enantioselectivity of subtilisin Carlsberg toward amino acid esters was reported to decrease with increased hydrophobicity of the reaction medium.⁴¹ The opposite relationship was observed when the enantioselectivity toward chiral nucleophiles, deacylating the enzyme, was investigated.⁴²

The observation that the flexibility of suspended enzymes in organic solvents, expressed in ϕ_{int} , changes upon changing the organic solvent, prompted us to look for a relationship between the enantioselectivity ($E = (k_{\text{cat}}/K_M)^L/(k_{\text{cat}}/K_M)^D$)⁴³ and the enzyme flexibility in different solvents. The ϕ_{int} of dansylsubtilisin Carlsberg was determined in various solvents and correlated with the enantioselectivity of the transesterification reaction of *N*-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester with 1-propanol (5 mM).⁴⁴ The recovered ϕ_{int} values have been plotted against the enantioselectivity expressed as $\Delta\Delta G^*$ derived from the relation $\Delta\Delta G^* = -RT \ln E$ (Figure 6A).

The results given in Figure 6A show that a shorter ϕ_{int} value correlates with a higher enantioselectivity of the enzyme in that solvent. Therefore, the enantioselectivity of subtilisin Carlsberg might indeed be controlled by the flexibility of the enzyme. Experimental evidence supporting this view has not been reported before for any enzyme. The quantitative relationship between enantioselectivity, expressed in $\Delta\Delta G^*$, and enzyme flexibility, expressed in ϕ_{int} , is not known. The same relationship as shown in Figure 6A is observed when our ϕ_{int} data are plotted against the enantioselectivities of subtilisin Carlsberg toward *N*-acetylalanine 2-chloroethyl ester in the same solvents containing 1 M 1-propanol, reported by Klivanov and co-workers⁴¹ (Figure 6B). The similarity of both figures suggests that the observed relationship does not depend on substrate structure or propanol concentration. Also, the fluorescence anisotropy decay of the single tryptophan residue of subtilisin Carlsberg was determined in various organic solvents. In this way, the flexibility of the enzyme can be determined at a distinct site.

Subtilisin Carlsberg suspended in dichloromethane, THF, or DMF gave anisotropy decay curves with ΔA values of 0.034, 0.051, and 0.055, respectively.⁴⁶ Since the enantioselectivity

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(44) This substrate was used because we observed a large dependence of the enzyme enantioselectivity on the reaction medium, and the enantioselectivities in all cases have values which could be determined accurately. It should be noted from the negative value of $\Delta\Delta G^*$ in Figure 6A that the enantioselectivity of subtilisin Carlsberg, suspended in cyclohexane, for the *N*-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester is inverted ($E = 0.37$).²⁶ A detailed analysis of the reaction conditions revealed that activation of the ester via introduction of fluoro atoms in the ethoxy group results in a lower $k_{\text{cat}}^L/k_{\text{cat}}^D$ ratio, while decreasing the propanol concentration from the commonly used 1 M^{41,45} to 5 mM especially decreased the K_M^D/K_M^L ratio. By using this approach, the enantioselectivity of three other serine proteases could be also inverted.²⁶

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(46) Suspending the native enzyme in acetone or 2-butanone resulted in quenching of the fluorescent signal, most likely due to a photochemical reaction between the excited tryptophan and the solvent, while an increase in anisotropy with time was observed when the enzyme was suspended in cyclohexane.

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Figure 6. (A) Relationship between the enantioselectivity ($\Delta\Delta G^\ddagger$) of subtilisin Carlsberg in reaction with *N*-acetyl-D,L-phenylalanine 2,2,2-trifluoroethyl ester and the internal rotational correlation time (ϕ_{int}) of dansylsubtilisin Carlsberg in various organic media at a fixed water activity.²⁵ Enantioselectivities have been determined in the various organic solvents containing only 5 mM 1-propanol at 25 °C. (B) Published⁴¹ enantioselectivities of subtilisin Carlsberg toward *N*-acetyl-alanine 2-chloroethyl ester in various solvents containing 1 M 1-propanol plotted against the ϕ_{int} values given in A. The standard errors, obtained from duplicate measurements, are in the range of 0.5–1 ns for correlation times > 1 ns (0.3 ns for correlation times < 1 ns).

of subtilisin Carlsberg increases in these three solvents in the same direction,⁴¹ the tryptophan depolarization data support our view that the enzyme becomes more enantioselective when the enzyme flexibility increases.

In computer simulations of the acylation of α -chymotrypsin by *N*-acetyl-D,L-tryptophanamide, Kollman and co-workers⁴⁷ noted that the enzyme only generates differences in binding

energy between the enantiomers if the active site residues can relax. When a rigid active site model was used, no discrimination between the enantiomers was calculated.

Conclusions

For the first time time-resolved fluorescence anisotropy techniques have been used to obtain information on the flexibility of α -chymotrypsin and subtilisin Carlsberg suspended in organic solvents. These techniques monitor the rotational mobility of a fluorophore in *suspended* enzymes. The internal diffusion correlation time ϕ_{int} decreases when the hydration level of the enzyme is increased.⁴⁸

The relationship observed for subtilisin Carlsberg that a shorter rotational correlation-time of the dansyl and tryptophan group correlates with a higher enzyme enantioselectivity might explain the relation between enzyme flexibility and enantioselectivity. A large enzyme flexibility, in the order of (sub)-nanoseconds, enables the rapid sampling of a large repertoire of enzyme conformations, and this enhances the probability of reaching a conformational state capable for binding and converting an enantiomeric substrate. Therefore, flexibility of the enzyme seems to be essential in order to maximize favorable interactions with the substrate in initial and transition state.⁵⁰ Since the largest number of these specific interactions are expected with the natural L-enantiomer⁵¹ an increase of flexibility of subtilisin Carlsberg will result in a higher activity toward the L-enantiomer.

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(48) Significant changes in ϕ_{int} were not observed when 18-C-6 is added to the reaction medium. This indicates that 18-C-6 does not induce changes in the enzyme flexibility on the nanosecond time scale. Therefore, the higher enzyme activity in organic solvents in the presence of crown ethers is most likely due to influencing enzyme parameter(s) other than the enzyme flexibility.^{26,45,49} The observation⁴⁵ that only the enzyme activity and not the enantioselectivity is significantly increased when 18-C-6 is present is in line with the conclusion that a higher enzyme flexibility correlates with a higher enantioselectivity.

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(51) Comparison of the enzyme activity in water and organic solvents shows that especially the activity toward the L-enantiomer is decreased in organic solvents.²⁶