Volume 236, number 2, 329-332 FEB 06242 August 1988

Circular dichroism and fluorescence of polyethylene glycol-subtilisin in organic solvents

P. Pasta, S. Riva and G. Carrea

Istituto di Chimica degli Ormoni, CNR, Via Mario Bianco 9, 20131 Milan, Italy

Received 29 June 1988; revised version received 12 July 1988

Subtilisin Carlsberg has been made soluble in organic solvents such as dioxane and acetonitrile by covalent linking to polyethylene glycol. Far-ultraviolet circular dichroism and intrinsic protein fluorescence have shown that subtilisin dissolved in dioxane, in which the enzyme is active and highly stable, maintains the native secondary structure as well as the native microenvironment for tyrosyl residues. In acetonitrile subtilisin undergoes conformational changes that cause enzyme inactivation and precipitation.

Subtilisin; Circular dichroism; Fluorescence spectroscopy; Organic solvent; Polyethylene glycol

1. INTRODUCTION

The use of hydrolytic enzymes in organic solvents for the specific synthesis of ester and peptide bonds has been steadily increasing [1–10]. Usually, reactions are carried out with vigorous stirring, the enzymes being suspended in the organic solvents in which they are not soluble. In a few cases, enzymes have been dissolved in organic solvents, in a transparent state, by covalent attachment to the amphipathic compound polyethylene glycol [8–10]. However, in spite of the great interest in enzyme catalysis in such unnatural media, we know of no spectroscopic investigations of the conformation of enzymes in organic solvents.

Recently, it has been demonstrated that subtilisin Carlsberg is catalytically active in a large number of anhydrous organic solvents, including several water-miscible solvents such as dioxane, dimethyl formamide, pyridine, acetone and acetonitrile [5–7]. Here, we have covalently linked polyethylene glycol to subtilisin and then investigated the conformation of the enzyme dissolved in dioxane and acetonitrile in a transparent state by means of far-ultraviolet circular dichroism (CD) and intrinsic protein fluorescence.

Correspondence address: G. Carrea, Istituto di Chimica degli Ormoni, CNR, Via Mario Bianco 9, 20131 Milan, Italy

2. MATERIALS AND METHODS

2.1. *Materials*

Crystalline subtilisin Carlsberg and methoxypolyethylene glycol-succinimidyl succinate (M_r 5000) were obtained from Sigma. 2,4,6-Trinitrobenzenesulfonic acid was purchased from Aldrich. All other reagents and chemicals were of analytical or spectrophotometric grade.

2.2. *Linking of polyethylene glycol to subtilisin*

To a solution of subtilisin (40 mg/4 ml) in 0.05 M sodium tetraborate buffer (pH 8), containing 0.2 mM $CaCl_2$, 800 mg of methoxypolyethylene glycol-succinimidyl succinate were added. The pH was readjusted to 8 with NaOH and the solution was incubated at 30°C for 6 h. To quench the residual activated polyethylene glycol, ethanolamine titrated to pH 8 was added (final concentration 0.1 M) and the solution incubated at 30°C for an additional 1 h. The solution was then gel filtered in the cold through a Sephadex G-100 column (2.5 × 90 cm) equilibrated with deionized water. The peak containing the modified enzyme was collected, 4 ml of 0.02 M sodium tetraborate buffer (pH 8) added and the whole lyophilized. The degree of enzyme modification was estimated by measuring the remaining free amino groups with 2,4,6-trinitrobenzenesulfonic acid [11]. The protein concentration was determined by amino acid analysis after hydrolysis with 6 M HCl.

2.3. *Enzyme activity*

The activity of subtilisin was determined spectrophotometrically (255 nm) at 25°C in 0.05 M sodium

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

tetraborate buffer (pH 8), containing 0.2 mM $CaCl_2$ and 1 mM *N*-α-benzoyl-L-arginine ethyl ester as the substrate. To determine the activity of subtilisin in dioxane, a trans-esterification reaction was utilized [7]. 1 ml dioxane containing trichloroethylbutyrate (0.1 M), *n*-butanol (0.4 M) and modified or unmodified enzyme (1 mg protein) was incubated with shaking at 45°C. The formation of butylbutyrate was monitored by GLC with a 5 m HP1 capillary silica gel column coated with methylsilicon gum (Hewlett-Packard).

2.4. *Spectroscopy*

CD spectra were recorded using a Jasco 500 A spectropolarimeter at 25°C. The concentration of subtilisin was 0.1 mg protein/ml, the optical path being 0.02 cm. Mean residue ellipticity values $[\theta]$ are expressed for all wavelengths as degree·cm^2·dmol^{-1} and were calculated from the equation $[\theta] = \theta_{obs} \times 99 \times 10^{-1} \times l^{-1} \times c^{-1}$, in which θ_{obs} is the ellipticity measured in degrees, 99 the mean residue molecular weight for the enzyme, c the protein concentration (in g/l) and l the light path (in cm). The intrinsic fluorescence of subtilisin (0.01 mg protein/ml) was recorded with a Jasco FP-550 spectrofluorimeter, at 25°C, after excitation of the samples at 280 nm.

3. RESULTS AND DISCUSSION

3.1. *Linking of polyethylene glycol to subtilisin*

A prerequisite for a reliable spectroscopic investigation of the conformation of subtilisin in organic solvents is that the enzyme should be soluble in these media. For this purpose, subtilisin was covalently linked to the amphipathic compound polyethylene glycol. Using an 11 M excess of the activated polymer over subtilisin amino groups, 47% of protein amino groups, which number 10 per molecule [12], were covalently linked to the polymer, as estimated from the trinitrobenzenesulfonic acid method. This means that approx. 5 polymer chains (M_r 5000 each) were bound per enzyme molecule (M_r 27000). The activity of the modified subtilisin was 71% that of the unmodified form when tested in an aqueous buffer with *N*-α-benzoyl-L-arginine ethyl ester as the substrate.

3.2. *Enzyme stability and activity*

The stability of the modified enzyme was compared to that of the native form in aqueous buffer and in solvents such as dioxane and acetonitrile which meet two essential requisites. First, they should be able to dissolve the modified enzyme (up to 1 mg protein/ml) and second, their spectroscopic characteristics must be compatible with far-ultraviolet CD and intrinsic protein fluorescence measurements.

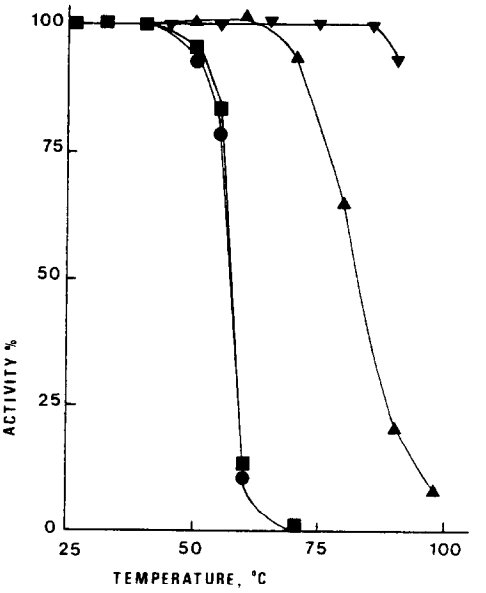

Fig.1. Inactivation of subtilisin as a function of temperature. Samples of native (■) and modified (●) subtilisin (0.5 mg protein/ml) were incubated in 0.05 M sodium tetraborate buffer (pH 8) for 15 min at the indicated temperatures. Aliquots were then withdrawn and their activity assayed spectrophotometrically at 25°C. Modified subtilisin was dissolved (0.5 mg protein/ml) in dioxane (▲) and treated as above. Samples of native subtilisin were suspended (0.5 mg protein/ml) in dioxane (▼) and incubated for 15 min at the indicated temperatures. The samples were then cooled and centrifuged, the supernatants discarded and the pellets dissolved in aqueous buffer and assayed spectrophotometrically for enzymatic activity.

Fig.1 shows the thermal inactivation of subtilisin. It can be seen that the modified and native enzymes have practically the same transition temperature in aqueous buffer whereas in dioxane the stability of the native enzyme is greater. It should be emphasized that the stabilities of both native and modified enzymes are greater in dioxane than in aqueous buffer. By contrast, the half-life of the modified enzyme was only 30 min in acetonitrile at 25°C and even shorter in dimethyl formamide, in which inactivation was almost immediate at 25°C. In these solvents as well, the stability of native subtilisin was greater (e.g. half-life of about 4 days in dimethylformamide, at 25°C). Therefore, the modification of subtilisin with polyethylene glycol decreases enzyme stability in organic solvents but not in aqueous buffer.

Table 1

Fluorescence of polyethylene glycol-subtilisin and *N*-acetyl-L-tyrosine ethyl ester[a]

Conditions	Polyethylene glycol-subtilisin		*N*-Acetyl-L-tyrosine ethyl ester	
	λ_{max} (nm)	Intensity(max) (relative)[b]	λ_{max} (nm)	Intensity(max) (relative)[b]
Buffer[c]	310	100	310	100
25% dioxane in buffer	310	104	310	185
50% dioxane in buffer	310	105	308	352
75% dioxane in buffer	310	101	306	500
Dioxane	310	98	305	1800
50% acetonitrile in buffer	310	148	308	268
Acetonitrile	310	228	305	1204

[a] Data represent mean for two determinations
[b] Relative to buffer taken as 100
[c] 0.05 M sodium tetraborate (pH 8)

The activity of modified subtilisin in dioxane, assayed by the trans-esterification reaction between trichloroethylbutyrate and *n*-butanol, was identical with that of native enzyme after correction for partial inactivation (29%) caused by covalent linking to polyethylene glycol.

3.3. *Spectroscopy*

The far-ultraviolet CD spectra of polyethylene glycol-subtilisin were recorded in aqueous buffer (0.05 M sodium tetraborate, pH 8) and in pure dioxane between 200 and 250 nm. Both spectra were practically coincident, indicating that the secondary structure of subtilisin is the same in buffer and dioxane. The mean residue ellipticities corresponding to the minima at 210 and 219 nm were -7900 and -7800 degree·cm^2·dmol^{-1}, respectively. Identical spectra were obtained in 75:25, 50:50 and 25:75 aqueous buffer-dioxane mixtures. Because of the poor transparency of dioxane below 210 nm, a cell of 0.02 cm path was used. It should be emphasized that modification of subtilisin with polyethylene glycol did not alter its CD spectrum in aqueous buffer.

When experiments were carried out in acetonitrile, the results were dependent on the time of incubation of modified enzyme in solvent. Solutions that were freshly prepared and rapidly analyzed (a few minutes) gave spectra fairly similar to those obtained in dioxane. However, the solutions became progressively turbid and after about 2 h almost no protein was detectable in solution. This denaturation-aggregation process is in agreement with the half-life of the activity in acetonitrile, namely 30 min.

The intrinsic fluorescence of modified enzyme was analyzed in aqueous buffer, dioxane and acetonitrile. Table 1 lists the wavelengths of the emission-curve maxima (λ_{max}) and the fluorescence intensities of polyethylene glycol-subtilisin and of the model compound *N*-acetyl-L-tyrosine ethyl ester. *N*-Acetyl-L-tyrosine ethyl ester was chosen as the model compound since subtilisin Carlsberg contains 13 tyrosyl residues and only 1 tryptophanyl residue [12]. It can be seen (table 1) that the λ_{max} and fluorescence intensity of subtilisin do not change on going from buffer to pure dioxane while for *N*-acetyl-L-tyrosine ethyl ester there is a blue-shift of λ_{max} and a very marked increase (up to 18-times) of the fluorescence intensity from buffer to dioxane. These data strongly suggest that in the enzyme the microenvironment of the tyrosyl residues remains the same when passing from buffer to dioxane and that there is no exposure of the aromatic fluorophores to the surrounding organic medium. In fact, the exposure of enzyme tyrosines to the solvent would cause a blue-shift of λ_{max} and an increase in fluorescence intensity similar to that of the reference compound. Instead, for enzyme freshly dissolved in acetonitrile and rapidly analyzed there is an increase in fluorescence intensity (table 1) which suggests that the enzyme undergoes changes in conformation that expose the tyrosyl residues to the organic medium. The enzyme then aggregates and precipitates. The precipitated enzyme did not dissolve in aqueous buffer and was inactive.

In conclusion, this spectroscopic investigation demonstrates that subtilisin dissolved in dioxane, in which the enzyme is active and highly stable, maintains the native secondary structure as well as the native microenvironment for tyrosyl residues. We hope that this study can contribute to the understanding of the behaviour of enzymes in organic solvents and prompt others to undertake further spectroscopic investigations.

Acknowledgement: We thank the Biotechnology Action Programme of the Commission of the European Communities for financial support of this work.

REFERENCES

[1] Klibanov, A.M. (1986) Chemtech 16, 354–359.
[2] Marlot, C., Lagrand, G., Triantaphylides, C. and Baratti, J. (1985) Biotechnol. Lett. 7, 647–650.
[3] Chen, C.-S., Wu, S.-H., Girdaukas, G. and Sih, C.J. (1987) J. Am. Chem. Soc. 109, 2812–2817.
[4] Sonnet, P.E. (1987) J. Org. Chem. 52, 3477–3479.
[5] Riva, S., Chopineau, J., Kieboom, A.P.G. and Klibanov, A.M. (1988) J. Am. Chem. Soc. 110, 584–589.
[6] Riva, S. and Klibanov, A.M. (1988) J. Am. Chem. Soc. 110, 3291–3295.
[7] Zaks, A. and Klibanov, A.M. (1988) J. Biol. Chem. 263, 3194–3201.
[8] Takahashi, K., Ajima, A., Yoshimoto, T., Okada, M., Matsushima, A., Tamaura, Y. and Inada, Y. (1985) J. Org. Chem. 50, 3414–3415.
[9] Inada, Y., Yoshimoto, T., Matsushima, A. and Saito, Y. (1986) Trends Biotechnol. 4, 68–73.
[10] Gaertner, H.F. and Puigserver, A.J. (1988) Protein 3, 130–137.
[11] Snyder, S.L. and Sobocinski, P.Z. (1975) Anal. Biochem. 64, 284–288.
[12] Ottesen, M. and Svendsen, J. (1970) Methods Enzymol. 19, 199–215.