

Effects of pH and urea on the conformational properties of subtilisin DY

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Subtilisin DY is very resistant to the denaturing action of urea: the conformational properties are not affected up to 4.5 M-urea, and even in the presence of 8 M-urea there is only a slow loss of ordered structure and caseinolytic activity. C.d. and fluorescence-emission studies also show that this proteinase is stable in the 5.5–10.0 pH range, whereas below pH 5.5 a sharp denaturation occurs that is complete at pH 4.5. Protein denaturation leads to a change of the emission quantum yield; in particular, in the native protein, indole fluorescence is quenched by some amino groups. Moreover, subtilisin DY possesses two classes of tyrosine residues: one class of exposed residues titrates normally, with $pK_{app.} = 10.24$, whereas one class of partially buried or hydrogen-bonded residues ionizes with $pK_{app.} = 11.58$. In general, such conformational properties resemble those of other subtilisins. However, some differences occur: e.g., subtilisin DY is less stable at acidic pH values and its tyrosine residues are more accessible to the solvent. Such differences are probably due to small variations of the three-dimensional structure; e.g., subtilisin DY has a slightly lower α -helix content.

Extracellular alkaline proteinases of bacterial origin differ from most globular proteins in displaying unusual conformational stability in the presence of both hydrogen-bond-breaking and hydrophobic-weakening agents such as urea, guanidine and 50% ethanol (Gounaris & Ottesen, 1965; Brown & Schleich, 1975). According to Svendsen (1976), both types of forces play an important role in stabilizing the native protein structure. However, there are some contrasting reports in the literature. Thus Stauffer & Sullivan (1971) stated that subtilisin Carlsberg is not denatured by 10 M-urea or 6 M-guanidine, whereas Markland (1969) and Brown & Schleich (1975) observed irreversible time-dependent conformational changes when the same protein is incubated with 5 M-guanidine. Such discrepancies may be partially due to the use of different types of enzymes (e.g. native or blocked at the active site) by the various authors as well as to the lack of kinetic analysis of the denaturation process. Moreover, some experiments (Herskovits & Fuchs, 1972) have been performed at acidic pH, where subtilisins are unstable even in the absence of denaturing agents.

In the preceding paper (Genov *et al.*, 1982) we showed that subtilisin DY displays several analogies with other subtilisins, in particular with subtilisin Carlsberg. It was therefore decided to investigate the effect of some denaturing agents on subtilisin DY, in order to compare its behaviour with that observed for other subtilisins. Studies at alkaline pH values were performed with Pms-subtilisin DY to avoid the fast autolysis that occurs with the native protein. On the other hand, Pms-subtilisins are unstable in the acidic pH region.

Materials and methods

The preparation and characterization of subtilisin DY, as well as of its Pms derivative, were described in the preceding paper (Genov *et al.*, 1982). Urea and guanidinium chloride were obtained from Riedel-De Haën (Hannover, West Germany) and were recrystallized from 100% ethanol. Methylammonium chloride was a Merck (Darmstadt, West Germany) product.

For studies at different pH values, the following buffer systems containing 0.2 M-KCl were used: 0.1 M-sodium citrate in the pH range 2.0–5.0; 0.1 M-Tris/HCl in the pH range 5.0–9.0; 0.1 M-sodium carbonate/bicarbonate at higher pH values.

Abbreviations used: Pms, phenylmethanesulphonyl; AcTrpNH₂, *N*-acetyl-L-tryptophan amide.

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pH values above 12.5 were obtained by addition of suitable amounts of KOH. The concentration of native and Pms-subtilisin DY was determined spectrophotometrically by using $\epsilon_M = 2.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm.

C.d. and fluorescence spectra were obtained as outlined in the preceding paper (Genov *et al.*, 1982). Spectrophotometric titrations of tyrosine residues were performed as described by Genov (1975), with a protein solution at pH 7.0 as a reference. Spectral measurements were made immediately after adjustment of the pH to the desired value, as well as after 4 h to allow a complete equilibration of the system. The pK values of the ionizable tyrosine residues were determined by the equation (Tachibana & Murachi, 1966):

$$[\text{H}^+] \Delta \epsilon = \Delta \epsilon_{\text{max}} \cdot K - \Delta \epsilon \cdot K$$

where $\Delta \epsilon_{\text{max}}$ is the difference in ϵ_M after complete tyrosine ionization, K is the apparent dissociation constant and $\Delta \epsilon$ is the difference in ϵ_M at a given pH value. The experimental results were treated by the method of least squares. In view of the similarity of the amino acid compositions, the isoelectric point of subtilisin DY should be close to that of subtilisin Carlsberg, i.e. 9.4 (Markland *et al.*, 1972); thus the electrostatic interaction factor will be small in moderately alkaline media. The number of ionized tyrosine residues was calculated by using $\Delta \epsilon_{295} = 2.33 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the ionization of a single phenolic group (Tachibana & Murachi, 1966).

The caseinolytic activity of subtilisin DY was assayed by the method of Kunitz (1935).

Results

The tryptophan and tyrosine emission intensity of subtilisin DY is essentially constant in the pH 5.5–8.0 region (Figs. 1 and 2). Below pH 5.5 the tryptophan quantum yield increases by about 60% whereas the tyrosine quantum yield appreciably decreases; this causes a shift of the emission λ_{max} to about 320 nm when the protein is excited at 275 nm. Therefore the tyrosine contribution can be obtained only by difference spectra (Genov *et al.*, 1982). As shown in Fig. 3, where the pH-dependence of the protein ellipticity at 220 nm is reported, a denaturation process occurs below pH 5.5, but the native conformation is preserved in the pH 5.5–10.5 range. All the readings reported in Figs. 1 and 2 were taken after 24 h incubation at each pH, since the changes of the tryptophan fluorescence were time-dependent.

From the c.d. spectra of subtilisin DY in the neutral pH region, a content of α -helix of 21% and of β -structure of 23% was estimated (Chen *et al.*, 1974). The validity of our procedure was checked by repeating the same calculations for three other subtilisins (Table 1). The α -helix value

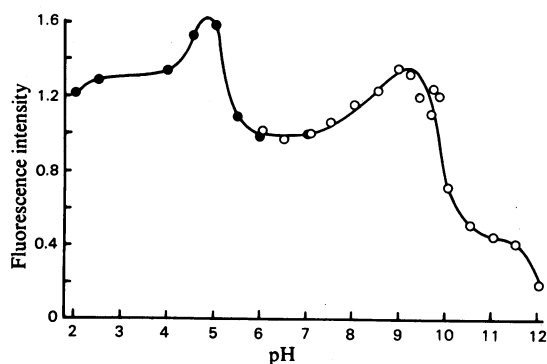


Fig. 1. pH titration of the tryptophan fluorescence emission of subtilisin DY after 24 h incubation of the protein at each pH

All intensity values are normalized to that at pH 7.0 for both native subtilisin DY (●) and Pms-subtilisin DY (○). For details see the text.

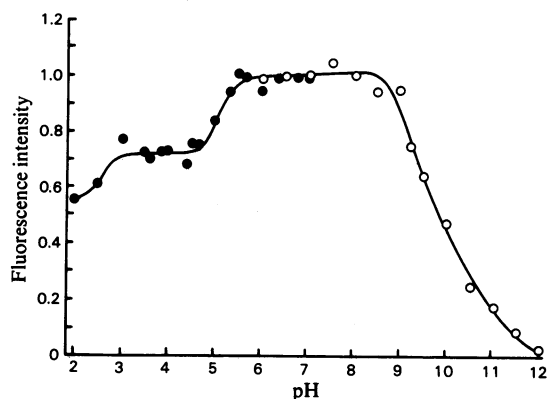


Fig. 2. pH titration of the tyrosine fluorescence emission of subtilisin DY

Tyrosine fluorescence was obtained as the difference between the 275 nm- and 300 nm-excited spectra after normalization at 400 nm. Experimental conditions and symbols were as indicated in Fig. 1.

Table 1. α -Helix and β -structure contents of some subtilisins

For details see the text.

Enzyme	α -Helix content (%)	β -Structure content (%)
Subtilisin DY	21	23
Subtilisin Novo	31	19
Subtilisin Carlsberg	26	15
Mesenterico-peptidase	26	21

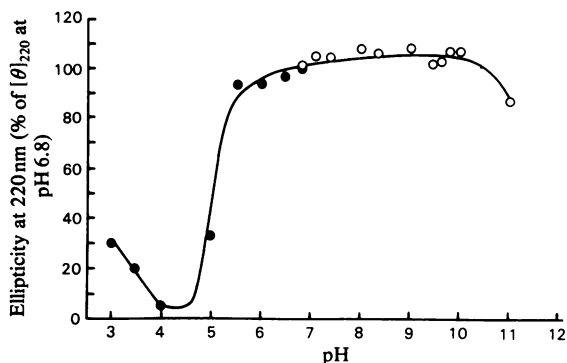


Fig. 3. pH-dependence of the ellipticity at 220 nm of native subtilisin DY (●) and Pms-subtilisin DY (○) after 24 h incubation of the protein at each pH. The ellipticity at pH 6.8 is taken as 100%. For details see the text.

of 31% for subtilisin Novo, which is identical with subtilisin BPN', agrees with the value obtained from the X-ray model of the latter proteinase (Wright *et al.*, 1969).

The pH titration curves of Pms-subtilisin DY, obtained by difference absorption spectroscopy, are shown in Fig. 4. In the pH 8.5–11.3 range, where the ionization is time-independent, 8 phenolic groups per molecule of protein are ionized, with a $pK_{app.}$ of 10.24. Above pH 11.3, where the tyrosine ionization is time-dependent, 5–6 residues/molecule are ionized, with a $pK_{app.}$ of 11.58. The reverse titration curve (curve E), obtained after 2 h exposure of Pms-subtilisin DY at pH 13.6, gives a $pK_{app.}$ of 10.37. pH-induced denaturation of the protein is irreversible and normalizes the ionization of all tyrosine residues in subtilisin DY.

Exposure of Pms-subtilisin DY to urea concentrations up to 8 M induces a time-dependent enhancement of the tryptophan quantum yield, whereas the tyrosine quantum yield is unchanged (Fig. 5). A value $Q_{Trp} = 0.124$ is obtained after 2 days' incubation in 8 M-urea, which is almost exactly the quantum yield value for AcTrpNH₂ in the same medium. Addition of methylamine (50 mM) diminishes the denaturing action of urea (Fig. 5), probably owing to reaction with cyanate impurities formed in urea solutions (Stark *et al.*, 1960). Cyanate readily modifies the free amino groups of proteins, thus enhancing the denaturing action of urea. In fact, treatment of Pms-enzyme with 20 mM-cyanate (i.e. the equilibrium concentration of cyanate in 8 M-urea) causes a 1.6-fold increase of Q_{Trp} . The magnitude of the process is comparable with that observed in 8 M-urea. A similar differential effect whether or

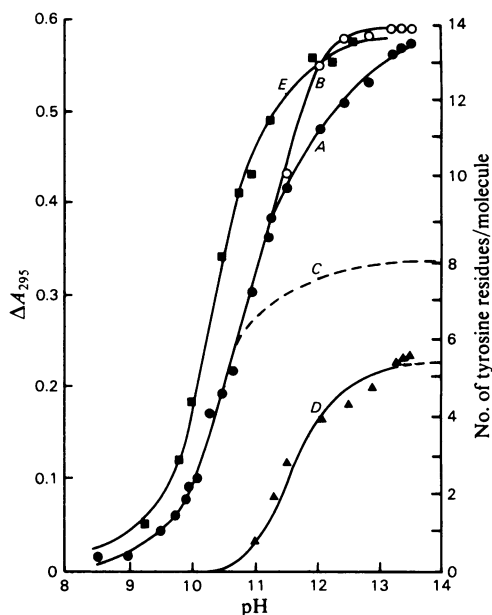


Fig. 4. Spectrophotometric titration of the tyrosine residues of subtilisin DY.

Curve A, forward titration after 1 min exposure to alkali (●); curve B, forward titration after 4 h exposure to alkali (○); curve C, theoretical ionization curve for the case of 8 tyrosine residues/protein molecule with $pK_{app.} = 10.24$; curve D, calculated difference plot between curves A and C (▲); curve E, reverse titration after 2 h exposure to pH 13.6 (■). For details see the text.

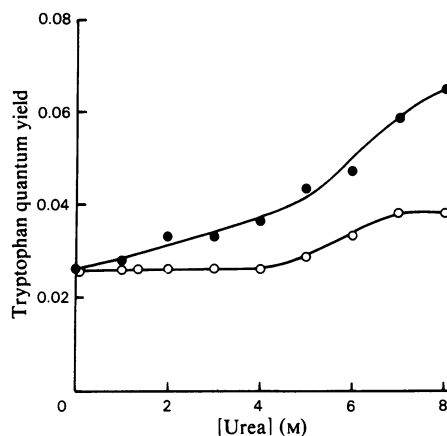


Fig. 5. Urea-concentration-dependence of tryptophan quantum yield in Pms-subtilisin DY in the presence (○) and in the absence (●) of 50 mM-methylamine in 0.1 M-Tris/HCl buffer, pH 7.0, containing 0.2 M-KCl. All data were taken with 300 nm excitation after 24 h incubation of the protein at each urea concentration. For details see the text.

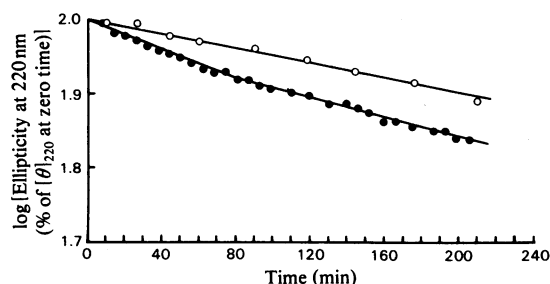


Fig. 6. Time course of 8 M-urea-induced denaturation of Pms-subtilisin DY in the presence (○) and in the absence (●) of 50 mM-methylamine, as monitored by the changes of the ellipticity at 220 nm

Spectra were taken in 0.1 M-Tris/HCl buffer, pH 7.0, containing 0.2 M-KCl. For details see the text.

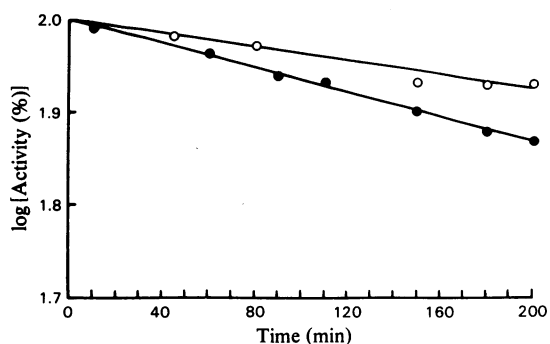


Fig. 7. Time-dependence of the caseinolytic activity of native subtilisin DY incubated with 8 M- or 9.5 M-urea. Conditions and symbols were as indicated in Fig. 6 legend.

not methylamine is present is shown by the action of urea on 220 molar ellipticity (Fig. 6) and caseinolytic activity (Fig. 7) of subtilisin DY. The process of the protein denaturation is very slow.

Discussion

Subtilisin DY resembles most other subtilisins in being very resistant to denaturation by various agents (see the introduction). Up to 4.5 M-urea, no effect on the subtilisin DY conformation is detectable, as shown by fluorescence and c.d. measurements. Even in the presence of higher urea concentrations, only relatively small and slow changes of the protein conformation take place (Figs. 5, 6 and 7).

The diminished action of urea in the presence of methylamine leads us to some interesting considera-

tions on the tryptophan microenvironment. Methylamine exerts no direct influence on the overall protein conformation, as shown by the identity of c.d. and fluorescence spectra of subtilisin DY whether or not methylamine is present. It is known that only alkylammonium salts bulkier than tetramethylammonium exert appreciable effects on protein molecules, acting as denaturing agents (Von Hippel & Wong, 1965). Rather, the present findings indicate that some amino groups act as strong quenchers of tryptophan emission. This hypothesis is supported by the pH-dependency of tryptophan emission intensity (Fig. 1) in the alkaline pH region: the increase of tryptophan fluorescence emission at pH values above 7 can be accounted for by the titration of an ionizable group with pK 8.1, which is within the ionization region of the α -amino group. The quenching of tryptophan emission by positively charged amino groups has been previously demonstrated for simple tryptophan-containing peptides (Edelhoch *et al.*, 1967). However, fluorescence-quenching studies with ionic species (Genov *et al.*, 1982) do not support the presence of positive groups adjacent to the tryptophan residue. Alternatively, the quenching process may involve electron transfer from the first excited singlet state of the tryptophan residue to the α -amino group or to an ϵ -amino group with abnormal pK , which can occur over appreciable distances (Grossweiner, 1977). On the other hand, c.d. spectra rule out the possibility that the changes in fluorescence intensity at moderately alkaline pH reflect large conformational changes of the protein, since the latter appears to be conformationally stable at least up to pH 10.0. Moreover, the tryptophan fluorescence enhancement of subtilisin DY in the acidic pH region where denaturation occurs (Figs. 1 and 2) is analogous to that observed on urea-induced denaturation (Fig. 5). Therefore our hypothesis that the abnormally low Q_{Trp} in native subtilisin DY is to be ascribed to conformation-dependent quenching processes appears to be justified.

As regards the pH-induced conformational changes, subtilisin DY differs from subtilisins Novo and Carlsberg, which were found to be stable down to pH 4 (Brown & Schleich, 1977).

The decrease in tryptophan fluorescence emission at pH above 9.0 is due to the formation of non-fluorescent tyrosinate residues (Figs. 2 and 4), thus allowing the occurrence of tryptophan-to-tyrosinate energy transfer (Weinryb & Steiner, 1971). This process may be facilitated by the occurrence of some conformational rearrangement bringing the aromatic donor and acceptor moieties into favourable distances and/or orientations. In fact, c.d. spectra (not shown) indicate small changes in the band shape at pH above 9.0.

On the basis of their titration behaviour, the 14 tyrosine residues of subtilisin DY can be grouped

into two classes. Eight residues titrate normally, with a pK_{app} of 10.24, which is close to the value for tyrosine in aqueous solutions. Since the ionic state of the titratable groups depends on the protein environment, the 8 tyrosine residues must be exposed on the protein surface and be fully accessible to the solvent. The remaining 6 phenolic groups ionize with a pK_{app} of 11.58, which suggests that they are buried to some extent in the internal regions of subtilisin DY or participate in hydrogen bonds. In the former case, the homogeneity of tyrosine fluorescence (Genov *et al.*, 1982) can be a consequence of internal-to-external tyrosine energy transfer. Participation of internal tyrosine residues in hydrogen-bonding, which renders them non-fluorescent (Cowgill, 1976), still restricts the fluorescence emission to external residues. The absence of tyrosine environmental heterogeneity, as deduced by fluorescence parameters, has been observed also for subtilisin Carlsberg (Brown *et al.*, 1977).

Although subtilisin DY shows strong analogies with other subtilisins (Genov *et al.*, 1982), some differences have been found. Thus this proteinase possesses the largest number of normally ionizing tyrosine residues and contains no phenolic groups titratable with a pK_{app} higher than 12.5 (Table 2). Therefore tyrosine residues in this enzyme are more accessible to the solvent than are the corresponding residues in subtilisin BPN', subtilisin Carlsberg and mesenterico-peptidase. The somewhat different

location in the protein molecule of tyrosine residues could explain the different tyrosine quantum yields found for subtilisins Carlsberg and DY (Genov *et al.*, 1982). Such differences in the microenvironment of tyrosine residues as well as the lower stability to acidic pH probably arises from local alterations of the three-dimensional structure, as exemplified by the lower content of α -helix for subtilisin DY with respect to other subtilisins.

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Table 2. Spectrophotometric titration data for some subtilisins

For details see the text.

Enzyme	No. of tyrosine residues/molecule	pK_{app}
Subtilisin DY	8.1	10.24
(present work)	5.5	11.58
Subtilisin BPN'	5.3	9.74
(Markland, 1969)	2.6	11.25
	2.0	12.50
Subtilisin Carlsberg	6.4	9.92
(Markland, 1969)	3.6	11.61
	3.0	12.50
Mesenterico-peptidase	6.8	9.92
(Genov, 1975)	4.5	11.96
	1.7	13.00