BBA 68276

RESOLUTION OF INDEPENDENTLY TITRATING SPECTRAL COMPONENTS IN THE ULTRAVIOLET CIRCULAR DICHROISM OF SUBTILISIN ENZYMES BY MATRIX RANK ANALYSIS

MICHAEL F. BROWN * and THOMAS SCHLEICH **

Division of Natural Sciences, University of California, Santa Cruz, Calif. 95064 (U.S.A.)

(Received February 14th, 1977)

Summary

The ultraviolet circular dichroism of di-isopropylphophoryl-subtilisins Carlsberg and Novo (EC 3.4.21.14) has been examined as a function of pH. The CD of these enzymes below 260 nm is invariant over the pH interval 4 to 12, below or above which spectral changes occur suggesting a transition to a random coil form. Above pH 8 contributions due to the ionization of tyrosyl residues appear in the CD above 260 nm as bands shifted to longer wavelengths. Three independently titratable components, obtained by matrix rank analysis, account for the observed CD spectral changes above 260 nm of Dip-subtilisin Carlsberg in the pH interval 8 to 12. By contrast, two components were derived for the Novo enzyme. The identities of the matrix rank components were surmised from their apparent pK_a values. One component of both subtilisin enzymes corresponds to the CD of the "buried" or irreversibly titratable tyrosyl residues of the enzyme. The other matrix rank components correspond to the CD of the "exposed" or freely ionizable tyrosyl residues. These residues are optically active only in the ionized state. Two types of "exposed" tyrosyl residues, arising because of differing sensitivity to the ionization of the "partially buried" or abnormally titrating tyrosyl residues, are evident in Dip-subtilisin Carlsberg. A pH-induced local conformational change in this enzyme is proposed to account for this behavior. The "partially buried" tyrosyl residues of both subtilisins appear to be devoid of optical activity in either the tyrosyl or tyrosylate form.

Introduction

The three dimensional structure of subtilisin Novo (EC 3.4.21.14) is known in considerable detail from high resolution X-ray studies [1,2]. Yet relatively little is known about the conformational details of subtilisin enzymes in solution and the X-ray structure of subtilisin Carlsberg (EC 3.4.21.14) has not been elucidated. With the exception of a single conservative substitution all of the 84 amino acid substitutions (30% of the total sequence) existing between subtilisins Novo and Carlsberg occur on the solvent accesible exterior of the Novo structure; hence, the conformations of these enzymes are believed to be very similar [1,2]. This viewpoint is further supported by CD measurements taken below 260 nm and secondary structure preductions, which suggest identical secondary structures for the subtilisin enzymes, except for the sequence region containing the Pro-56 deletion in subtilisin Carlsberg [3]. By contrast, significant differences exist in the CD of these enzymes above 260 nm which imply differences in the conformations or environments of the aromatic residues. In order to investigate the identity of the optically active aromatic residues the CD was perturbed by changes in pH and decomposed into independently titrating spectral components by matrix rank analysis. The differences in aromatic residue ionization behavior in subtilisins Carlsberg and Novo and the low and high pH induced denaturation of these enzymes are discussed in terms of conformational homology and the X-ray structure of Dip-subtilisin Novo.

Materials and Methods

Crystalline subtilisins Carlsberg (lot no. 70-3) and Novo (lot no. 120-2) were obtained from Novo Industri, Copenhagen, Denmark, inactivated by reaction with Dip-F and purified as described previously [4]. Sodium acetate and glycine-NaOH buffers (0.06 M) were employed in the pH intervals 4—7 and 7—12, respectively. pH measurements were made in the CD cells immediately before and after recording spectra using a combination microelectrode (Instrumentation Laboratory, Inc., Lexington, Mass.) and a Radiometer Model 26 pH meter. CD spectra were recorded using a Durrum-Jasco J-20 spectropolarimeter at an ambient temperature of 22—23°C as described previously [4] and are reported in terms of mean residual molar ellipticity (deg cm^2 dmol^{-1}). Protein concentrations were ca. 0.1 and 2 mg/ml for measurements made in the far and near ultraviolet spectral regions, respectively. Cells of path lengths 0.1 and 1.0 cm were used. The maximum absorbance did not exceed 1.5. All the reported spectra represent the average of at least two scans. Spectra were digitized manually at 1 nm intervals and smoothed using a nine point cubic convolution least squares program [5]. Matrix rank analysis was performed using a modification of a published algorithm [6]. Linear regression fits of the matrix rank components to the experimental spectra were computed using a modification of the program REGRES [7].

Results

pH dependence of Dip-subtilisin Carlsberg CD spectra. The CD of Dip-subtilisin Carlsberg above 260 nm is insensitive to changes in pH in the interval

4 to 8, above which contributions from ionized tyrosyl residues are apparent. By contrast, the CD below 260 nm of this enzyme is invariant over the pH range 4—12. The changes that occur in the CD of Dip-subtilisin Carlsberg below pH 4 are shown in Fig. 1A. Since the spectra exhibit a slow time dependence, over a period of hours, the observed changes do not represent equilibrium behavior. In such cases spectra were recorded 30—45 min after solution preparation, before the occurrence of significant time dependence changes [4]. The native structure is replaced at low pH by a form exhibiting a single minimum at ca. 198 nm, with a broad shoulder extending to longer wavelength. An isosbestic point at 206—208 nm is evident, suggesting the occurrence of a transition between two independent spectral forms. The CD above 260 nm displays a proportional loss of optical activity at all wavelengths at pH values less than 4. The low pH induced changes in the optical activity of Dip-subtilisin Carlsberg are partially reversible.

Changes are also evident in the CD of Dip-subtilisin Carlsberg at pH values greater than 12, as shown in Fig. 1B. The shape changes are similar to those ob-
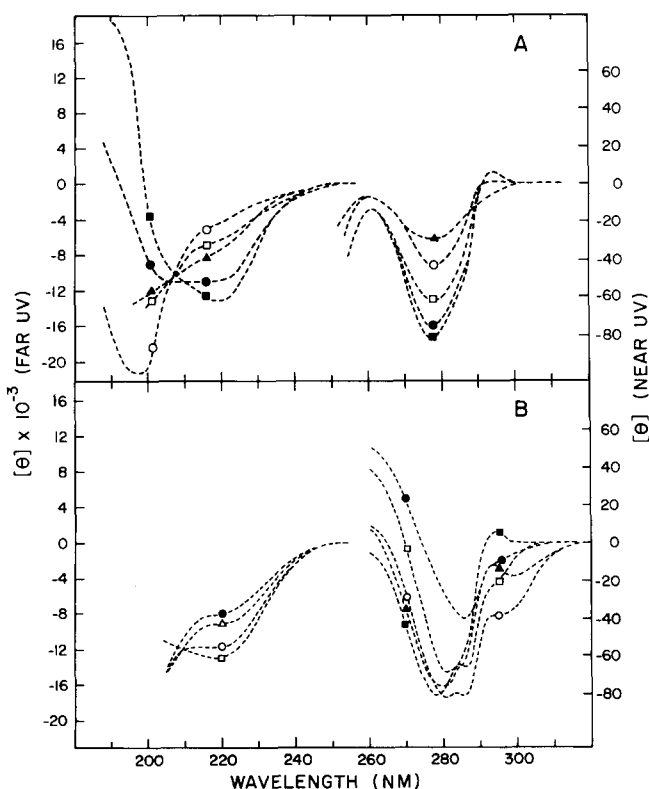

Fig. 1. A. Changes in the CD of Dip-subtilisin Carlsberg induced by decreasing pH. Far ultraviolet (UV) spectral region: pH 4—7, (■); pH 3.25, (●); pH 2.79, (□); pH 2.69, (○); pH 1.69, (▲). Near ultraviolet spectral region: pH 4—7, (■); pH 3.69, (●); pH 3.08, (□); pH 2.70, (○); pH 2.14, (▲). B. Effect of increasing pH on the CD of Dip-subtilisin Carlsberg. Far ultraviolet spectral region: pH 7—12, (□); pH 12.20, (○); pH 12.49, (△); pH 12.87, (●). Near ultraviolet spectral region: pH 7.0, (■); pH 9.08, (▲); pH 10.58, (○); pH 12.17, (□); pH 12.90, (●).

served below pH 4, with an isosbestic point at 206–208 nm. A diminution in optical activity is apparent at all wavelengths above 260 nm at pH values greater than 12. The high pH induced transition is time dependent and, in con-
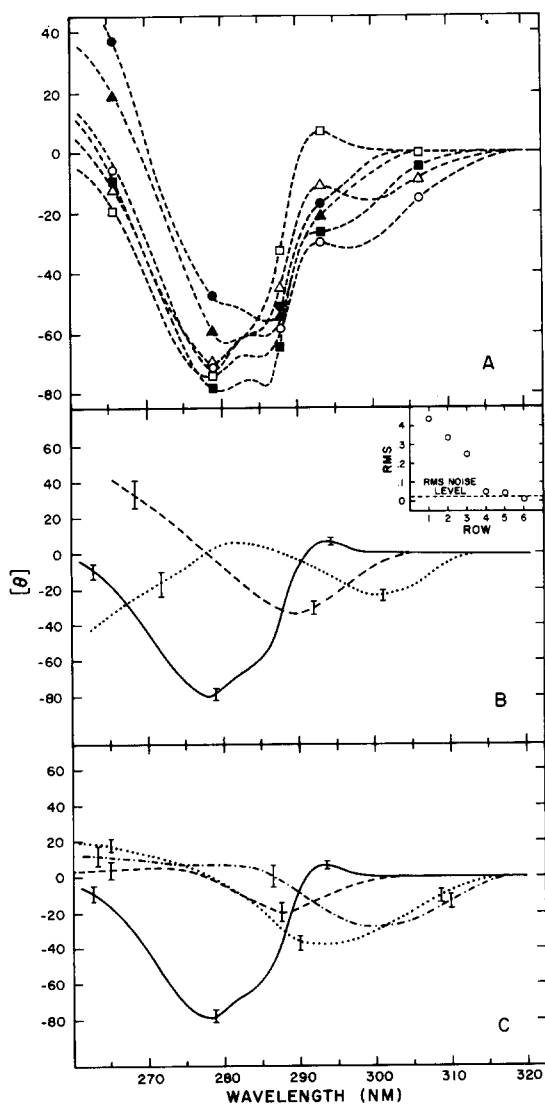

Fig. 2. A. The CD of Dip-subtilisin Carlsberg vs. pH. pH 7.85, (□); pH 9.08, (△); pH 9.88, (○); pH 11.38, (■); pH 12.17, (▲); pH 12.51, (●). B. Independently varying spectral components resolved from the spectra shown in Panel A by matrix rank analysis. The first basis spectrum is at pH 7.85, normalized at 278 nm; the second basis spectrum is at pH 12.17 normalized at 290 nm. Components are numbers in order of their derivation in the rank analysis: component 1 (———); component 2, (- - - - -); component 3, (· · · ·). The RMS of each spectrum in the reduced matrix is shown in the inset. The RMS noise level of the experimental spectra is indicated by the dashed line. C. Independently varying spectral components resolved from the spectra shown in Panel A by matrix rank analysis. The first basis spectrum is at pH 7.85, normalized at 278 nm; the second basis spectrum was derived from the pH 9.88 spectrum, normalized at 302 nm. Components are numbered in order of their derivation in the rank analysis: component 1, ———; component 2, · · · ·; component 3, - - - - - -. The difference between components 2 and 3, normalized at 288 nm, is shown as the fourth spectrum — · — ·.

trast to the low pH induced transition, completely irreversible.

Matrix rank analysis of Dip-subtilisin Carlsberg CD changes. Titration of the tyrosyl residues of Dip-subtilisin Carlsberg results in the appearance of new spectral bands shifted to longer wavelength with respect to the neutral pH spectrum. Representative spectral changes are shown in Fig. 2A. In the pH interval 8—10.5 CD bands appear in the wavelength region 295—305 nm and the minimum at 278 nm in the neutral pH spectrum is displaced to the red and split into two components centered at 281 and 286 nm. These minima persist to pH 12, as does the small positive band at 293 nm. The optical activity at 300 nm does not further increase above pH 10.5, but instead diminishes almost to zero as the pH is increased. These CD changes are reversible to pH values as high as 12, above which the previously mentioned irreversible changes become apparent.

The complex band shifts associated with increasing pH in the CD of Dip-subtilisin Carlsberg above 260 nm and the absence of isosbestic points suggests the presence of a number of intermediate species exhibiting characteristic CD bands. We analyzed the CD shape changes induced by variations in pH using matrix rank analysis [3,6,8,9] to obtain the number and spectral shape of the independently titrating components. Alternative procedures to matrix rank analysis have been described by Magar [9]. In the matrix rank method a set of experimental spectra that exhibit shape changes due to perturbation are digitized and arranged as the row vectors of a $D \times N$ matrix $[\theta]_j(\lambda_k)$, where λ_k denotes the discrete wavelength (column) of the jth spectrum (row). One of the spectra is chosen as a first basis spectrum, is normalized, and subtracted from all the subsequent spectra in the matrix to yield an equivalent matrix with the first basis spectrum removed, denoted by $[\theta]'_j(\lambda_k)$.

$$[\theta]'_1(\lambda_k) = [\theta]_1(\lambda_k) \tag{1}$$

$$[\theta]'_{j>1}(\lambda_k) = [\theta]_j(\lambda_k) - N_j[\theta]_1(\lambda_k) \tag{2}$$

where $N_j = [\theta]_j(\lambda_n)/[\theta]_1(\lambda_n)$ is a normalization constant evaluated at a specific wavelength λ_n. One of the rows in the equivalent matrix is then selected as a new basis spectrum and the matrix reduction is again iterated according to eqns. 1 and 2. This reduction process is repeated until no statistically significantly shape differences exist among the set of reduced spectra. One of these spectra is then retained as a final basis spectrum. The rank of the spectral matrix is given by the number of nonzero rows in the equivalent reduced matrix and is equal to the number of linearly independent spectral components contained in the original set of spectra. If approximations discussed elsewhere are applicable [3,9] the rank analysis also yields the shape of the independent spectral components. Since the basis set of spectra derived from matrix rank analysis is complete, the experimental spectra can be fit as a superposition of these components

$$[\theta]_i^{obs}(\lambda_k) = \Sigma_j C_{ij}[\theta]_j^{(j-1)'}(\lambda_k) \tag{3}$$

where C_{ij} is the coefficient of the jth basis spectrum in the ith experimental spectrum, and $[\theta]_j^{(j-1)'}(\lambda_k)$ is the jth row in the reduced matrix. The relative statistical significance of the matrix rank components can be assessed by com-

paring the root mean square (RMS) of each row in the reduced matrix with the RMS error of the experimental spectra.

Three statistically significant spectral components account for the observed pH-induced spectral changes of Dip-subtilisin Carlsberg within experimental error (Fig. 2B, inset).

We chose a spectrum obtained at pH 7.9 (i.e., no ionized tyrosyl contribution) as the first basis spectrum (designated component 1) since the spectral shape is constant from pH 4 to 8. Our choice of normalization wavelengths was governed by plots of ellipticity vs. pH for various wavelengths, which suggested certain spectral regions (278, 290 and 302 nm) to be monitors of the three independently titrating spectral components. By normalizing at 278 nm and subtracting the pH 7.9 spectrum from the other spectra a set of spectra is obtained with component 1 removed.

Our choice of a second basis spectrum from the equivalent matrix of reduced spectra was governed by an examination of the ratio of ellipticity at 302 and 290 nm plotted as a function of pH (Fig. 3). Since the ratio $[\theta]_{302}/[\theta]_{290}$ is relatively constant over the pH range 9—10.5 the remaining two components must be linearly dependent over this pH interval and associated with a distinct physical species or a stable mixture of difference species. Above pH 10.5, however, the ellipticity ratio changes, implying that the red shifted spectral components are linearly independent above this pH. The constant ratio $[\theta]_{302}/[\theta]_{290}$ above pH 12 implies the presence of another distinct physical species (with respect to tyrosyl ionization) at higher pH values. The transition between the physical species existing at ca. pH 10 and above 12 obeys the Henderson-Hasselbalch equation (pK_{app} = 11.6). The fact that the CD at 302 nm is very small above pH 12 implies that spectra recorded above this pH most likely correspond to a single spectral component upon reduction and removal of component 1. We performed the rank analysis using two choices for the second basis spectrum, corresponding to the species which exist at pH values above and below the pK_{app} 11.6 transition. Similar spectral components were derived in both cases. Using a spectrum obtained at pH 12.2 (taken before the occurrence of significant time dependent CD changes) as a second basis spectrum in the rank analysis and normalizing at 290 nm we obtained the components shown in
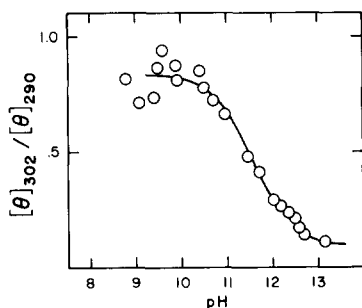

Fig. 3. Mean residual molar ellipticity ratio $[\theta]_{302}/[\theta]_{290}$ vs. pH determined from CD spectra of Dip-subtilisin Carlsberg recorded at different pH values. The solid line is calculated using the Henderson-Hasselbalch equation assuming a pK_{app} of 11.6.

TABLE I

INDEPENDENTLY TITRATING SPECTRAL COMPONENTS RESOLVED BY MATRIX RANK
ANALYSIS

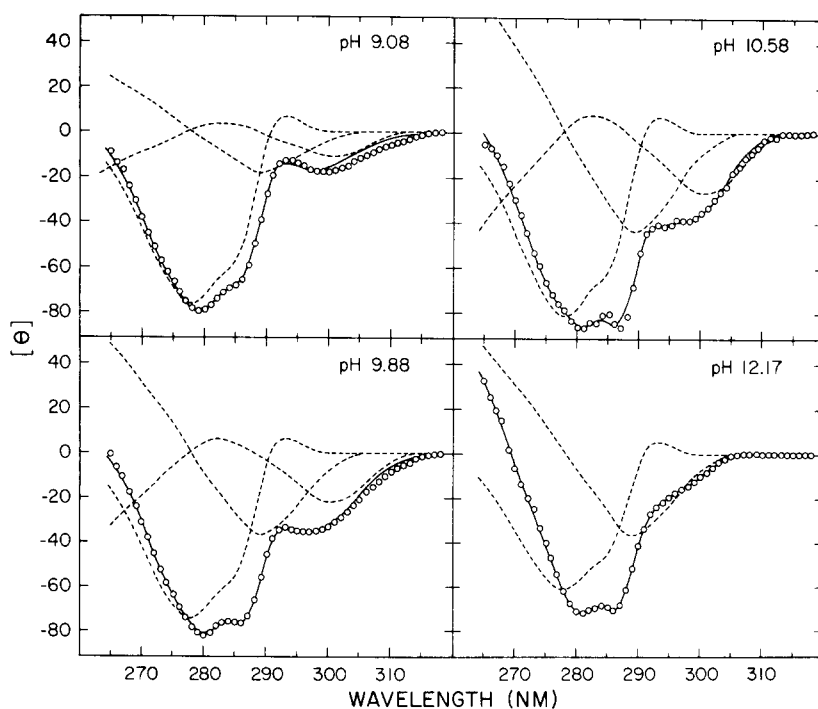| Component	Wavelength extrema (nm)	$[\theta]$ (deg cm^2 dmol^{-1})
Carlsberg		
1	278	$-\ 75 \pm 10$
	286	$-\ 53 \pm\ \ 7$
	293	$+\ \ \ 7 \pm\ \ 3$
2	289	$-\ 35 \pm 10$
3	300	$-\ 25 \pm 10$
Novo		
1	278	-145 ± 20
	286	-124 ± 16
2	296	$-\ 84 \pm 10$

Fig. 4. Multiple linear regression fit of Dip-subtilisin Carlsberg experimental CD spectra with the matrix
rank components shown in Fig. 2B. The spectra were fitted as a linear combination of the form: $[\theta]^{obs}$
$(\lambda) = f_1[\theta]_1(\lambda) + f_2[\theta]_2(\lambda) + f_3[\theta]_3(\lambda)$ where f_1, f_2, and f_3 are the normalized coefficients for the three
basis spectra derived from the regression analysis. The amplitudes of the matrix rank components are
indicated by the dashed lines, the solid lines represent the calculated sum of the components and the open
circles represent the experimental data points.

Fig. 2B. The matrix reduction was also performed using the pH 9.9 reduced spectrum as the second basis spectrum with normalization at 302 nm. The three derived components are shown in Fig. 2C. In this case the second matrix rank component is a linear combination of the components derived from the rank analysis using the pH 12.2 spectrum as the second basis spectrum. If we normalize component 3 to component 2 (Fig. 2C) at 290 nm and subtract it, we obtain a CD spectrum similar to component 3 of Fig. 2B. Since we are able to derive similar spectral components independently of our choice of the second basis spectrum the uniqueness of the derived components is established. The wavelength extrema and amplitudes of the matrix rank components are summarized in Table I.

The experimental spectra were fit as a superposition of the derived basis spectra by multiple linear regression. A few representative fits are shown in Fig. 4. The derived components are both necessary and sufficient to describe the experimental spectra; if components are removed from the basis set the error of fit is increased significantly.

pH dependence of the amplitudes of the matrix rank components of Dip-subtilisin Carlsberg. It is possible to follow changes in the matrix rank components directly from the ellipticity of the experimental spectra at 278 (component 1), 290 (component 2) and 302 nm (component 3), respectively. Plots of the amplitude of each matrix rank component present in the experimental spectra as a function of pH are depicted in Fig. 5. The amplitude of com-
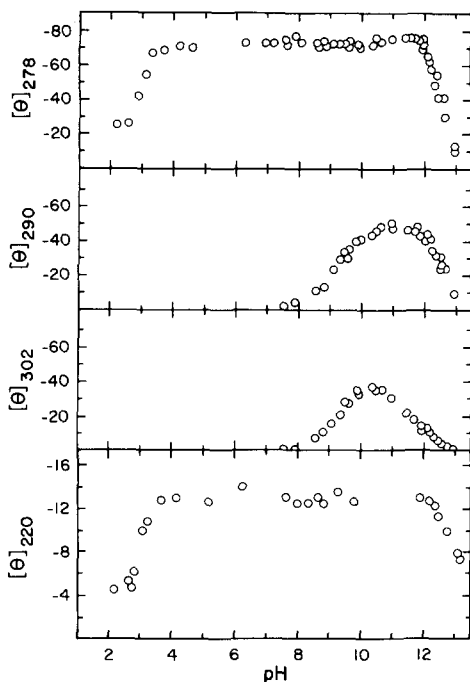

Fig. 5. The pH dependence of the Dip-subtilisin Carlsberg matrix rank components measured from experimental spectra at 278 nm (component 1), 290 nm (component 2), and 302 nm (component 3). The pH dependence of the CD at 220 nm is indicated in the lower panel.

ponent 1 is invariant from pH 4 to 12; below or above these values an irreversible loss in optical activity of all matrix rank components occurs. The pH midpoint for the low-pH induced denaturation is ca. 3 and the value for the high-pH mediated transition is ca. 12.5. Component 2 appears with a pK_{app} of ca. 9.3 and persists to pH 12, above which a decrease occurs. Component 3 appears with the identical pK_{app} as component 2 but does not persist up to the point of irreversible denaturation; a loss of optical activity is apparent at pH values greater than 10.5; decreasing to almost zero at pH 12. The pK_{app} for this decrease is ca. 11.6. Rotational strength is not conserved upon tyrosyl ionization, i.e., the appearance of components 2 and 3 is not paralleled by a proportional decrease in the amplitude of component 1. The changes in the CD at 220 nm (Fig. 5) parallel exactly those of component 1 at all pH values and component 2 at high pH.

pH dependence of Dip-subtilisin Novo CD spectra. The CD below 260 nm of Dip-subtilisin Novo is essentially identical to that of Dip-subtilisin Carlsberg and is constant within experimental error over the pH range 4—12. The CD of Dip-subtilisin Novo above 260 nm is different from that of Dip-subtilisin Carlsberg in that it lacks the 293 nm (+) band and is pH-dependent over the range 4—8. Above pH 8 contributions from tyrosyl residue ionization are apparent in the CD of both enzymes.

Below pH 4 and above pH 12 time-dependent changes occur in the CD of Dip-subtilisin Novo. The native spectral form is replaced below pH 4 by a form exhibiting a minimum at ca. 198 nm, with an isosbestic point at 206—208 nm (Fig. 6). A proportional diminution in optical activity occurs at all wavelengths greater than 260 nm with decreasing pH. The low-pH induced transition of Dip-subtilisin Novo is irreversible, on contrast to the behavior of Dip-subtilisin Carlsberg.

The CD spectra of Dip-subtilisin Carlsberg at a series of pH values above neutrality are depicted in Fig. 6B. Changes analogous to those observed at low pH are evident above pH 12. The high-pH induced transition is time dependent and, as in the case of Dip-subtilisin Carlsberg irreversible.

Matrix rank analysis of Dip-subtilisin Novo CD changes. The existence of an isosbestic point at 283 nm for the spectral changes occurring in the pH interval 8—12 (Fig. 6B) implies that the changes observed in the pH region of tyrosyl ionization are due to a pH-induced transition between two spectral forms. No such isosbestic point is evident in the CD spectra of Dip-subtilisin Carlsberg recorded in the same pH interval. The observed spectral changes are reversible to pH values as high as 12. No time dependence of the CD above 260 nm was noted in this pH range.

The spectra of the linearly independent matrix rank components were derived in a straightforward manner. Since two spectral components account for the observed pH-induced changes (Fig. 7, inset) it is necessary only to find a spectrum which is unperturbed and can be normalized to the set of perturbed spectra at a wavelength accurately reflecting the amount of the first basis spectral contribution in the set of remaining perturbed spectra. Using a spectrum recorded at pH 5.7 and normalizing it to the set of other spectra at 278 nm we obtain upon subtraction the components shown in Fig. 7. The wavelength extrema and amplitudes of the two derived matrix rank components are sum-
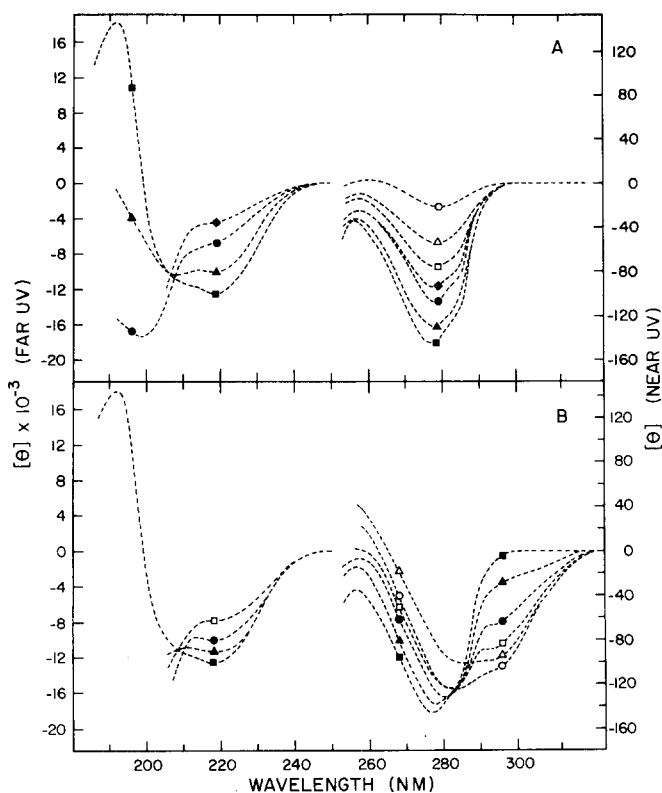
Fig. 6. A. Changes in the CD of Dip-subtilisin Novo induced by decreasing pH. Far ultraviolet (UV) region: pH 4—8, (■); pH 2.76, (▲); pH 2.37, (●); pH 1.86, (♦). Near ultraviolet spectral region: pH 5.98, (■); pH 4.67, (▲); pH 4.26, (●); pH 3.72, (♦); pH 3.01, (□); pH 2.70, (△); pH 2.32, (○). B. Effect of increasing pH on the CD of Dip-subtilisin Novo. Far ultraviolet spectral region: pH 8—12, (■); pH 12.43, (▲); pH 12.94, (●); pH 13.02, (□). Near ultraviolet spectral region: pH 7.74, (■); pH 9.08, (▲); pH 9.95, (●); pH 10.54, (□); pH 11.62, (○); pH 12.46, (△).
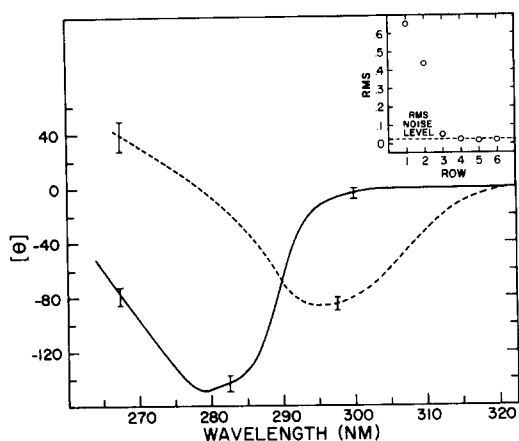

Fig. 7. Independently varying spectral components for Dip-subtilisin Novo resolved from the spectra shown in Fig. 6B by matrix rank analysis. Components are numbered in order of their derivation in the rank analysis: component 1, ————; component 2, - - - - - -. The first basis spectrum was recorded at pH 5.74 and normalized at 278 nm. The RMS of each spectrum in the reduced matrix is shown in the inset. The RMS noise level of the experimental spectra is indicated by the dashed line.
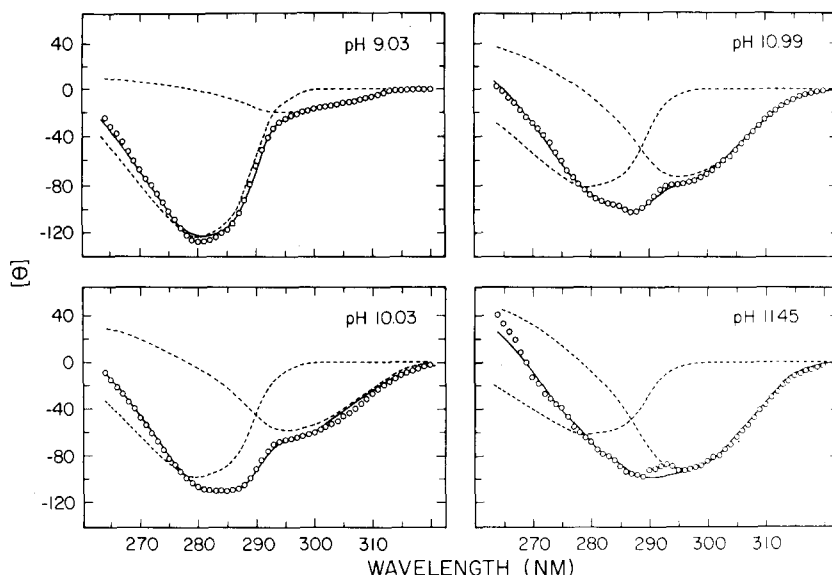
Fig. 8. Multiple linear regression fit of Dip-subtilisin Novo experimental CD spectra with the matrix rank components shown in Fig. 7. The amplitudes of the matrix rank components are indicated by the dashed lines, the solid lines represent the calculated sum of components and the open circles represent the experimental data points.
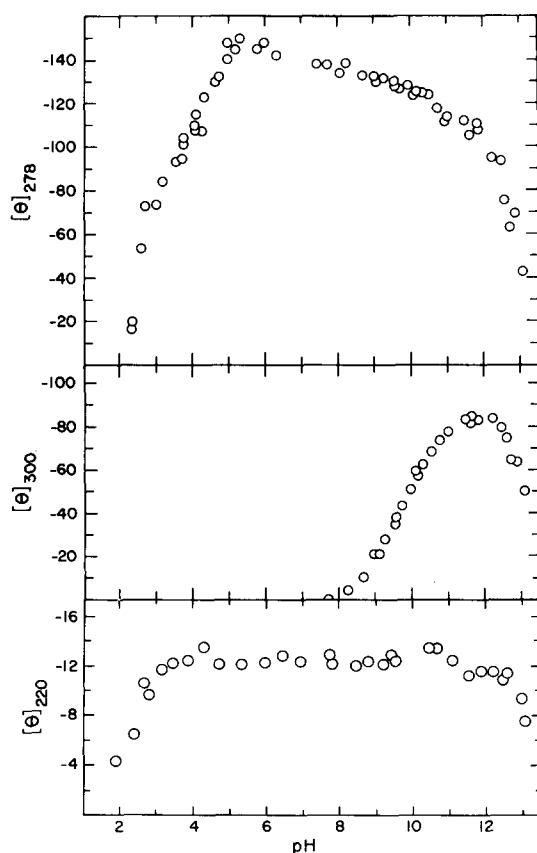

Fig. 9. The pH dependence of the Dip-subtilisin Novo matrix rank components measured from experimental spectra at 278 nm (component 1) and 300 nm (component 2). The pH dependence of the CD at 220 nm is indicated in the lower panel.

marized in Table I. The experimental spectra were fit as a superposition of the two derived basis spectra using multiple linear regression. The derived components are sufficient to account for the observed CD spectral changes within experimental error. Representative fits are shown in Fig. 8.

pH dependence of the amplitudes of the matrix rank components of Dip-subtilisin Novo. The presence of the two-matrix rank components can be followed from the experimental ellipticity at 278 and 300 nm (Fig. 9). The CD due to component 1 appears sensitive to the ionization of the protein over the entire pH range considered. Below pH 4 and above pH 12 irreversible structural transitions occur characterized by pH midpoint values of ca. 3 and 12.5, respectively. In the pH interval 4—12, where the CD below 260 nm is stable, the ellipticity at 278 nm (component 1) is maximal at pH 5—7, decreasing more or less continuously from pH 7—12. Component 2 (300 nm) appears with a pK_{app} of 9.6 and persists to pH 12. The CD changes at 220 nm parallel those of component 1 at low and high pH and component 2 at high pH.

Discussion

Interpretation of the CD matrix rank components. The CD of Dip-subtilisins Carlsberg and Novo above 260 nm originates from the tyrosyl and tryptophyl residues, since phenylalanine and its derivatives are devoid of optical activity above 270 nm [10,11] and cysteine is absent. To discriminate between those optically active residues sensitive to pH changes and those that are not, we utilized the known titration behavior of the tyrosyl residues [12] to perturb the CD and decomposed the spectra using matrix rank analysis into linearly independent sets of titratable components. Superposition of two spectral components accounts for the observed CD spectra (recorded above 260 nm) of Dip-subtilisin Novo at all pH values in the region of tyrosyl ionization (pH 8—12), whereas three components are necessary for Dip-subtilisin Carlsberg.

Since subtilisin Novo contains 3 tryptophyl residues whereas subtilisin Carlsburg has only one tryptophyl residue, the similar shape of the CD of both Dip-subtilisin enzymes between 260 and 290 nm suggests minimal tryptophyl contributions in this spectral region. The similarity of the CD of Dip-subtilisins Novo and Carlsberg above 260 nm to that of ribonuclease and tyrosyl model compounds [13,14] further strengthens our interpretation of a predominant tyrosyl contribution. The 0—0 and 0—800 cm^{-1} vibronic transitions of tyrosine are apparently manifested in the CD of Dip-subtilisins Carlsberg and Novo at 286 nm and 278—279 nm. The 0—0 band appears as a shoulder due to the contributions of higher vibronic components to the CD in the 280 nm region [14] and has not been observed at wavelengths longer than 289 nm in proteins [14, 15]. Thus it is reasonable to assign the 293 nm band of Dip-subtilisin Carlsberg to the single tryptophyl residue at position 113. Since Trp-113 is conserved in both homologous subtilisins the absence of the 293 nm band in the CD of Dip-subtilisin Novo implies a somewhat different environment for this residue, although fluoresence studies suggest the indole ring of Trp-113 is "buried" in both enzymes [16].

The pH dependence of component 1 of Dip-subtilisin Novo in the pH interval 5—8 probably reflects the ionization of two of the six histidyl residues [17]

while the dependence in the range 8—12 may reflect the ionization of accessible tyrosyl or lysyl residues, or may be due to the diminution of small contributions to the ellipticity at 278 nm from the reversibly ionizable tyrosyl residues. The CD of Dip-subtilisin Carlsberg due to component 1 displays no such dependence in the pH interval 4—12. The major contribution to the neutral pH CD of both enzymes appears to be due to the "buried" tyrosyl residues since the amplitude of the neutral pH component 1 persists to pH 12, above which a large decrease in optical activity is evident.

The higher matrix rank components represent the CD spectra of ionized tyrosyl residues in the native protein structures. The sign of the tyrosyl CD of subtilisins is negative and unchanged upon ionization, as observed for tyrosyl model compounds and ribonuclease [13,14,18]. The second matrix rank component of Dip-subtilisin Novo corresponds to the CD of tyrosyl residues that are freely ionizable with a pK_{app} of 9.6, in agreement with the value of 9.7 determined by ultraviolet difference spectroscopy [12]. The appearance of red-shifted CD bands due to tyrosyl ionization is not paralleled by a proportional decrease in the amplitude of component 1 at 278 nm, implying that the freely ionizable tyrosyl residues have no, or very weak, optical activity in the unionized state. The relatively low pK_{app} values derived in this study for the freely ionizable tyrosyl residues of Dip-subtilisins Novo and Carlsberg are consistent with stabilization of the tyrosylate form. An examination of the X-ray structure of Dip-subtilisin Novo reveals that many of the tyrosyl residues are in close proximity to the basic amino acids lysine and arginine. It is possible that charge-charge interactions occur upon tyrosyl ionization which enhance optical activity.

The derivation of three-components by matrix rank analysis of the Dip-subtilisin Carlsberg pH-induced spectral changes in the CD above 260 nm is due to the sensitivity of the "exposed" or freely ionizable tyrosyl residues to the ionization of the "partially buried" tyrosyl residues. The appearance of both components 2 and 3 is characterized by a pK_{app} of 9.3 which is somewhat lower than that expected for a freely ionizable tyrosyl residue. A pK_{app} of 9.9 for the "exposed" tyrosyl residues of Dip-subtilisin Carlsberg using ultraviolet difference spectroscopy has been reported [12]. It is interesting that components 2 and 3 of Dip-subtilisin Carlsberg have identical pK_{app} values and yet are distinguishable in terms of their red shifts and behavior at higher pH values. Component 2 persists up to pH 12—12.2 and decreases in amplitude only upon titration of the "buried" tyrosyl residues and irreversible disruption of the protein structure at high pH. The amplitude of compoenent 3, however, steadily decreases at pH values greater than 10.5. The fit of $[\theta]_{302}/[\theta]_{290}$ to the Henderson-Hasselbalch equation over the pH range where component 2 is invariant suggests that a single proton ionization is responsible for the disappearance of component 3, although multiple independent ionizations are not ruled out. The pK_{app} for this process is 11.6 and corresponds to the pK_{app} determined by Markland [12] for the titration of the "partially buried" tyrosyl residues. Components 2 and 3 must therefore both correspond to the CD of the "exposed" tyrosyl residues of Dip-subtilisin Carlsburg. The "partially buried" tyrosyl residues of both subtilisin enzymes appear to be devoid of optical activity in either the tyrosyl or tyrosylate forms.

A local conformational change in the vicinity of some of the "exposed" tyrosyl residues triggered by the ionization of one or more "partially buried" tyrosyl residues is suggested to account for the difference in pH behavior of components 2 and 3 of Dip-subtilisin Carlsberg. The resolution of only two components by rank analysis of the Dip-subtilisin Novo CD changes suggests that the above mentioned conformational change is peculiar to the Carlsburg structure and does not occur in the subtilisin Novo molecule. The "buried" class of tyrosyl residues is responsible for the optical activity of both subtilisin enzymes at neutral pH (component 1), as previously discussed, and is the only group of tyrosyl residues which displays optical activity in the unionized state. Disruption of the native protein conformations by highly alkaline solutions results in the titration of the "buried" tyrosyl residues and loss of optical activity at all wavelengths above 260 nm.

Low- and high-pH induced denaturation of subtilisin enzymes. At pH values below 4 and greater than 12 conformational transitions occur to a form whose CD at short wavelengths is similar to that exhibited by denatured proteins [19]. The parallel changes that occur in the CD above 260 nm are consistent with disruption of secondary and tertiary structure.

The midpoint of the low-pH induced denaturation of Dip-subtilisins Novo and Carlsberg (ca. 3) suggests the participation of carboxyl and/or imidazole side chains. The potentiometric titration curve of phenylmethanesulfonyl-subtilisin Novo between pH 5 and 8 is best fit by two histidyl residues which are protonated only upon acid induced denaturation of the protein [17]. It is also possible that carboxylate side chains are involved in the acid denaturation of subtilisins, since the potentiometric titration curve of phenylmethanesulfonyl-subtilisin Novo is consistent with the ionization of four carboxyl groups which remain protonated to high pH values [17].

The irreversible denaturation of subtilisin enzymes above pH 12 results in the ionization and disruption of interactions involving a small number of tyrosyl residues, although the role of overall electrostatic factors in the high pH induced denaturation is not precluded by the experimental data available at present. The tyrosyl residues of subtilisin Novo are located at or near the surface of the enzyme [1,2] and are highly exposed to solvent [20,21]. The phenolic hydroxyl groups of four tyrosyl residues (6, 104, 171 and 162) appear hydrogen bonded to the peptide backbone [2]. The donor and acceptor moieties of these hydrogen bonds occur in sequentially distal regions of the polypeptide chain and are invariant in subtilisins Novo and Carlsberg, despite the high degree of "surface" residue substitution. It is possible that these tyrosyl residues constitute the "partially buried" and "buried" groups of Dip-subtilisin Novo. This suggestion appears to be consistent with studies of the nitration and iodination of subtilisins Novo and Carlsberg [22,23].

Since the abnormally titrating tyrosyl residues of subtilisin Novo are most likely at or very near the solvent-protein interface the participation of phenolic side chains in the high pH induced denaturation of subtilisins draws attention to the importance of surface interactions in the stability of these and possibly other proteins. The arbitrary nature of the adjectives "exposed" and "buried" in reference to abnormally ionizing functional groups in proteins is well illustrated by the subtilisin enzymes.

Acknowledgments

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