Spectroscopic Studies of the Exposure of Tyrosine Residues in Proteins with Special Reference to the Subtilisins*

(Received for publication, August 5, 1970)

BRYANT MYERS, II, AND A. N. GLAZER

From the Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024

SUMMARY

The solvent perturbation behavior and acetylation characteristics of the tyrosine residues in the subtilisins have been examined. The availability of the phenolic side chains both to acetylation and solvent is high, in agreement with the proposed x-ray structure for subtilisin BPN'.

Spectroscopic studies on N,N-diacetyltyrosine, N-acetyltyrosine, and 3-nitrotyrosine in water-dioxane mixtures show that the spectra of these compounds are very sensitive to solvent composition. Consequently, when determination of the degree of acetylation, or nitration, is performed spectrophotometrically on native proteins, significant errors may occur due to differences in the microenvironments of individual tyrosine side chains. Relevant data from the literature are discussed which indicate that phenolic groups in apolar locations undergo substitution preferentially, and that reactivity with N-acetylimidazole or tetranitromethane is an uncertain (and, in some cases, misleading) index of exposure of a phenolic side chain to the solvent.

Circular dichroism studies on subtilisins Novo and Carlsberg indicate some differences in the arrangement of the aromatic residues in these two enzymes, and, possibly, small differences in the conformation of the polypeptide backbones as well.

The limitations of the techniques currently in vogue for the study of protein structure in solution can best be assessed in those cases where the three-dimensional structure of the protein under investigation is known. It would be expected that in most, if not all, cases, the gross conclusions of studies in solution would be compatible with the structure based on x-ray crystallographic analysis at high resolution.

From their study of the structure of subtilisin BPN' at 2.5 A resolution and knowledge of its amino acid sequence (1), Wright, Alden, and Kraut (2) concluded that all of its 10 tyrosine and 3 tryptophan residues reside on the surface of the molecule. However, nitration and iodination (3) studies, as well as spectrophotometric studies (4), on subtilisin BPN' or Novo (these are assumed to be identical (5)) show a drastic departure in the behavior of certain of the tyrosine residues from that exhibited by small tyrosine derivatives in aqueous solution. Thus, in subtilisin Novo, 2 tyrosine residues resist modification by tetranitromethane or iodine, and exhibit pK_app >12.5 (see Table I), although the same 2 residues need not be assumed to be involved in each case.

The results of Wright et al. (2) support the prediction that native subtilisin Novo would exhibit a very high degree of exposure of the aromatic residues to solvent. The relevant solvent perturbation studies are presented here. In addition, from a comparison of the amino acid sequences, Wright et al. (2) concluded that all 13 tyrosine residues in Carlsberg subtilisin would also be at the surface of the molecule, if a close correspondence between the three-dimensional structures of subtilisins Novo and Carlsberg was assumed. This prediction has also been examined here by solvent perturbation. The results emphasize some of the difficulties encountered in evaluating the significance of differences observed between the actual experimental results obtained with this technique and the predictions from the x-ray crystallographic study.

Since N-acetylimidazole has been proposed as a reagent for the differentiation between buried and exposed tyrosine residues, a study of its reaction with the subtilisins, as well as ribonuclease and insulin, was performed. In the course of this study, it became apparent that no clear correlation could be demonstrated between the pK values of the tyrosine residues in the subtilisins and their reactivity with N-acetylimidazole. Further, insufficient attention has been given in many published studies to the significant influence of the local dielectric constant on the spectra of O-acetyltyrosine and 3-nitrotyrosine, and hence on the spectrophotometric determination of these derivatives in native proteins. A brief study of this problem is presented here.

EXPERIMENTAL PROCEDURE

Materials

Crystalline bacterial proteinase Novo (lot 58) and lyophilized subtilisin Carlsberg were a gift from Novo Industries, Copenhagen. Exhaustively dialyzed solutions of these enzymes were used in all experiments. Ribonuclease (lot 622) and phosphate-free ribonuclease A (lot GFA) were from Worthington, and insulin (lot 26B-0700) was from Sigma. Protein concentrations were determined with E_492_mg (280 m\(\mu\)) values of 8.6 (7), 6.0 (10), and 1

1 Recently, a higher E_492_mg (280 m\(\mu\)) of 9.8 has been reported for Carlsberg subtilisin (8). However, the value of 8.6 (7) employed for the calculations reported here is in good agreement with an E_492_mg (280 m\(\mu\)) of 8.3 calculated for this protein on the basis of the known content of tyrosine (13 residues, em_280 = 1330) and tryptophan (1 residue, em_280 = 5600 (9)).
Methods

Preparation of Phenylmethanesulfonyl-subtilisins Novo and Carlsberg—These derivatives were prepared by the addition of a 10-fold molar excess of PMSF \(^2\) (dissolved in a minimum volume of \(n\)-propyl alcohol) to the enzyme dissolved in 0.1 \(M\) Tris-0.02 \(M\) CaCl\(_2\) at pH 7.0 at 26\(^\circ\)C. An additional 10-fold molar excess of PMSF was added after 45 min. After 2 hours, no detectable esterase activity toward \(N\)-acetyl-\(L\)-tyrosyl ethyl ester remained. The reaction mixture was exhaustively dialyzed against de-ionized water and lyophilized. Amino acid analyses of the derivatives were identical with those reported for the native enzymes \((1, 6)\). Disopropylphosphoryl-subtilisin was prepared as previously described \((12)\).

Circular Dichroism—Circular dichroism spectra were obtained with a Jasco-Durrum model J-10 recording spectropolarimeter at 26\(^\circ\)C. Spectra from 260 to 320 \(\text{nm}\) were determined with a constant slit of 0.7 \(\text{mm}\), a light path length of 10 \(\text{mm}\), on the most sensitive scale (1 millidegree of ellipticity per \(\text{cm}\) of chart paper). The reaction mixture was exhaustively dialyzed against de-ionized water and lyophilized. Amino acid analyses of the derivatives were identical with those reported for the native enzymes \((1, 6)\). Disopropylphosphoryl-subtilisin was prepared as previously described \((12)\).

Solvant Perturbation—Solvant perturbation experiments were performed by the procedure of Herskovits and Laskowski \((14)\) in 20\% \((\text{v/v})\) ethylene glycol or in varying concentrations of dioxane. Pairs of matched cylindrical tandem cells (Pyrocell Manufacturing Company, Inc., Westwood, New Jersey) were used to subtract directly the solvent contributions to the difference spectra. The conditions chosen for each experiment are described in the figure legends. The figures represent the average of four difference spectra converted to \(\Delta M\) values as a function of wave length. All difference spectra were determined with a Cary model 15 recording spectrophotometer equipped with a 0 to 0.1 optical density unit slide-wire.

A preliminary estimate of the exposure of tyrosyl and tryptophyl residues in the subtilisins was made by solving Equations 1 and 2

\[
\Delta M_{292-220} \text{(protein)} = a \Delta M_{292-220} \text{(Trp)} + b \Delta M_{292-220} \text{(Tyr)} \tag{1}
\]

\[
\Delta M_{295-280} \text{(protein)} = a \Delta M_{295-280} \text{(Trp)} + b \Delta M_{295-280} \text{(Tyr)} \tag{2}
\]

where \(a\) and \(b\) represent the apparent number of exposed tryptophyl (Trp) and tyrosyl (Tyr) residues in the protein studied, and the \(\Delta M\) values refer to the molar absorptivity differences of the protein and free tyrosine and tryptophan model compounds \((15)\), for the perturbant used, at the 291 to 293 \(\text{nm}\) and the 286 to 288 \(\text{nm}\) difference spectral maxima designated by the subscripts. The initial estimates of \(a\) and \(b\) were refined by determining the best fit of the observed complete protein difference spectra with the calculated spectra plotted according to the equation

\[
\Delta \theta = a \Delta \theta \text{(Trp)} + b \Delta \theta \text{(Tyr)} \tag{3}
\]

employing the model compound data of Herskovits and Sorensen \((15)\) at each wave length.

Preparation of \(N\)-\(O\)-Diacytlytyrosine and Determination of Its Spectral Characteristics—A prerequisite for the accurate spectrophotometric determination of the extent of acetylation of proteins by acetylimidazole is the availability of the exact characteristics of the spectrum of \(N\)-\(O\)-diacytlytyrosine, and, in particular, of the \(\Delta M\) values in the range of 275 to 280 \(\text{nm}\). No detailed spectrum of this compound has hitherto been published. Further, Simpson, Riordan, and Vallee \((16)\) reported an \(\Delta M\) (275 \(\text{nm}\)) value for this compound of 85 \(\text{m}^2\text{cm}^{-1}\text{mol}^{-1}\). Since the presence of small amounts of \(N\)-\(O\)-diacytlytyrosine would produce large errors in the determination of the \(\Delta M\) (275 \(\text{nm}\)) values, crystalline \(N\)-\(O\) diacytlytyrosine free of deacylated material was prepared and its spectral characteristics determined.\(^8\)

\(N\)-\(O\)-Diacytlytyrosine (\(m.p. 171^\circ\), literature \(170-171^\circ (18, 19)\) was prepared by the method of Bergmann and Zervas \((18)\). No Puay-positive material was detected after paper chromatography of the material in \(n\)-butyl alcohol-acetic acid-water \((200: 30: 75, \text{by volume})\). The extinction coefficient at \(\lambda_{\text{max}} 262 \text{nm}\) was found to be 251 \(\text{m}^2\text{cm}^{-1}\text{mol}^{-1}\), which is in excellent agreement with the value of 260 \(\text{m}^2\text{cm}^{-1}\text{mol}^{-1}\) reported by Simpson et al. \((16)\) and Riordan et al. \((17)\). However, the value of \(\Delta M\) (275 \(\text{nm}\)) was found to be 60 \(\text{m}^2\text{cm}^{-1}\), suggesting that the higher values reported earlier \((16, 17)\) reflected contamination by small amounts of \(N\)-\(O\)-diacytlytyrosine.

Acetylation of Tyrosine Residues with \(N\)-Acetylimidazole—Acetylation of the tyrosine phenolic group causes a shift in the tyrosine absorption maximum to a lower wave length and a

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\(^2\) The abbreviations used are: PMSF, phenylmethanesulfonylfluoride; DIP, disopropylphosphoryl; PMS, phenylmethanesulfonyl; CD, circular dichroism.

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\(^8\) Samples of \(N\)-\(O\)-diacytlytyrosine from two commercial suppliers were found to contain small amounts of \(N\)-\(O\)-diacytlytyrosine.

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**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme used</th>
<th>Number of tyrosines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn acid analysis(^a)</td>
<td>Native</td>
<td>10</td>
</tr>
<tr>
<td>Nitration(^a) (exhaustive)</td>
<td>Native</td>
<td>9</td>
</tr>
<tr>
<td>Iodination(^b) (exhaustive)</td>
<td>Native</td>
<td>8</td>
</tr>
<tr>
<td>Spectrophotometric titration(^d)</td>
<td>DIP-derivative(^e)</td>
<td>6.4</td>
</tr>
<tr>
<td>Class I ((\text{pK}_{\text{int}}) = 9.2-9.4)</td>
<td>5.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Class II ((\text{pK}_{\text{int}}) = 9.9-10.3)</td>
<td>2.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Class III ((\text{pK}_{\text{app}}) &gt; 12.5)</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Markland and Smith \((1)\) and Smith et al. \((6)\).

\(^b\) Svendsen \((3)\).

\(^c\) Not exhaustive.

\(^d\) Markland \((4)\).

\(^e\) DIP, disopropyl-.
FIG. 1. Far-ultraviolet circular dichroism spectra of subtilisins Novo and Carlsberg. All spectra were obtained in 0.02 M phosphate buffer at pH 7.0 and 26°, at a protein concentration range of 0.26 to 1.1 mg per ml, with a light path length of 1 mm. I, dialyzed subtilisin Novo (□), PMS-subtilisin Novo (●), and DIP-subtilisin Novo after 2 hours (▲) and 24 hours (Δ) under the above conditions. II, dialyzed subtilisin Carlsberg (□) and its PMS derivative after 2 hours (▲) and 24 hours (Δ) under the above conditions.

The decrease in absorbance at 278 mU may be used as a measure of the extent of conversion of tyrosine to the O-acetyl derivative. The number of residues modified per mole of protein may be determined from the equation (20)

\[ N = \frac{\Delta A_{278} \text{ mol wt}}{\Delta A_{278} \times c} \]

where \( \Delta A_{278} \) is the decrease in absorbance at 278 mU, mol wt is the molecular weight of the protein, and \( c \) is its concentration in milligrams per ml, and \( \Delta A_{278} \) is the molar absorptivity difference between N-acetyl- and N,O-diacyl-tyrosine.

The molar absorptivity differences between N-acetytyrosine (\( \epsilon_{M, 278} = 1370 \text{ M}^{-1} \text{ cm}^{-1} \)) and N,O-diacyltyrosine (\( \epsilon_{M, 278} = 260 \text{ M}^{-1} \text{ cm}^{-1} \)) were determined from difference spectra to be \( \Delta \epsilon_{M, 278} = 1210 \text{ M}^{-1} \text{ cm}^{-1} \) and \( \Delta \epsilon_{M, 278} = 1310 \text{ M}^{-1} \text{ cm}^{-1} \), respectively, and thus differ significantly from the previously reported \( \Delta \epsilon_{M} \) value of 1100 mU cm for both wavelengths (16, 17).

An independent determination of the \( \Delta \epsilon_{M} \) values was also performed by acetylatyng acetyl-L-tyrosine ethyl ester at pH 7.5 with a 10-fold molar excess of N-acetylimidazole. At the end of 2 hours, the reaction mixture was acidified to pH 4.5 to promote complete hydrolysis of excess reagent. The \( \epsilon_{M, 278} \) of the acetylated product was 64 M\(^{-1}\) cm\(^{-1}\), and \( \Delta \epsilon_{M, 278} \) and \( \Delta \epsilon_{M, 278} \) were 1200 mU cm\(^{-1}\) and 1310 mU cm\(^{-1}\), respectively, in excellent agreement with the values obtained by comparison of model compounds.

In acetylation experiments, the desired concentration of N-acetylimidazole was obtained by drying a suitable aliquot of the stock solution of the reagent in benzene, redissolving the dry material in 0.5 ml of water, and immediately adding 0.15 ml to a sample cell containing a buffered protein solution and to a reference cell containing buffer only. The final volume in each cell was 2.5 ml. All experiments were performed in 0.016 M Veronal buffer at an initial pH of 7.5. The decrease in absorbance at 278 mU was followed until no further change was observed over a period of 20 min. The time required for completion of the reaction depended on the initial N-acetylimidazole concentration and ranged from 70 to 180 min at 25°. A close first approximation to the number of tyrosine residues acetylated could be obtained by using the value of \( \Delta A_{278} \) obtained from Equation 5 (below) in Equation 4

\[ \Delta A_{278} = A_{\text{initial}}^{278} - A_{\text{final}}^{278} \]

where \( A_{\text{initial}}^{278} \) is the absorbance of the protein solution versus the absorbance of the buffer, prior to addition of N-acetylimidazole, and \( A_{\text{final}}^{278} \) is the absorbance of the reaction mixture versus the absorbance of the reagent control, as determined at the end of the absorbance change and corrected for the dilution by the reagent. A more accurate calculation of the number of tyrosine residues acetylated was then performed by compensating for the difference in residual reagent concentration in the sample and reference cells. The absorbance of the solution in the reference cell, denoted by \( A_{\text{final}}^{278} \), was measured at the conclusion of the experiment and the value multiplied by the ratio of the approximate number of tyrosine residues acetylated, \( z_{2} \), to the initial molar ratio of reagent to protein used, \( y \). The difference in residual reagent concentrations in the two cells is then given by

\[ \Delta A_{278, \text{corr.}} = \frac{x_{2} A_{\text{final}}^{278}}{y} \]

\( \Delta A_{278} \) was then recalculated from the relation

\[ \Delta A_{278} = A_{\text{final}}^{278} - (A_{\text{final}}^{278} + \Delta A_{278, \text{corr.}}) \]
Because of the large molar excess of N-acetylimidazole used relative to tyrosine residues acetylated, this correction was small, ranging from 1.5 to 3%. The decrease in absorbance has not been corrected for changes due to acetylation of lysine residues. However, even if it is assumed that all of the lysine residues in the subtilisins are acetylated under the conditions used, the maximum error in the estimation of tyrosine acetylation would be 0.2 residue per molecule.

The above procedure and that of Riordan and Vallee (20) were compared under conditions for complete acetylation of tyrosine residues in ribonuclease A and insulin in 8 M urea. Both procedures yielded concordant results.

RESULTS

Circular Dichroism Studies—The CD spectra of Novo and Carlsberg subtilisins, as well as those of the corresponding PMS and DIP derivatives, are shown in Figs. 1 and 2. It is apparent that the introduction of these groups on the serine residue at the active site does not perturb the far-ultraviolet CD spectra, nor the near-ultraviolet Cotton effects arising from the aromatic residues. This is consistent with the x-ray diffraction results which show only a small local perturbation by the PMS group, not involving aromatic chromophores, of the structure of subtilisin BPN' (2).

Wright et al. (2) state that “based on the assumption that the over-all three-dimensional folding of a protein molecule is predominantly determined by the interior residues, and on the observed spatial distribution, in the model, of the side-chains that differ between the BPN' and Carlsberg subtilisins, we conclude that the two enzymes must have very similar three-dimensional structures. All differences, with one exception, occur in exterior chain segments. The exception is Ile 31 which changes to a leucine in the Carlsberg enzyme.”

Subtilisin Novo exhibited an ellipticity of about -10,000 deg cm² per decimole of residue at 217 mp compared to -7,800 for subtilisin Carlsberg. This suggests either a small difference in the amount of α helix or β structure in the two proteins, or, possibly, a difference in the orientation of the various segments of the backbone to each other. A surprising difference was noted in the magnitude of the Cotton effect centered at 277 mp (Fig. 2), which was 45% smaller for subtilisin Carlsberg than Novo. This is particularly noteworthy since subtilisin Carlsberg contains 13 residues of tyrosine, whereas subtilisin Novo contains 10 (6).

Solvent Perturbation—The solvent perturbation difference spectra produced by 20% (v/v) aqueous ethylene glycol, for native subtilisins Novo and Carlsberg, are shown in Fig. 3. The values for the exposure of tyrosine and tryptophan residues calculated from these spectra, using the model compound data and procedures of Herskovits and Sorensen (15), are given in Table II. Whereas the calculated and observed difference spectra show only approximate agreement, these results are comparable to those reported in similar studies on pepsin, aldolase, and serum albumin (21), and cytochrome c and myoglobin (22).

The extent of exposure of tyrosine residues indicated by our

![Fig. 3. Comparison of the solvent perturbation difference spectra for native subtilisin Novo (A) and Carlsberg (B) produced by 90% ethylene glycol. The solid curves represent the experimental data, and the dashed curves represent the theoretical spectra calculated from Equation 3, with a = 1.6 and b = 8.8 for subtilisin Novo (A), and a = 0.8 and b = 8.8 for subtilisin Carlsberg (B). The spectra were obtained in 0.1 M Tris buffer containing 0.02 M CaCl₂ at pH 7.8 and 25°C, at protein concentrations of 5.2 × 10⁻⁴ M and 1 × 10⁻⁴ M for subtilisin Novo and Carlsberg, respectively.](http://www.jbc.org/)

![Fig. 4. Acetylation of proteins with N-acetylimidazole. The experimental details are given in the text. A ΔM (278 mp) value of 1210 m² cm⁻¹ was used to calculate the extent of tyrosine modification. O, subtilisin Novo (3.5 ± 0.5 × 10⁻⁴ M) and N-acetylimidazole concentration range of 1.7 to 7.8 mM; C, subtilisin Carlsberg (3.86 ± 0.05 × 10⁻⁴ M) and N-acetylimidazole from 1.7 to 8.0 mM; A, ribonuclease A (6.7 ± 1.2 × 10⁻⁴ M) and N-acetylimidazole from 26 to 75 mM; B, insulin (1.1 × 10⁻⁴ M) and N-acetylimidazole from 2.4 to 14 mM. The data for bovine β-lactoglobulin (Δ) and goat β-lactoglobulin (A) are taken from Reference 29.](http://www.jbc.org/)
data is high, in agreement with the location assigned these residues by Wright et al. (2) at the protein surface. However, the exposure of tryptophan side chains to the solvent is definitely very incomplete in subtilisin Novo. This was most clearly seen on comparison of the solvent perturbation difference spectra obtained with native and autolyzed subtilisin Novo. The latter exhibited a pronounced peak at 291 m\(\mu\) and a difference spectrum consistent with the exposure of all three tryptophan side chains to the solvent, whereas in the native enzyme, a maximum of 2 residues appears available to the solvent. Presumably, the full solvent perturbation difference spectrum is developed only on adequate exposure of the indole chromophore to the solvent, and the observed spectrum may well be the consequence of partial exposure of 3 residues.

Obviously, the values obtained from solvent perturbation data represent the summation of contributions from totally exposed side chains, as well as those presenting only a portion of the chromophore to the solvent, and consequently, the numbers given in Table II need not be equated with the actual number of residues perturbed. It is noteworthy, however, that the tyrosine contribution to the difference spectrum for native subtilisin Novo does approach that expected from the tyrosine content of this protein (8.8 out of 10), in agreement with the proposed distribution of these side chains (2). A particularly interesting feature of the results is that the number of exposed tyrosine residues appears to be the same in subtilisin Novo and Carlsberg (see Table II), even though the latter enzyme contains 3 more tyrosine residues.

Acetylation of Subtilisins Novo and Carlsberg—Acetylation of the inactive PMS derivatives of the subtilisins was performed as described under “Experimental Procedure.” The active enzymes could not be used since they catalyzed the hydrolysis of the reagent at a rate far in excess of the rate of tyrosine acetylation. The extent of acetylation, plotted as a function of the initial molar ratio of N-acetylimidazole to protein, for subtilisins Novo and Carlsberg is shown in Fig. 4. At the end of each experiment, the decrease in absorbance at 278 m\(\mu\) was completely reversed by a 20-min incubation of the sample with an equal volume of 2 M hydroxylamine adjusted to pH 7. As may be seen in Fig. 4, at adequately high N-acetylimidazole concentrations, all 10 tyrosine residues in subtilisin Novo and all 13 in subtilisin Carlsberg could be acetylated. However, because of the smooth dependence of the degree of acetylation on N-acetylimidazole concentration, it must be noted that no arbitrary concentration of the reagent could be chosen to yield the number of free tyrosine residues as previously claimed (17). Further, comparison of the acetylation results with those of spectrophotometric titration (4) cited earlier (Table I) leads to the conclusion that N-acetylimidazole modified readily titratable residues, as well as those with high pK values, without discernable discrimination. A comparison of the dependence of the degree of acetylation of N-acetylimidazole concentration observed with the subtilisins with that obtained with several other proteins (Fig. 4) is interesting. Clearly, all of the tyrosine residues in the two subtilisins can be acetylated at N-acetylimidazole concentrations lower than those necessary for the acetylation of normally ionizing tyrosine residues in insulin or ribonuclease A.

Effect of Dioxane on Spectra of Certain Tyrosine Derivatives—The studies on the subtilisins show that acetylation of tyrosine residues, exhibiting abnormal ionization behavior, takes place under relatively mild conditions. Clearly, the microenvironment of such residues is unusual, and it is probable that some are in a local environment of considerably lower polarity than that of water. Since the spectrophotometric determination of
TABLE III
Extinction coefficients of N-acetyl-, N,O-diacetyl-, and 3-nitrotyrosine in water-dioxane mixtures

<table>
<thead>
<tr>
<th>Compounds and conditions</th>
<th>Wave length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>dioxane, v/v</td>
</tr>
<tr>
<td>N-Acetyltyrosine (0.05 M borate, pH 7.5)</td>
<td>275</td>
</tr>
<tr>
<td>N,O-Diacetyltyrosine (0.05 M borate, pH 7.5)</td>
<td>275</td>
</tr>
<tr>
<td>Difference</td>
<td>275</td>
</tr>
<tr>
<td>N-Acetyltyrosine (0.05 M borate, pH 7.5)</td>
<td>278</td>
</tr>
<tr>
<td>N,O-Diacetyltyrosine (0.05 M borate, pH 7.5)</td>
<td>278</td>
</tr>
<tr>
<td>Difference</td>
<td>278</td>
</tr>
<tr>
<td>3-Nitrotyrosine (0.1 N CO$_2$-free NaOH)</td>
<td>428</td>
</tr>
<tr>
<td>N-Acetyltyrosine (0.1 N CO$_2$-free NaOH)</td>
<td>293</td>
</tr>
</tbody>
</table>

* From Reference 17.
* See text.
* From Reference 24.
* From Reference 25.

Fig. 7. Dependence of extinction coefficients of N-acetyltyrosine, N,O-diacetyltyrosine, and 3-nitrotyrosine on dioxane concentration. Increase in $e_M$ (275 nm) for N-acetyltyrosine at pH 7.5 (A) and at pH 13 (B); increase in $e_M$ (428 nm) for 3-nitrotyrosine at pH 13 (C); increase in $\Delta e_M$ (275 nm) (D) and in $\Delta e_M$ (278 nm) for N-acetyltyrosine versus N,O-diacetyltyrosine at pH 7.5 (E).

The difference spectra obtained by solvent perturbation, as well as the results of the acetylation experiments, support the conclusion based on the x-ray crystallographic study on subtilisin BPN' (2) that the aromatic residues exhibit a high degree of exposure in the subtilisins. In the latter study, the description of an aromatic residue as exposed was based on the availability of a portion of the side chain to the solvent. Examination of the model of subtilisin BPN' reveals that in a number of cases only a small portion of the phenolic or indole chromophore is, in fact, accessible to the solvent, the remainder being in van der Waals' contact with other side chains of the protein. In view of this, the results of the solvent perturbation experiments with subtilisin Novo are surprisingly good.

All of the tyrosine residues in both subtilisin Novo and Carlsberg can be readily acetylated. Unfortunately, the necessity of performing these studies on the inactive PMS-subtilisins precluded determination of the effect of full acetylation on the enzymatic activity, and thus the possibility of local conformational changes cannot be ruled out. However, this possibility appears remote, since exhaustive nitration modified 8 of the 10 tyrosine residues in subtilisin Novo with no change in enzymatic activity (3), and since carbamylation (26), or succinylation (27), of all lysine residues is likewise without effect on the activity.
The examination of the model of subtilisin BPN′, as well as consideration of the results of iodination, nitration, and titration studies (Table I), clearly indicates that the tyrosine residues are present in grossly different microenvironments. Since both N-acetylimidazole and tetranitromethane are very soluble in apolar solvents, it is highly probable that tyrosine residues in hydrophobic environments may in fact be preferentially modified in some cases. Relevant examples will be presented later. Since the quantitation of the extent of tyrosine modification is based on the extinction coefficients for O-acetyltyrosine and 3-nitrotyrosine in aqueous solution, it was of interest to examine the influence of dielectric constant on the spectra of these compounds. As shown in Figs. 5 to 7 and Table III, the nature of the solvent is of considerable importance. Thus, the $\Delta \varepsilon_\text{M}$ (278 nm) for N,O-diacetyltyrosine versus N-acetyltyrosine changes by 20% on going from water to 46% (v/v) aqueous dioxane. The $\Delta \varepsilon_\text{M}$ (428 nm) for 3-nitrotyrosine changes by 15% over the same range. Thus, for proteins with 5 or more tyrosine residues per molecule, if residues in apolar environments are modified, considerable error may be present in spectrophotometric determinations of the extent of modification on the native protein.

The reaction of tetranitromethane with residues in apolar environments appears to have been demonstrated in several instances. Beaven and Gratzer (28) have shown that tyrosine residues in subtilisin Carlsberg exhibit considerable modification on the native protein. The examination of the model of subtilisin BPN′, as well as consideration of the results of iodination, nitration, and titration studies (Table I), clearly indicates that the tyrosine residues are present in grossly different microenvironments. Since both N-acetylimidazole and tetranitromethane are very soluble in apolar solvents, it is highly probable that tyrosine residues in hydrophobic environments may in fact be preferentially modified in some cases. Relevant examples will be presented later. Since the quantitation of the extent of tyrosine modification is based on the extinction coefficients for O-acetyltyrosine and 3-nitrotyrosine in aqueous solution, it was of interest to examine the influence of dielectric constant on the spectra of these compounds. As shown in Figs. 5 to 7 and Table III, the nature of the solvent is of considerable importance. Thus, the $\Delta \varepsilon_\text{M}$ (278 nm) for N,O-diacetyltyrosine versus N-acetyltyrosine changes by 20% on going from water to 46% (v/v) aqueous dioxane. The $\Delta \varepsilon_\text{M}$ (428 nm) for 3-nitrotyrosine changes by 15% over the same range. Thus, for proteins with 5 or more tyrosine residues per molecule, if residues in apolar environments are modified, considerable error may be present in spectrophotometric determinations of the extent of modification on the native protein.

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The above remarks obviously apply with equal force to nitration and acetylation. Our studies show that with the subtilisins no obvious distinction in reactivity could be made between residues with a very wide range of pK values. In many published studies, conclusions as to the accessibility of tyrosine residues to acetylation have been based on experiments performed at arbitrarily chosen ratios of N-acetylimidazole to protein (or tyrosine). It should be emphasized that it is not the ratio of a labile reagent used in large excess, but its initial concentration, which will determine the degree of modification. Valid conclusions as to the difference in reactivity of various tyrosine residues with the reagent can only be made from examination of plots of the degree of acetylation as a function of N-acetylimidazole concentration. Reliance cannot be placed on results obtained at an arbitrary concentration of reagent. For example, it was concluded from acetylation studies with carboxypeptidase A that 6 to 7 tyrosine residues are on the surface of the molecule and 11 to 12 tyrosine residues are buried (17). In actual fact, examination of the three-dimensional structure of carboxypeptidase A derived from x-ray diffraction studies (32) reveals that the phenolic hydroxyl groups of at least 16 of the 19 tyrosine residues are available to the solvent.

The studies presented here permit some general conclusions regarding the relationship of the three-dimensional structures of subtilisins Novo and Carlsberg to each other. The difference in the far-ultraviolet CD bands at 208 nm of the two enzymes is relatively small and may reflect the summation of small differences in the folding of the backbone, perfection of α helices, or their mutual orientation. The differences in the near-ultraviolet CD band, resulting from the aromatic residues (subtilisins do not contain half-cystine), are more intriguing. The 277 nm CD band of subtilisin Carlsberg exhibits half the rotatory strength of that of Novo. This is particularly interesting since the former enzyme has a higher tyrosine content and only a single tyrosine residue. The percentage of exposure of the tyrosine residues in subtilisin Carlsberg to ethylene glycol is lower than that observed in Novo (Table II). The observed difference may either reflect this difference in the environment of the tyrosine residues and their mutual orientation in the two enzymes, or, possibly, a greater motility of these side chains (or, indeed, of the whole molecule) in subtilisin Carlsberg.

Acknowledgments—We are grateful to Professors R. E. Dickerson and W. N. Lipscomb for permission to cite their unpublished results. We should also like to thank Professor Joseph Kraut for the opportunity to examine the model of subtilisin BPN′.

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Spectroscopic Studies of the Exposure of Tyrosine Residues in Proteins with Special Reference to the Subtilisins
Bryant Myers II and A. N. Glazer


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