The Inactivation of \textit{Bacillus subtilis} $\alpha$-Amylase by N-Acetylimidazole and Tetranitromethane

REACTION OF TYROSYL RESIDUES

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SUMMARY

\textit{Bacillus subtilis} $\alpha$-amylase is inactivated by treatment with N-acetylimidazole. Full catalytic activity is restored when the acetylated protein is treated with hydroxylamine. Spectral changes associated with the loss and recovery of activity indicate that exposed residues of tyrosine react. If the enzyme is acetylated in the presence of substrate (soluble starch) the modified protein retains 90\% of its catalytic activity and one less tyrosyl residue per 24,000 g of protein reacts, compared with the control which has only 20\% activity remaining.

The apparent $pK$ of the 3-nitrotyrosine in the modified enzyme is 7.7 which is higher than that of the free amino acid derivative ($pK_{app} = 6.8$). Peptide maps of a tryptic digest of the modified protein show a single yellow spot. This tryptic peptide contains 1 eq of 3-nitrotyrosine and corresponds with a tyrosine-containing peptide in the map of the native enzyme. These data are interpreted to indicate that in \textit{B. subtilis} $\alpha$-amylase tyrosyl residues are required for enzymic activity. Attempts to relate the tyrosyl residue protected by substrate during acetylation with that residue nitrated by tetranitromethane were made, but are not conclusive.

The present communication reports experiments in which \textit{B. subtilis} $\alpha$-amylase is treated with the following three reagents: (a) N-acetylimidazole which readily acetylates exposed tyrosyl residues and free amino groups; subsequent mild treatment with hydroxylamine removes the acetyl groups from only the tyrosyl residues (2, 3); (b) maleic anhydride which reacts with free amino groups (4); and (c) tetranitromethane which nitrates exposed tyrosyl residues to yield acid-stable, chromophoric 3-nitrotyrosyl residues (5). N-Acetylimidazole (2) and tetranitromethane (5, 6) have been used to advantage in the examination of the tyrosyl residues in carboxypeptidase A.

Grant (7) has obtained evidence from peptide mapping studies of \textit{B. subtilis} $\alpha$-amylase that the physical entity of about 48,000 mol wt is apparently composed of two subunits; the maps show only about one-half as many peptides as would be expected for the larger unit. In this laboratory we have extended this study by purifying and analyzing each of the peptides seen on two-dimensional maps and find that together they account for the total amino acid composition of the protein and hence confirm her finding. The present studies on the chemical inactivation of the enzyme are considered in terms of the hypothesis that the chemical minimum molecular weight of \textit{B. subtilis} $\alpha$-amylase is about 24,000.

EXPERIMENTAL PROCEDURE

\textit{B. subtilis} $\alpha$-amylase (Lot 26B 1050, Sigma) was used without further treatment for the present work. The enzyme had a specific activity of 6 units per mg, as assayed by the method of Coleman and Elliott (8); it sedimented as a single symmetrical boundary in the ultracentrifuge and gave a single peak on DEAE-cellulose chromatography. A molecular weight of 24,000 for the $\alpha$-amylase is used throughout this paper for the calculation.
tion of results, concentration of reagents, and molar absorbances. N-Acetylimidazole was obtained from Pierce Chemical Company, Rockford, Illinois, tetratromethane from Aldrich, 3-nitro-L-tyrosine from Nutritional Biochemicals, 3-amino-L-tyrosine-2HCl from Cyclo Chemical Corporation, soluble starch (Analur) and dextrin ('precipitated by alcohol') from British Drug Houses, Toronto, and trypsin (twice crystallized) from Worthington.

4H-Sulphanilic acid was obtained from The Radiochemical Centre, Amersham, England, and 4C-N-acetylimidazole from Calbiochem. Maleic anhydride (British Drug Houses) was sublimed at about 70°C immediately before use. N,O-Diacetylimidazole was obtained from Pierce Chemical Company, Rockford, Illinois, tetranitromethane from Aldrich, 3-nitro-n-

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Acetylation by N-Acetylimidazole—Acetylation was performed at 25°C with a protein concentration of 2 mg per ml in 0.02 M Tris chloride buffer, pH 7.5. The N-acetylimidazole (500-fold molar excess; 40 mM) was added as a solid because in the above buffer it has a half-life of about 40 min. The reaction time was varied from 2 to 60 min; these conditions generally produced samples with the required range of loss of activity but occasionally the molar excess of reagent was varied. When starch (4 g per 100 ml, a concentration approximately 10 times greater than Kd) or dextrin (1 to 2 g per 100 ml, a concentration approximately 10 times greater than Kd) was present during the acetylation, dilute enzyme solutions of about 60 μg per ml were used but the N-acetylimidazole was maintained at about 40 mM; the appropriate enzyme controls were treated at the same concentrations. The starch or dextrin was removed by DEAE-cellulose column chromatography, with a linear gradient of 0.02 M Tris chloride, pH 7.5, to 0.02 M Tris chloride, pH 7.5, plus 0.5 M NaCl. The protein emerged in the first few milliliters. The modified enzyme was dialyzed exhaustively at 4°C against distilled water and lyophilized. A Cary model 15 automatic recording spectrophotometer were used to determine the spectral properties of the modified protein. All spectra were determined in 0.02 M Tris chloride, pH 7.5, with the same buffer as blank.

Hydroxylamine solution, 2.0 M, was prepared by dissolving hydroxylamine hydrochloride and adjusting to pH 7.5 with NaOH. Labile acetyl groups were determined as hydroxamates by the method of Balls and Wood (12). The number of amino groups acetylated was determined by the method of Moore and Stein (13), with alanine as ninhydrin color standard.

Titration of tyrosyl groups of the α-amylase was performed by measuring the increase in absorbance at 245 and 300 μm, as a function of pH. The titration was carried out on a Radiometer pH meter at 23°C. Samples were read within 2 min of reaching the desired pH and read again after standing in buffer for 30 and 60 min. Tris chloride buffer was used in the range pH 7.0 to 8.0, glycine-NaOH buffer for pH 8.5 to 10.5, and lysine-NaOH buffer for pH 11.0 to 12.5.

Maleylation—Maleic anhydride was added to the enzyme (2 mg per ml) in 0.1 M NaHCO3 buffer, pH 9.0 at 2°C for 5 min. Samples were then diluted into 0.3 M sodium acetate buffer, pH 5.5, and the number of lysine residues which were still available for reaction with ninhydrin was determined (13).

Nitrification by Tetramethane—The tetramethane was diluted with 95% ethanol and an aliquot added to a solution of α-amylase (10 mg per ml) in 0.05 M Tris chloride, pH 8.0 at 25°C. The protein was separated from the reaction mixture by passage through a column of Sephadex G-25, dialyzed against distilled water at 4°C and lyophilized.

Samples for analysis were hydrolyzed with 6 N HCl, in sealed evacuated tubes (14-under 110°C for 22 hours. Amino acid analyses were performed by the procedure of Spackman, Stein, and Moore (15) on a Beckman model 120B amino acid analyzer. Determination of the pKₐ of the 3-nitrotyrosyl residue in the modified protein was made in a 0.5-cm cell at 428 μm and 20°C. The buffers were 0.01 ionic strength. KHPO₄-Na₂HPO₄ buffer was used in the range pH 6.4 to 7.6, and Tris chloride in the range pH 7.8 to 8.2.

Reduction of 3-nitrotyrosyl to 3-aminotyrosyl residues was performed with sodium dithionite at pH 8.0 and room temperature by the method of Sokolovsky, Riordan, and Valle (16). Peptide Mapping—The modified protein was oxidized with performic acid (17), hydrolyzed by trypsin (1% of protein by weight) in 0.5% ammonium bicarbonate buffer at 37°C, for 4 hours, and lyophilized. Peptide maps of 2-μg samples of the hydrolysate were prepared on Whatman No. 3MM paper. The first dimension was electrophoresis in pyridine acetate, pH 4.7, at 25°C, with a protein concentration of 2 mg per ml in 0.02 M Tris chloride, pH 7.5, plus 0.5 M NaCl. The protein emerged in the first few milliliters. The modified enzyme was dialyzed exhaustively at 4°C against distilled water and lyophilized. A Cary model 15 automatic recording spectrophotometer were used to determine the spectral properties of the modified protein. All spectra were determined in 0.02 M Tris chloride, pH 7.5, with the same buffer as blank.

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Samples for analysis were hydrolyzed with 6 N HCl, in sealed evacuated tubes (14) at 110°C for 22 hours. Amino acid analyses were performed by the procedure of Spackman, Stein, and Moore (15) on a Beckman model 120B amino acid analyzer. Determination of the pKₐ of the 3-nitrotyrosyl residue in the modified protein was made in a 0.5-cm cell at 428 μM and 20°C. The buffers were 0.01 ionic strength. KH₂PO₄-N₂H₂PO₄ buffer was used in the range pH 6.4 to 7.6, and Tris chloride in the range pH 7.8 to 8.2.

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range 0.06 to 2 mg per ml.

The absorption spectra of samples of enzyme acetylated to varying extents were recorded between 240 and 320 µm. Differences showed a maximum decrease at 278 µm, which is interpreted as an indication that tyrosyl residues are being acetylated. The difference in extinction at 278 µm increased with increasing inactivation of the enzyme. Simpson, Riordan, and Vallee (2) calculated that a difference in molar absorbance at 278 µm of 1160 corresponds to 1 mole of N,O-diacetyltyrosine deacetylated. The number of tyrosyl residues acetylated was calculated with this value and Fig. 2 shows that a linear relationship was found between the number of tyrosyl residues acetylated and the extent of inactivation of the enzyme.

The inactive acetylated enzyme, when exposed to hydroxylamine at pH 7.5 and 25° for 20 min, regains its catalytic activity (Table I). Concomitantly the spectral properties of the modified enzyme are restored to those of the native enzyme control. The number of moles of acetylhydroxamate formed when hydroxylamine removes the O-acetyl groups from tyrosine, is in fair agreement with the determination, by spectral difference, of the number of tyrosyl residues acetylated.

Table I includes the number of tyrosyl residues acetylated in the presence of starch or dextrin and can be compared with the value obtained when inactivation occurs in the absence of substrate or competitive inhibitor.

Exposed amino groups on the enzyme react during the acetylation, however, the acetyl groups are not removed by treatment with hydroxylamine under the conditions which restore enzymic activity. Estimates of the number of amino groups available at each stage for reaction with ninhydrin are shown in Table I.

Supporting evidence, for the formation of O-acetyl tyrosine residues when α-amylase is treated with N-acetylimidazole, comes from a study of pH absorbance curves of the native and acetylated enzymes. The titration curve of the inactive acetylated protein differs from that of the native protein as is shown in Fig. 3 (the values reported are for 300 µm; a similar pattern was obtained at 245 µm; both these wave lengths are away from those affected by ionization of tyrosine). Fewer tyrosyl residues are available for titration at pH 11.5 when the absorbance is read within 2 min of reaching the pH value. However, when the acetylated protein is allowed to stand at pH 11.5 for 60 min the molar absorbance approaches that of the native enzyme (Table II). N,O-Diacetyltyrosine is hydrolyzed on standing at pH 11.5, the absorbances at 245 and 300 µm are those expected for N-acetyltyrosine after 30 min.

When acetylated protein is exposed to high pH, enzymic activity is partially regained as shown in Fig. 4. The native protein is stable up to pH 12.0 when assayed within 2 min of reaching that pH. When a sample of acetylated enzyme, which has 40% activity, is diluted into the assay mixture within 2 min of

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**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity</th>
<th>Tyrosyl residues acetylated</th>
<th>Acetylhydroxamates</th>
<th>Free amino groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native α-amylase</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl α-amylase</td>
<td></td>
<td></td>
<td></td>
<td>8.3</td>
</tr>
<tr>
<td>α-Amylase acetylated in presence of starch</td>
<td>20</td>
<td>3.5</td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>α-Amylase acetylated in presence of dextrin</td>
<td>90</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native α-amylase + hydroxylamine</td>
<td>65</td>
<td>2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl α-amylase + hydroxylamine</td>
<td>100</td>
<td>0</td>
<td></td>
<td>4.5</td>
</tr>
</tbody>
</table>

The inactive acetylated enzyme, when exposed to hydroxylamine at pH 7.5 and 25° for 20 min, regains its catalytic activity (Table I). Concomitantly the spectral properties of the modified enzyme are restored to those of the native enzyme control. The number of moles of acetylhydroxamate formed when hydroxylamine removes the O-acetyl groups from tyrosine, is in fair agreement with the determination, by spectral difference, of the number of tyrosyl residues acetylated.

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When acetylated protein is exposed to high pH, enzymic activity is partially regained as shown in Fig. 4. The native protein is stable up to pH 12.0 when assayed within 2 min of reaching that pH. When a sample of acetylated enzyme, which has 40% activity, is diluted into the assay mixture within 2 min of
reaching pH 11.5 some activity is regained. However, when it has been exposed to pH 12.5 for 2 min the activity increases to 70% of its original value. After 1 hour at pH 11.5 the acetylated protein regains up to 85% of its original activity. At pH 12.5 the native enzyme is unstable retaining only 15% of its activity after 1 hour and so the initial gain in activity for the acetylated enzyme is not observed.

Maleic Anhydride—B. subtilis α-amylase was treated with various amounts of maleic anhydride; the results are presented in Table III. When a 500-fold molar excess of reagent is used the enzyme loses amino groups equivalent to 4.7 eq of alanine (ninhydrin color reaction) while retaining 61% of its original activity.

Tetranitromethane—When a 100-fold molar excess of tetranitromethane is used to modify B. subtilis α-amylase at an enzyme concentration of 10 mg per ml, 70% of the enzyme activity is lost in 2 hours. Amino acid analyses show 1.2 residues of 3-nitrotyrosine, while the absorption spectrum at 428 m\(\mu\) indicates 1.0 residue of 3-nitrotyrosine per 24,000 molecular weight subunit (Table IV). The amino acid analyses show that no other amino acid residues are apparently affected by the tetranitromethane treatment.

When the molar excess of tetranitromethane was increased to 250-fold no greater loss of activity was observed in 2 hours. However, on standing overnight 90% of the activity was lost. Details of the results are presented in Table IV. In preliminary experiments with a 100-fold excess of reagent with 2 mg per ml of enzyme only 20% of activity was lost in 2 hours. The high enzyme concentration and long time for reaction which appear necessary for nitration meant that it was not feasible to use the substrate, starch, as a protective agent against nitration as it was all hydrolyzed during the first minutes of the treatment. When the competitive inhibitor, dextrin, was present, the enzyme appeared to be inactivated more rapidly than the control (Table IV).

The apparent pK of the 3-nitrotyrosyl residue in modified B. subtilis α-amylase was determined spectrophotometrically at 428 m\(\mu\) to be 7.74, as compared with a pK\(_{\text{app}}\) of 6.8 for 3-nitrotyrosine and 7.0 for N-acetyl-3-nitrotyrosine (18). In the presence of starch or dextrin the solutions tended to be cloudy and unsuitable for determining whether there was a change in the apparent pK of the 3-nitrotyrosyl residue when the substrate or competitive inhibitor is bound.

The 3-nitrotyrosyl residue in the inactive nitrated protein was reduced to the 3-amino derivative by sodium dithionite. The reaction was complete in 15 min; the absorbance in the region of
TABLE IV

Nitration of tyrosyl residues in B. subtilis α-amylase treated with tetranitromethane

Samples were treated as described under "Experimental Procedure." Enzymic activity assayed by the method of Coleman and Elliott (8). 3-Nitrotyrosine residues were determined by amino acid analysis (15) and from the absorbance at 428 nm (5); chemical molecular weight, 24,000. Dextrin when present was at a concentration of 15 mg per ml.

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>Molar excess of reagent</th>
<th>Time of treatment</th>
<th>Enzymic activity remaining</th>
<th>Residues of 3-nitrotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td>hr</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>2</td>
<td>30</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>250</td>
<td>2</td>
<td>30</td>
<td>1.0</td>
</tr>
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<td>10</td>
<td>250</td>
<td>16</td>
<td>10</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>2</td>
<td>80</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>6</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>2 + Dextrin</td>
<td>100</td>
<td>2</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2 + Dextrin</td>
<td>100</td>
<td>6</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

428 nm was eliminated. There was no change in the enzymic activity of the sample after reduction. Amino acid analysis showed that 3-nitrotyrosine was no longer present; however a new peak was readily detected before lysine in the Technicon autoanalyzer system (133-cm column, Chromo-bead Type A resin, autograd elution, 60°). The position of this peak corresponds to that of 3-aminoxylyrosine. The analysis showed that the reduction had been quantitative, while no other amino acid was affected by the dithionite treatment.

Acetylation Followed by Nitration—B. subtilis α-amylase which had been inactivated by acetylation with N-acetylimidazole was then treated with tetranitromethane. Amino acid analysis showed that a maximum of 0.1 residue of 3-nitrotyrosine had been formed, while 12 residues of tyrosine were recovered intact.

When the sample of α-amylase was acetylated in the presence of dextrin (60% activity remaining) and then reacted with tetranitromethane (activity down to zero) less than 0.1 residue of 3-nitrotyrosine was detected on amino acid analysis, however only 10 residues of tyrosine were recovered. There was no peak in the position expected for 3,5-dinitrotyrosine (5) and no evidence of oxidation during hydrolysis.

Peptide Mapping—Peptide maps of the tetranitromethane modified B. subtilis α-amylase were prepared. One yellow peptide containing a single residue of 3-nitrotyrosine was detected. The position of this peptide is shown in Fig. 5. A more intense color is obtained when the chromophore is exposed to ammonia vapor; however, no other yellow spot became evident on the peptide map under these conditions. The amino acid composition of this peptide was determined in duplicate with an alternative range card in the recorder of the analyzer, so as to obtain increased sensitivity (approximately 5 moles of peptide applied to each column). The analyses showed the molar ratios to be: aspartic acid, 2.2; threonine, 1.2; serine, 1.8; glutamic acid, 2.5; proline, 1.0; glycine, 3.1; valine, 1.1; methionine (sulfone), 0.9; phenylalanine, 1.0; 3-NO₂-tyrosine, 1.0; and lysine, 1.0. This corresponds with the peptide which occurs in the same relative position in a map of the native enzyme except that it then contains 1 residue of tyrosine; molar ratios: aspartic acid, 1.9; threonine, 1.0; serine, 1.6; glutamic acid, 2.0; proline, 1.1; glycine, 3.5; valine, 1.3; methionine (sulfone), 1.0; phenylalanine, 1.1; tyrosine, 1.0; and lysine, 1.0. It is considered that any unmodified peptide would have overlapped the nitratated peptide; thus, as there was no tyrosine detected in the analyses of the yellow peptide and 1.0 eq of 3-nitrotyrosine was obtained compared with lysine, the particular tyrosine residue apparently reacted quantitatively with the tetranitromethane.

Attempts were made to identify the particular tyrosyl residue indicated by the difference observed between samples of α-amylase acetylated in the presence or absence of starch. These attempts, with ¹⁴C-N-acetylimidazole, were unsuccessful. Two approaches were employed. In one, samples of the enzyme were labeled in the presence and absence of starch, and in the other the enzyme was previously treated with nonradioactive N-acetylimidazole in the presence of starch and then, after removal of the starch, inactivated by ¹⁴C-N-acetylimidazole. In neither case was the protein labeled to the expected level and peptides were only weakly labeled. This may have been caused by difficulties associated with the instability of the reagent, although the experimental procedure was varied to try to overcome the problem.

Tryptic peptide maps of α-amylase which had been inactivated by diazotized sulfanilic acid were prepared, again with the aim...
of seeing if there was a relationship between the tyrosyl residues involved in this and the tetratromethane treatment. The inactivation was performed with a 25:1 molar ratio of reagent to enzyme for 20 to 30 min, 80% loss of activity (see Reference 1 for diazotization procedure). Amino acid analysis showed that 1.5 residues of lysine and 0.8 residue of tyrosine per 24,000 g of protein had apparently reacted. Analyses of other samples treated with different molar ratios and for different times all showed that the tyrosyl residues reacted to a greater extent than tyrosine and that 1.2 residues of tyrosine had reacted when 95% of the enzymic activity had been lost. On the peptide maps, yellow bands near the origin were obtained but amino acid analyses showed that the peptides were not pure. On attempted purification the yellow color was lost. When 35S-sulfanilic acid and autoradiography were used for detection the analyses did not correspond with any known tryptic peptide. The derivatives of neither tyrosine nor lysine are stable to acid hydrolysis and so where a}

\[ \text{DISCUSSION} \]

The results obtained with N-acetylimidazole, maleic anhydride, and tetratromethane implicate tyrosyl residues as being important for the catalytic activity of \textit{B. subtilis} \( \alpha \)-amylase, while reaction of free l-lysyl residues does not have a marked effect upon activity. The question arises as to whether the particular residue of tyrosine labeled by tetratromethane is related to the tyrosyl residues indicated by other treatments and what role these residues might be considered to have.

Consider first the evidence obtained from each reagent used separately. N-Acetylimidazole is obviously acetylating both tyrosyl and l-lysyl residues. However, the restoration of activity by hydroxylamine or alkali, with the concomitant reversal of spectra, indicates that tyrosyl residues are important for activity while the lysyl residues, which remain acetylated after complete recovery of activity with hydroxylamine, are clearly not required. The linear relationship between the percentage loss of activity and the number of tyrosyl residues acetylated by N-acetylimidazole shows that apparently all the exposed tyrosines are reacting at the same rate. Thus, if there is one tyrosine with which activity is directly associated, that tyrosine is not acetylated any faster, or slower, than the other exposed tyrosines, as shown by this general acetylation agent.

Isemura and Imanishi (19) have titrated the tyrosyl residues in \textit{B. subtilis} \( \alpha \)-amylase. Their results, calculated on the basis of 12 tyrosyl residues for a chemical molecular weight of about 24,000, show that about 4 residues ionize with a normal pK between pH 9.0 and 11.5 while the remaining 8 tyrosyl residues display a time dependent ionization at pH values between 11.5 and 13, and are considered to be "buried." The pH absorbance curve for the acetylated protein (Fig. 3) has fewer tyrosyl residues which ionize up to pH 11.5 and would indicate that it is these normal, exposed tyrosyl residues which have been modified. The number of tyrosines acetylated approaches the number of tyrosines considered to be exposed.

The protectively effective of the substrate starch and the difference of 1 residue of tyrosine per 24,000 g of protein, when the inactive and protected acetylated proteins are compared, does suggest a special role for 1 residue of tyrosine.

The effect of dextrin, a competitive inhibitor of \( \alpha \)-amylase activity, was more complex. While it protected the enzyme during mild treatments, it was much less effective for more severe acetylation. The results point to a possible difference in the mode of binding of starch and dextrin. Starch must still be bound effectively by the active N-acetylated protein, after removal of O-acetyl groups from tyrosines and when the enzyme is protected by starch, the active enzyme has all but the particular tyrosyl residue acetylated. If this tyrosyl residue is actually at the catalytic site, or a site essential for binding or correct conformation, then it would be expected to be directly protected by the substrate. Dextrin may both the exposed l-lysyl and tyrosyl residue for binding (or the presence of acetyl groups may interfere with binding), so that binding is weakened and protection lost with increased acetylation; or alternatively, in whatever way dextrin is bound it may not protect the catalytic site, or other site essential for hydrolysis of starch, from inactivation by small molecules if these are present in sufficient concentration. The latter view, with dextrin providing only partial steric hindrance of the hydroxyl group of the tyrosine, would be favored in the interpretation of the effect of dextrin upon inactivation by tetratromethane (below), although in that case steric hindrance of the aromatic ring would not be considered to occur.

The results obtained with maleic anhydride complement those obtained with N-acetylimidazole in that modification of l-lysyl residues is not alone sufficient for complete loss of enzymic activity; only when extensive modifications, which include the substitution of acidic for basic charges, are made does the enzyme lose over 50% of its original catalytic activity.

The results of peptide mapping of the tetratromethane-treated protein indicate that a unique tyrosine is involved in this loss of activity and that the over-all analysis which showed 1 tyrosine nitrated per 24,000 g of protein is not caused by reaction of a fraction of each of the exposed tyrosyl residues. This is a remarkable occurrence compared with acetylation, since all the exposed tyrosyl residues appear to have the same reactivity towards N-acetylimidazole.

The higher apparent pK of the 3-nitrotyrosyl residue in its environment in the modified protein, compared with that of the free amino acid derivative, is in direct contrast to the lower pK of the 3-nitrotyrosyl residue in modified carboxypeptidase A. In that case the tyrosyl residue appeared to be especially reactive, requiring much less reagent for reaction in a shorter time than observed for the \( \alpha \)-amylase. The simplest interpretation is that if the 3 nitrotyrosyl derivative has a lower apparent pK, then the tyrosyl residue involved also has a lower pK caused by the same environment, and hence in carboxypeptidase A that particular tyrosine is more reactive. Similarly, in the present case the higher pK would seem to indicate that the tyrosyl residue should be less reactive than the average. The fact that this was not so may mean that the higher pK is within the normal range of values expected for the tyrosyl derivative and that the selective reactivity observed may be caused by partial accessibility to tetratromethane. Alternatively, the nitration may have caused a conformational change such that the 3-nitrotyrosine is in a more hydrophobic environment than the original tyrosine and not reflect the properties of that tyrosine residue before it reacted. An increase in the apparent pK of the 3-nitrotyrosine residue in nitratated carboxypeptidase was observed when a competitive inhibitor was added to the modified enzyme and one explanation given for this was that a conformational change...
may have moved the nitrotyrosine group into a more hydrophobic region (18).

As explained under “Results,” it is not practicable to use starch as a protective agent because the concentration of enzyme required hydrolyzes the starch before any reaction could occur. The apparent effect of dextrin increasing the extent of inactivation by tetranitromethane could possibly be an example of “induced fit” (20) with the small reagent being able to approach the reactive aromatic ring which had been activated by a change in the conformation of the protein (and the microenvironment of the hydroxyl group) when the competitive inhibitor is bound. However, with the high $K_i$ (approximately 1.4 mg per ml) for dextrin and its limited solubility (saturated at about 20 mg per ml, the solution tending to be viscous), the enzyme is present at about the same concentration as the inhibitor, so that the system is not an attractive one and the observation was not pursued. Unfortunately, B. subtilis α-amylase possesses only very limited affinity for low molecular weight substrates or inhibitors (21) which would be preferable for such studies. In this regard starch is not an ideal substrate and so no attempt has been made to determine whether the 30% activity remaining after the standard treatment with tetranitromethane (1 to 1.2 residues of tyrosine nitrated in 2 hours) is caused by a change in $K_m$ or $V_{max}$ for the hydrolysis.

The samples treated with tetranitromethane after acetylation were examined in order to see whether there is a relationship between the tyrosyl residues involved in the two reactions. The inactive acetylated sample clearly is no longer susceptible to nitration. The sample which had been partially protected from acetylation, by dextrin, did not yield a significant amount of 3-nitrotyrosine but the total recovery of tyrosine was low. The result from the first sample would suggest that the residue normally nitrated is one of those acetylated. The second sample is the more interesting and technically more difficult to prepare, but the result obtained is equivocal. It is possible that the nitration proceeded to give some form of tyrosine which did not survive acid hydrolysis, or that the loss of activity could have been caused by a reaction at a different site although analysis did not show loss of any other amino acid. It will be necessary to study the rate of inactivation of partially acetylated samples by tetranitromethane and see if there is a consistent loss of tyrosine.

Failure to obtain reasonable labelling of the protein with $^{14}$C-N-acetyl imidazole prevented identification of the tyrosine residue involved and precluded correlation with the tyrosine modified in the nitrated protein. Examination of the maps of enzyme that had been inactivated by diazotized sulfanilic acid confirmed the analytical results that lysyl residues had been modified; this modification interfered with hydrolysis by trypsin such that a comparison of the tryptic map with that of the nitrated protein was not possible.

Although a correlation between the tyrosyl residues indicated by the various treatments has not been shown, the loss of activity observed in each case is compatible with the enzyme requiring the free hydroxyl group of a particular tyrosine. When acetylated (or reacted with diazotized sulfanilic acid) the hydroxyl is no longer available, while with nitration the pK of the hydroxyl is lowered several units and in addition a large grouping is introduced (the reduction of the nitro to an amino group, would still affect the pK and size of the original tyrosine). It is, however, not possible with the present evidence to distinguish between the alternatives that the tyrosyl residue is required for catalysis, for effective binding of substrate or for the enzyme to achieve an active conformation.

The observation of a single modified peptide on peptide maps of tetranitromethane inactivated enzyme strengthens the evidence from peptide mapping that the chemical minimum molecular weight of B. subtilis α-amylase is about 24,000. As the active enzymic entity has a molecular weight of about 48,000 and, on the basis of the reactivity towards inactivators, the 2 tyrosyl residues (1 per 24,000 mol wt) are equivalent, a point of interest is whether the active centers in the larger unit are independent and intrachain or interchain interactions of parts of each chain. The latter would offer a means of preventing activity of the subunit as synthesized within the cell, the extracellular enzyme not acquiring activity until two subunits associate with the calcium required for stability and activity.

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REFERENCES
