Environmentally Sensitive Tyrosyl Residues. Nitration with Tetranitromethane*

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ABSTRACT: Spectral titrations and the effects of β-phenylpropionate indicate that a tyrosyl residue of native carboxypeptidase and the single nitrotyrosyl residue of nitrocarboxypeptidase are sensitive to their environments. Mononitrocarboxypeptidase has 170% esterase and less than 10% peptidase activity relative to the native enzyme. Spectral titrations indicate an apparent pK of 6.3 for the nitrotyrosyl residue in this enzyme, compared to 7.0 for N-acetyl-3-nitrotyrosine. However, nitration of carboxypeptidase in the presence of β-phenylpropionate does not change the activities, and the apparent pK of the nitrotyrosyl residues is now 6.9, similar to that of the model compound. Apparently a nitration-susceptible active center tyrosyl residue of the native enzyme has an abnormally low pK, perhaps due to features of its immediate chemical environment which induce its ionization.

The esterase activity of nitrocarboxypeptidase can be inhibited by β-phenylpropionate, and spectral titrations in the presence of this inhibitor now indicate a pK of 7.0, suggesting that this reagent alters the immediate chemical environment of the active center nitrotyrosyl residue. This may be due to direct interaction with that group or to conformational changes which move the nitrotyrosyl group into a more hydrophobic region.

We have recently demonstrated that tetranitromethane[1] is a selective and mild reagent for the nitration of tyrosine (Riordan *et al.*, 1966a) and tyrosyl residues in peptides and proteins (Sokolovsky *et al.*, 1966). Since the product of the reaction (3-nitrotyrosine) is an ionizable chromophore, nitration could be employed to probe the immediate chemical environment of nitration-susceptible tyrosyl residues which participate in the biological activities of proteins. In such instances nitration might provide a means to indicate the chemical basis of the functional characteristics. If the resultant nitroprotein is an enzyme which retains activity, *e.g.*, carboxypeptidase A (Riordan *et al.*, 1966b), perturbation of the nitrotyrosyl spectrum through the binding of substrates or inhibitors might allow an exploration of the active center topology.

In carboxypeptidase one functional tyrosyl residue is nitrated preferentially, an apparent consequence of environmental influences on its ionization and a possible guide to the chemical basis of its catalytic role. This is suggested by the effects of a competitive inhibitor, β-phenylpropionate, on both the spectral properties and the apparent pK of the nitrotyrosyl residue of the nitroenzyme.

Materials

Carboxypeptidase A, prepared by the method of Anson (1937) and obtained from Worthington Biochemical Corp., had a zinc:protein ratio between 0.98 and 1.03 g-atoms/mole based on a molecular weight of 34,600 (Bargetzi *et al.*, 1963). TNM was obtained from the Aldrich Chemical Co. The preparation of N-acetyl-3-nitrotyrosine has been described (Sokolovsky *et al.*, 1966). Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957).

Methods

Nitration of carboxypeptidase was carried out in 0.05 M Tris–1 M NaCl buffer, pH 8.0, using a fourfold molar excess of TNM per mole of enzyme. Stock TNM was diluted tenfold with 95% ethanol and a suitable aliquot was added to the enzyme (10–20 mg/ml). The reaction mixture was kept at room temperature (20°) for 45 min and then separated on a Bio-Gel P-4 column to remove nitroform and unreacted reagent. In those instances where spectral titrations were to be performed, the column was equilibrated with 0.005 M Tris–1 M NaCl, pH 8.0. The degree of nitration was determined either by measuring the absorbance of the nitroenzyme at 381 or 428 mμ or by amino acid analysis.

Spectral titrations were performed on aliquots of the nitrotyrosyl-containing material diluted with 0.2 M Tris–0.2 M acetate–0.5 M NaCl buffers at various pH values by measuring the change in absorbance at 428 (for the protein) or 427 mμ (for N-acetyl-3-nitrotyrosine).

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[1] Abbreviation used: TNM, tetranitromethane.

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Cyclo Chemical Corp.) (Coleman and Vallee, 1960), and is expressed as an apparent proteolytic coefficient, C, defined as log a_0/a per minute per micromole of enzyme, where a_0 and a represent the concentration of substrate at times zero and t, respectively (Simpson *et al.*, 1963). The assays were performed at 0° in 0.02 M sodium Veronal–1.0 M NaCl buffer, pH 7.5, and C was calculated from the linear portion of the first-order reaction plots before hydrolysis exceeded 15%.

Esterase activity was determined by pH titration (Snoke *et al.*, 1948) with 0.1 M NaOH of the hydrogen ions released on hydrolysis using a pH-Stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagin). Assays were performed at 25° with 3 ml of 0.01 M hippuryl DL-β-phenyllactate (Cyclo Chemical Corp.) in 0.2 M NaCl–0.005 M Tris buffer, pH 7.5. Activities are expressed as zero-order velocity constants, k, with units of equivalents of H^+ released per minute per mole of enzyme.

Protein concentration was measured by the absorbance at 278 mμ. The molar absorptivity of native carboxypeptidase A is 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Absorption Spectra. A Zeiss PMQ II spectrophotometer was used for absorbance measurements at single wavelengths, and spectra were obtained with a Cary Model 15 MS or a Unicam Model SP 800 automatic recording instrument.

pH was measured with a Radiometer pH meter (Model pH M4) equipped with a Radiometer GK 2021 electrode.

Amino acid analyses were carried out with a Spinco Model 120B automatic amino acid analyzer by the technique of Spackman *et al.* (1958). Acid hydrolysis was carried out in sealed, evacuated tubes with 6 N HCl at 105° for 24 hr.

Results and Discussion

The visible absorption spectrum of *N*-acetyl-3-nitrotyrosine in 0.2 M Tris–0.2 M acetate–0.5 M NaCl is markedly dependent upon pH (Figure 1). Below pH 5, the un-ionized phenol absorbs maximally at 360 mμ and ε is 2790 M^{-1} cm^{-1}, while above pH 9, the nitrophenolate ion absorbs maximally at 427 mμ and ε is 4100 M^{-1} cm^{-1}. Spectral titrations based on the change in absorbance at 427 mμ have indicated that under these buffer and salt conditions the apparent pK for the ionization of the phenolic hydroxyl group is 7.0. However, a variety of factors which alter the electrochemical characteristics of the medium can predictably influence the degree of dissociation. Thus, an increase in salt concentration lowers, whereas addition of a nonpolar solvent, *e.g.*, dioxane, raises the pK to higher values.

Either the absorption of 3-nitrotyrosine at 427 mμ, λ_{max}, or at 381 mμ, an isosbestic point of the phenol–phenolate spectral family, can be employed to quantitate the degree of nitration of proteins (Sokolovsky *et al.*, 1966). The ease of quantitation together with the high selectivity of tetranitromethane for tyrosyl residues provide very effective means to study the role of such residues in biological function.

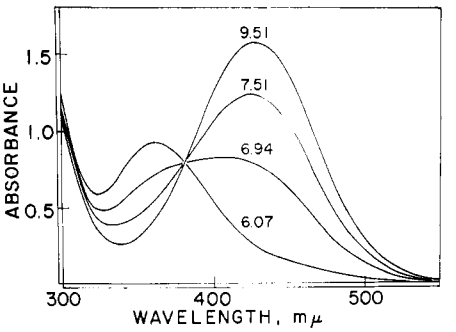

FIGURE 1: Absorption spectra of *N*-acetyl-3-nitrotyrosine (2.5×10^{-4} M) in 0.2 M Tris–0.2 M acetate–0.5 M NaCl at the pH indicated.

Within 30 min after the addition of a fourfold molar excess of TNM to a 2×10^{-4} M solution of carboxypeptidase A in 0.05 M Tris–1 M NaCl, pH 8.0, 20°, peptidase activity decreased to below 10% of the control (Table I). Concomitantly, esterase activity increased to about 170% in this experiment. Approximately 1.2 moles of nitrotyrosine/mole of protein were formed, as determined either by spectral or by amino acid analysis (Sokolovsky *et al.*, 1966). If the nitration reaction is carried out in the presence of the inhibitor, β-phenylpropionate, these changes in activity do not occur and one less tyrosyl residue is nitrated. Apparently, such changes in carboxypeptidase activity are due to the nitration of a single residue.

The pH dependence of tyrosyl nitration suggests an ionic mechanism involving the phenolate anion and the nitronium cation. Hence, the specific nitration of a single tyrosyl residue in carboxypeptidase might be attributable to an unusually low pK for this residue. The preferential deacetylation of the active center tyrosyl residues of acetylcarboxypeptidase by hydroxylamine observed previously (Simpson *et al.*, 1963) would be consistent with a low pK for these tyrosyl residues (Bruice *et al.*, 1962). Since the enzyme contains a total of 19 tyrosyl residues, the detection of one particular acidic residue by conventional spectrophotometric titration could pose problems. However, the same vicinal factors which may induce ionization in the native enzyme might also be expected to operate in the nitroenzyme. The distinct visible absorption of nitrotyrosine would permit direct observation of this phenomenon.

Spectral titration of mononitrocarboxypeptidase was carried out in 0.2 M Tris–0.2 M acetate–0.5 M NaCl by measuring the absorbance at 428 mμ as a function of pH. The apparent pK for the nitrotyrosyl residue is 6.3 (Table II), about 0.7 unit lower than that found for *N*-acetyl-3-nitrotyrosine.

TABLE I: Nitration of Carboxypeptidase with TNM in the Presence and Absence of β-Phenylpropionate. Effect on Activities.[a]

	Protected[b]			Unprotected[c]		
Time (min)	NO_2-Tyr[d] (mole/mole)	Esterase ($k \times 10^{-3}$)	Peptidase C	NO_2-Tyr[d] (mole/mole)	Esterase ($k \times 10^{-3}$)	Peptidase C
0	—	7.2	40	—	7.2	40
5	—	7.2	40	0.25	8.6	30
10	0.14	7.1	40	0.62	10.5	20
20	0.21	7.1	39	0.88	11.4	13
30	0.3	7.2	40	1.25	12.5	5
45	0.3	7.2	39	1.2	12.5	4

[a] Nitration was carried out with a fourfold molar excess of TNM, 20°, 0.05 M Tris–1 M NaCl, pH 8.0. Peptidase assays were performed according to Coleman and Vallee (1960) and esterase assays according to Snoke *et al.* (1948). [b] In the presence of 0.1 M β-phenylpropionate. [c] In the absence of β-phenylpropionate. [d] Determined both spectrally and by amino acid analyses (Sokolovsky *et al.*, 1966).

This pK value, abnormally low compared with *N*-acetyl-3-nitrotyrosine. is not characteristic either of proteins in general or of the specific conditions employed in these experiments. Carboxypeptidase was nitrated with a 32-fold molar excess of reagent in the presence of 0.1 M β-phenylpropionate. After removal of the inhibitor by gel filtration, the product exhibited esterase and peptidase activities virtually identical with those of the native enzyme, but now contained 2.7 nitrotyrosyl residues. Spectral titration in 0.2 M Tris–0.2 M acetate–0.5 M NaCl revealed that these nitrotyrosyl residues exhibit an apparent pK of 6.9, close to that for *N*-acetyl-3-nitrotyrosine (Table II).

TABLE II: Apparent Ionization Constants of Various Nitrophenols.[a]

Substance	pK_{app}
o-Nitrophenol	7.2
3-Nitrotyrosine	6.8
N-Acetyl-3-nitrotyrosine	7.0
Mononitrocarboxypeptidase[b]	6.3
Mononitrocarboxypeptidase[c] + 0.1 M β-phenylpropionate	7.0
Nitrocarboxypeptidase[d]	6.9

[a] All spectra were measured in 0.2 M Tris–0.2 M acetate–0.5 M NaCl at a concentration of 2.5×10^{-4} M. [b] Carboxypeptidase nitrated with a fourfold molar excess of TNM. [c] Carboxypeptidase nitrated with a fourfold molar excess of TNM. Spectra were measured in the presence of 0.1 M β-phenylpropionate. [d] Carboxypeptidase nitrated with a 32-fold molar excess of TNM in the presence of 0.1 M β-phenylpropionate.

Although the peptidase activity of nitrocarboxypeptidase is low, the esterase activity is considerable and can still be inhibited by β-phenylpropionate. Since β-phenylpropionate protects an active center tyrosyl residue from nitration, it might still interact with that group, perhaps indirectly, even after it has been nitrated. Such an interaction might be detected by spectral changes.

Hence, the effect of β-phenylpropionate on the spectrum of nitrocarboxypeptidase was examined (Figure 2). Increasing concentrations of inhibitor decrease the absorbance at 428 mμ. The magnitude of the changes in absorbance at 428 mμ, brought about by a given concentration of β-phenylpropionate, correlates closely with the degree of esterase inhibition observed with the same concentration of reagent (Figure 3).

The spectral changes are similar to those observed on conversion of nitrophenolate to nitrophenol. Since the pH was maintained constant at 8.0 for each of the solutions employed, the data suggest that the effect is due to an alteration in the apparent pK of the nitrotyrosyl group. Spectral titration of nitrocarboxypeptidase, in the presence of 0.1 M β-phenylpropionate, results in an apparent pK of 7.0, virtually identical with that determined in buffer for the protected nitroenzyme or for the model compound. The same concentration of inhibitor neither affects the spectral titration of the protected nitroenzyme nor of *N*-acetyl-3-nitrotyrosine. Hence, it would appear that the inhibitor only alters the immediate chemical environment of the specific functional nitrotyrosyl residue of carboxypeptidase here under consideration. This effect on the spectrum of nitrocarboxypeptidase is not restricted to β-phenylpropionate but has been observed both with several other inhibitors and with two peptide substrates, *i.e.*, glycyl-L-leucine and glycyl-L-phenylalanine.

Environmentally sensitive protein-bound chromophores, recently termed "reporter groups" (Burr and

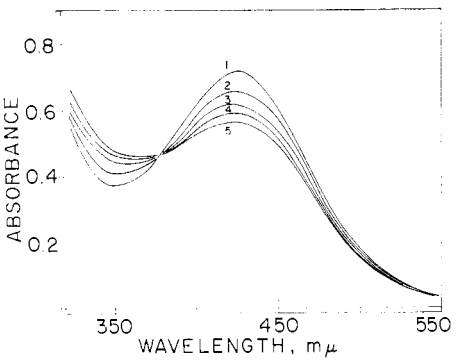

FIGURE 2: The effect of β-phenylpropionate on the spectrum of nitrocarboxypeptidase. Spectra were measured in 0.2 M Tris–0.2 M acetate–0.5 M NaCl buffer, pH 8.0. The concentrations of β-phenylpropionate were as follows: 1 (control), none; 2, 0.01 M; 3, 0.025 M; 4, 0.05 M; and 5, 0.1 M.

Koshland, 1964), have been employed previously in a number of studies of protein structure (Weber, 1952; Klotz and Ayers, 1957; Burr and Koshland, 1964; Kirtley and Koshland, 1966). The introduction of nitro groups into tyrosyl residues of proteins by means of TNM represents an important and convenient addition to these techniques. The mild reaction introduces a small substituent which is unlikely to create configurational anomalies on the basis of steric hindrance. The resultant spectral characteristics are generated by converting an amino acid residue in the primary structure of the enzyme into a visible chromophore.

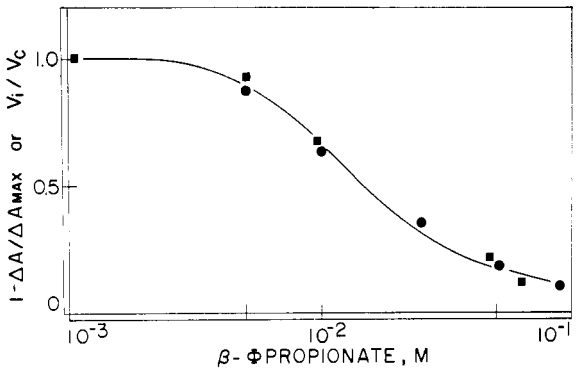

FIGURE 3: The effect of β-phenylpropionate on the esterase activity (■) and on the absorbance at 428 mμ (●) of nitrocarboxypeptidase. $\Delta A/\Delta A_{max}$ represents the fractional decrease in absorbance at 428 mμ, observed in the presence of the concentration of β-phenylpropionate indicated, while ΔA_{max} is the maximal decrease in absorbance at 428 mμ calculated by extrapolation to infinite concentration of β-phenylpropionate.

The present data suggest that β-phenylpropionate, other inhibitors, and substrates may alter the immediate chemical environment of the active center tyrosyl residue of carboxypeptidase either by direct interaction with that group or as a result of conformational changes which move the tyrosyl group into a more hydrophobic region.

The apparent activation of the functional tyrosyl residue by its environment, as evidenced by changes in the apparent p*K*, suggests a possible chemical basis for its catalytic role. Such alteration of the apparent p*K* of this residue can also be brought about by means of chemical modification, such as the reduction of the nitro to an amino group recently accomplished by us. This particular modification gives rise to the opportunity to perform a wide variety of subsequent ones (M. Sokolovsky, J. F. Riordan, and B. L. Vallee, in preparation) which will allow predictable alterations of the phenolic p*K*. The results of such selective modifications, when accompanied by activity changes, should aid greatly in understanding the mechanism of this and perhaps of other enzymes.

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References

Anson, M. (1937), *J. Gen. Physiol. 20*, 663, 777.

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. T., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Burr, M., and Koshland, D. E., Jr. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1017.

Bruice, T. C., Fife, T. H., Bruno, J. J., and Brandon, N. E. (1962), *Biochemistry 1*, 7.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Kirtley, M. E., and Koshland, D. E., Jr. (1966), *Biochem. Biophys. Res. Commun. 23*, 810.

Klotz, I. M., and Ayers, J. (1957), *J Am. Chem. Soc. 79*, 4078.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966a), *J. Am. Chem. Soc. 88*, 4104.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966b), Abstracts, 152nd National Meeting of the American Chemical Society, Sept, New York, N. Y.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Spackman, D. H., Stein. W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Thiers, R. E. (1957), *Methods Biochem. Analy. 5*, 273.

Weber, G. (1952), *Biochem. J. 51*, 155.