CONVERSION OF 3-NITROTYROSINE TO 3-AMINOTYROSINE IN PEPTIDES AND PROTEINS*

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Specific chemical modifications have helped to establish the participation of particular amino acid side chains of proteins in biological function. However, the experimental delineation of chemical factors which might account both for biological specificity and selective, chemical reactivity remains difficult.

A given residue can be modified by chemically different means leading alternately to changes in its charge, size, or other physicochemical properties. Such varied modifications of a particular type of residue represent one approach to discern the basis for the chemical reactivity and, further, its relationship to biological specificity. We have begun to examine tyrosine from this point of view (Riordan et al., 1965; Simpson and Vallee, 1966), and nitration with tetranitromethane (Riordan et al., 1966; Sokolovsky et al., 1966) has augmented the means to study the role of this amino acid in enzyme catalysis.

The number of potential derivatives of tyrosine has now been extended further by reducing the nitro group of 3-nitrotyrosine to an amino group, using sodium hydrosulfite (Grandmougin, 1906; Wasmuth et al., 1964). Thus, for instance, this amino can be converted to an azo group or substituted with

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a wide variety of reagents. The functional consequences of such modifications will be reported later.

When a 5 to 6-fold molar excess of sodium hydrosulfite ($Na_2S_2O_4$) is added to 3-nitrotyrosine or N-acetyl-3-nitrotyrosine in 0.05 M Tris buffer, pH 8.0, the characteristic nitrotyrosyl absorption band, λ_{max} = 428 mμ, is abolished within two minutes. A new maximum corresponding to 3-aminotyrosine appears at 288 mμ, ε = 2800. Between pH 6 and 9 the rates of reduction are essentially constant. Chromatography of the reduction mixture on the 15 cm column of the Spinco Model 120 B amino acid analyzer, operated at 25°, reveals a substance which elutes in the position of authentic 3-aminotyrosine, i.e., just after the acid and neutral and separated from the basic amino acids (Figs. 1A and 1C). Aminotyrosine is stable in 6 N HCl at 105° for 24 hours and is recovered in better than 95% yield, allowing direct determination of the aminotyrosine content of proteins.

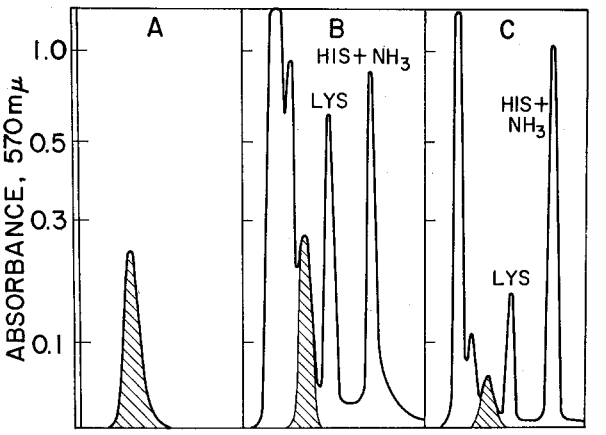

Figure 1 A: Elution profile of 3-aminotyrosine on chromatography on the short column of the Spinco Model 120 B amino acid analyzer at 25°.

1 B: Elution profile of an acid hydrolyzate of aminotyrosyl-lysozyme. The first peak contains the acidic and neutral amino acids; the second is phenylalanine; the third, indicated by the shading, is 3-aminotyrosine; the fourth is lysine; and the last contains both histidine and ammonia.

1 C: Elution profile of a mixture of 17 common amino acids (Type I calibration mixture, Beckman Instruments, Spinco Division, Palo Alto, California) to which 3-aminotyrosine has been added.

To examine a specific case, lysozyme (Worthington Biochemical Corp.) was nitrated with a 32-fold molar excess of tetranitromethane. The resultant nitrolysozyme, containing 2.6 nitrotyrosyl and approximately 0.4 tyrosyl residues per mole, was reduced with a 24-fold molar excess of sodium hydrosulfite at pH 8.0 in 0.05 M Tris buffer; this reaction was complete within ten minutes. Amino acid analysis of the reduced protein revealed 2.5 moles of 3-aminotyrosine per mole of lysozyme (Fig. 1B), no trace of nitrotyrosine, and no change in any of the other residues. Similarly, the nitro groups introduced into other proteins have been reduced quantitatively.

Sodium hydrosulfite is known to reduce disulfide bonds in proteins (Windus and Turley, 1941; Harris and Brown, 1947). When these conditions were employed for oxidized glutathione little if any disulfide bond cleavage was found. A 5-fold molar excess of sodium hydrosulfite which completely reduces 3-nitrotyrosine is equally effective in the presence of oxidized glutathione. The latter was reduced only slightly by this amount of hydrosulfite, as determined by thin-layer chromatography of the reaction mixture, using 70% propanol as solvent. The effect of $Na_2S_2O_4$ on disulfide reduction may vary, of course, from protein to protein and should therefore be investigated in each instance.

The properties of aminotyrosyl residues are distinct from those of other reactive groups of proteins. The absorption spectrum of 3-aminotyrosine exhibits a maximum at 288 mμ between pH 7.4 - 9.0. In dilute HCl this maximum shifts to 275 mμ, ε = 1600, and in dilute NaOH it shifts to 302 mμ, ε = 4200 (Fig. 2). Spectrophotometric titration yields pK' values of 4.75 and 10.0, analogous to similar data reported for o-aminophenol (Sims, 1959) and corresponding to the amino and hydroxyl groups, respectively. Since the background absorption due to tyrosyl and other aromatic residues can interfere significantly, such titrations are not readily performed in many proteins. Instead angiotensin II, an octapeptide containing one tyrosyl residue, was used as a model. After nitration, reduction, and purification by gel

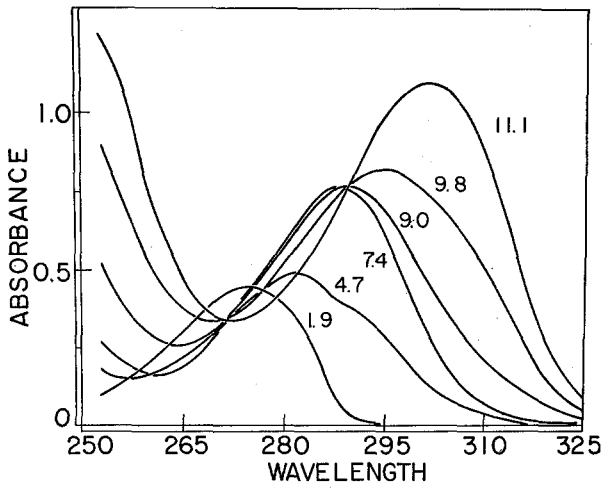

Figure 2: Absorption spectrum of 3-aminotyrosine, 2.67 x 10^{-4} M, in 0.2 M acetate - 0.2 M Tris - 0.5 M NaCl at the pH values indicated.

filtration, the pK' values were 5.1 and 10.0, similar to those of 3-aminotyrosine and o-aminophenol.

Tyrosyl residues may participate in the function of certain enzymes through reactions involving the phenolic hydroxyl groups; alterations in their pK' might be achieved by chemical modifications and reflect in the pH-dependence of their activities. The substituent aromatic amino group can be modified resulting in changes in the pK' of the phenoxy group. It can be preferentially acylated owing to its low pK', for example. Thus, a 15-fold molar excess of acetic or succinic anhydride was added to a 2.5 x 10^{-4} M solution of 3-aminotyrosine and the mixture was stirred and kept at pH 5.0 and 0° for 30 minutes. The aromatic group was modified completely. Spectrophotometric titration demonstrated that the product exhibited a single pK' of 9.22, both for the succinyl and acetyl derivatives. As judged by reaction with ninhydrin, the α-amino group was substituted about 20%. The spectral characteristics of the product showed that the hydroxyl group was not acylated. Table 1 summarizes the pK' values for the hydroxyl group of several modified tyrosines. It should be possible to extend systematically

the variation in pK' by substitutions of the amino group of aminotyrosine with additional reagents. When applied to tyrosyl enzymes these could then be related to their catalytic activities. Such studies are in progress.

TABLE I

pK' Values for Phenolic Hydroxyl Ionization in Tyrosine and Tyrosyl Derivatives

Compound	pK'
Tyrosine	10.1[a]
3-Aminotyrosine	10.0[b]
3-Acetylaminotyrosine	9.22[b]
3-Succinylaminotyrosine	9.22[b]
3-Azotetrazole-tyrosine	8.81[b]
3,4-Dihydroxyphenylalanine	8.7[c]
3-Iodotyrosine	8.2[a]
3-Nitrotyrosine	7.2[b]
3.5-Diiodotyrosine	6.4[a]

[a] Edelhoch, H., J. Biol. Chem. 237, 2778 (1962).

[b] This study.

[c] Biochemist's Handbook, C. Long, Ed., Van Nostrand, Princeton, New Jersey (1961).

The present procedure introduces aminophenols into the primary sequence of proteins. Their unique properties, exemplified by the chelation of metal ions (Sims, 1959) may serve a particularly useful function.

Several aminophenol metal chelates are colored, for instance, thereby allowing identification of the group and possibly the recognition of vicinal effects and perturbation through spectral studies. Further, metals can be introduced into these specified locations of proteins resulting in some of the experimental advantages, characteristic of metalloproteins (Dennard and Williams, 1966; Vallee and Coleman, 1964). Such modifications

would represent an extension of possible procedures to prepare derivatives for X-ray diffraction.

The ready formation of aminophenols here described and the application of this method to protein chemistry adds a novel chemical modification of tyrosyl residues to those already on reacord and provides new permutations in the study of structure-function relationships.

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