CONTROL OF REACTIVITY OF TRYPTOPHAN RESIDUES IN α-CHYMOTRYPSIN BY pH[1]

Narendra B. Oza and Charles J. Martin

Division of Enzymology, Institute for Medical Research,

The Chicago Medical School, Chicago, Illinois 60612

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Koshland *et al.* (1964) and Horton and Koshland (1965) have reported that the chromophoric reagent, 2-hydroxy-5-nitrobenzyl bromide, shows preferential specificity for the tryptophan residues of proteins in acid solution. Although the precise nature of this reaction is not yet known, it has been shown (Koshland *et al.*, 1964; Barman and Koshland, 1966) that for chymotrypsin and other proteins a quantitative relationship exists between the moles of covalently bound chromophoric groups as determined spectrally and the disappearance of tryptophan based on amino acid analyses. Thus, this reagent appears to offer considerable promise as an analytical reagent for the determination of total tryptophan and as a probe for the accessibility of tryptophan residues in proteins in varying solution environments (Bewley and Li, 1965; Yamagami and Schmid, 1966). As such, the reactivity of tryptophan residues to 2-hydroxy-5-nitrobenzyl bromide can complement the use of N-bromosuccinimide for similar purposes (Spande *et al.*, 1966) and the solvent perturbation technique (Williams *et al.*, 1965). Recently, we have employed 2-hydroxy-5-nitrobenzyl bromide to determine the accessibility of the tryptophan residues in chymotryp-

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sin at various pH values. It will be shown that the pH controls the relative size of two populations of enzyme molecules in which all of the tryptophan residues of one population are free to react with the reagent. The other population group contains most of the tryptophyls in an inaccessible state.

Experimental - All reactions have been performed by the addition of 100 μmoles 2-hydroxy-5-nitrobenzyl bromide to a solution (5.0 ml) of α-chymotrypsin (2.0 μmoles, 3x-crystallized, Worthington Biochemical Corp., Lot CDI-6142-3) at 30^0 in the absence of buffers. The desired pH was maintained by a pH-stat and initially, the reagent was present in only a partially soluble state. Liberation of HBr was complete in about 30 minutes or less. Each sample was then adjusted to pH 3.0 and dialyzed for 24 hours against two changes (2 liters each) of 1 mM HCl in the cold. The material (2.0 ml) was then placed on a Sephadex G-25 column (0.9 x 30 cm) and developed with 1 mM HCl. This procedure effectively separated the modified protein from solvolysis products of the reaction. In some cases, larger scale reactions were carried out and the final product lyophilized. The modified protein fraction so obtained was then subjected to the following analyses: a) spectral determination of moles chromophoric groups introduced per mole protein (Koshland *et al.*, 1964); active site concentration (Schonbaum *et al.*, 1961); and c) specific activity (0.01 M N-acetyl-*L*-tyrosine ethyl ester, 0.1 M $CaCl_2$, pH 8.0). Protein concentration was determined by dry weight since the label absorbs appreciably at 280 mμ. Specific activity is expressed as μmoles substrate hydrolyzed per minute per mg protein or per mg active enzyme.

Results and Discussion - The effect of pH on the extent of tryptophan labeling by the reagent is shown in Table 1. Within experimental error, all of the tryptophan residues in chymotrypsin

(Hartley (1964) has reported eight tryptophan residues in chymo-

TABLE I

TRYPTOPHAN EQUIVALENTS LABELED BY REACTION OF CHYMOTRYPSIN WITH 2-HYDROXY-5-NITROBENZYL BROMIDE AT VARIOUS pH VALUES

pH	Tryptophan Equivalents Labeled
5.6	0.7
4.0	1.0
3.5	1.3
3.0	2.1
2.7	4.4
2.0	7.5

trypsinogen) reacted with the reagent at pH 2.0. In the region of pH 4, only one group was accessible to the reagent.

An approximately linear relationship occurred between the tryptophan equivalents labeled per mole protein and the active site concentration of the modified protein (Fig. 1). Although

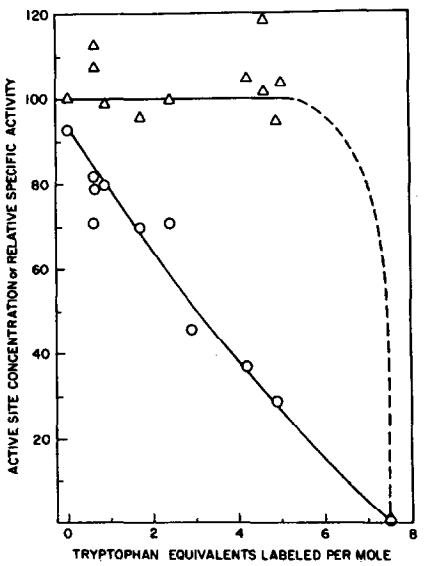

Figure 1 - The relationship between the tryptophan equivalents of chymotrypsin labeled by reaction with 2-hydroxy-5-nitrobenzyl bromide and the active site concentration remaining (0) and the specific activity per mg active enzyme (Δ).

enzyme activity decreased as the extent of reaction with the reagent increased, the activity per active site was closely equivalent to that of the native enzyme up to at least five moles label introduced per mole protein. The modified protein was enzymically inactive upon introduction of seven to eight chromophoric groups per mole. These results suggested that under conditions of reaction with the reagent, chymotrypsin existed as two population types. The one with all tryptophan residues reactive toward the reagent and becoming enzymically inactive thereby; the other with the tryptophan residues unreactive or essentially so toward the reagent and with very little loss in activity.

To determine the correctness of the above expressed viewpoint, an attempt was made to isolate the products of the reaction of the presumed population types with 2-hydroxy-5-nitrobenzyl bromide by gel filtration of the modified protein on Sephadex G-75.

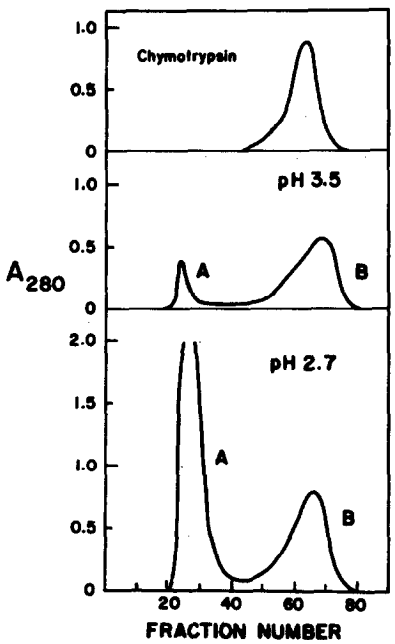

Figure 2 - Elution profiles of chymotrypsin and the modified protein on Sephadex G-75. Chymotrypsin was reacted with 2-hydroxy-5-nitrobenzyl bromide at the indicated pH.

Results of two such experiments are shown in Figure 2. The material that emerged first from the column (Fraction A) increased in amount as the pH was lowered. Fraction B behaved on the column essentially as did chymotrypsin and its concentration was inversely related to the amount of Fraction A formed. Analytical information on these fractions is given in Table II. Fraction A (6% of total at pH 3.5; 65% of total at pH 2.7) was found to be fully la-

TABLE II

ANALYTICAL DATA FOR PRODUCTS SEPARATED BY CHROMATOGRAPHY OF MODIFIED PROTEIN ON SEPHADEX G-75

pH of Reaction	Fraction	Weight %	Tryptophan Equivalents Labeled	Active Sites (%)	Specific Activity per mg protein	Specific Activity per mg active enzyme
3.5	A	6	8.4	0	0	
	B	94	0.65	83	270	325
2.7	A	65	7.0	0	0	
	B	35	0.9	87	260	300
Native enzyme		-	-	93	294	317

beled (within the limits of the overall errors involved) and enzymically inactive. Fraction B contained less than one chromophoric group per mole protein and the specific activity (per mg active enzyme) was equal to that of chymotrypsin. From the data of Table II, one can calculate what the tryptophan equivalents labeled per mole *modified protein* should be at either pH. At pH 3.5: *calc.*, 1.1; *found*, 1.3. At pH 2.7: *calc.*, 4.9; *found*, 4.6. The fact that Fraction B contains both a slightly lower active site concentration and specific activity per mg protein than chymotrypsin may indicate contamination with Fraction A. The presence of 9 to 13% of fully labeled material in the B fractions would account for the data obtained.

When the reaction between chymotrypsin and reagent was done

at pH 2.0, only fully labeled material (Fraction A) was obtained although a small amount of material with column behavior intermediate between A and B was obtained. At pH 5.6, reaction product distribution was close to that obtained at pH 3.5.

At pH 4.0, only one chromophoric group is introduced per mole chymotrypsin. At the same pH, reaction of the enzyme (0.21 μmoles per 5 ml vol.) with N-bromosuccinimide resulted in 7.2 to 7.8 tryptophan residues oxidized. This agrees with the data of Spande *et al.* (1966). The difference in protein concentration may well account for the greater reactivity of the tryptophyl groups toward N-bromosuccinimide.

Egan *et al.* (1957) have published Svedberg values for chymotrypsin as a function of pH at the same concentration (10 mg per ml) as used in the experiments reported herein for reaction with 2-hydroxy-5-nitrobenzyl bromide. A plot of their data (S_{20} *versus* pH) is closely superimposable on a plot of relative Fraction A concentration *versus* pH (Oza and Martin, *results to be published).* As a working hypothesis then, it is suggested that the difference in reactivity of the two population types of chymotrypsin to the chromophoric reagent is related to their differing states of polymerization. If this is correct, the amount of Fraction B obtained at any pH would reflect the concentration of an aggregate species in which all or essentially all of the tryptophan residues are inaccessible for reaction with the reagent. Since the degree of polymerization (dimer state?) of chymotrypsin is greatest at about pH 4.5 and the enzyme is in the monomer form at a pH of 2.3 (Egan *et al.*, 1957), the formation of Fraction A (greater as the pH is decreased) may represent the reaction of the reagent with the monomeric form of the enzyme.

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