CROSSLINKING DURING THE NITRATION OF BOVINE INSULIN WITH TETRANITROMETHANE

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Received December 12, 1969

ABSTRACT

The treatment of bovine insulin with tetranitromethane at pH 8 resulted in the nitration of 2.5 of the four residues of tyrosine in the molecule. Only 0.1 residue of tyrosine was found in the derivative, indicating the modification of 1.4 tyrosine residues by a reaction which did not produce 3-nitro-tyrosine. No other amino acid residue besides tyrosine seemed affected by the reaction. Gel chromatography of the derivative in 7 *M* urea demonstrated the existence of monomers, dimers and larger aggregates of the insulin molecule, an indication of intermolecular covalent crosslinking. The crosslinking also occurred when glycyl-L-tyrosine was treated with tetranitromethane at pH 8.

INTRODUCTION

The products of the reaction of TNM* with proteins were described by Riordan *et al.* (1,2), who reported the conversion of tyrosine to 3-nitro-tyrosine in Tris buffer at pH 8. Since then TNM has been used as a specific modifying reagent for tyrosine residues in many proteins. In several reports there was a good balance between the tyrosine content of the starting material and the nitro-tyrosine plus tyrosine content of the nitrated product, indicating that the tyrosine residues were either nitrated or remained unmodified (3,4,5). However, the nitration of collagen and γ-globulin resulted in the formation of a precipitate which the authors attributed to crosslinking (6). Recently it was noted with trypsin that the amount of tyrosine plus nitro-tyrosine in the TNM treated protein was less than the amount of tyrosine in the untreated protein (7). This implies that some of the tyrosine was converted to something besides 3-nitro-tyrosine on TNM treatment of the protein. In similar studies performed

*Abbreviations used in this paper: TNM, tetranitromethane; Tris, tris(hydroxymethyl)aminomethane.

on insulin, less than the theoretical amount of 3-nitro-tyrosine was produced but no analyses for unreacted tyrosine were reported (1,8).

We have found that essentially all of the tyrosine in the insulin molecule is modified at pH 8 as judged by the disappearance of tyrosine, but only part of the residues are accounted for by the appearance of 3-nitro-tyrosine. The remianing residues are apparently involved in a cross-linked compound.

EXPERIMENTAL

The nitration of insulin: Insulin·HCl (50 mg Eli Lilly and Co., lot OLVOO, converted to the hydrochloride as previously described (9)) was dissolved in 10 ml 0.05 *M* Tris buffer, pH 8.0 and 10 µl of TNM (Aldrich Chem. Co.) in 0.5 ml ethanol was added. After the specified reaction time the mixture was acidified to pH 3 with conc. HCl, dialyzed extensively against water and lyophilized.

The nitration of glycyl-L-tyrosine. Method A: Glycyl-L-tyrosine (32.5 mg, Mann Research Lab.) was dissolved in 50 ml 0.05 *M* Tris buffer, pH 8.0. TNM (50 µl) in 2.5 ml ethanol was added and the mixture was stirred. At specified times, aliquots were removed, acidified to pH 3 with conc. HCl, extracted twice with two volumes of ether to remove excess TNM and then lyophilized. No attempt was made to remove buffer salts. *Method B:* The dipeptide was nitrated according to the procedure of Atassi and Habeeb (3). In this method the buffering capacity of the mixture is rapidly overpowered by the acid produced in the reaction with the consequence that most of the nitration proceeds in an acidic media. Under these acidic conditions the rate of disappearance of tyrosine is less than 1/100th of that at pH 8.0.

Amino acid analyses were obtained using a Spinco model 120B automatic amino acid analyzer equipped with a high sensitivity range card and long path length cuvettes. Acid hydrolysis was carried out in sealed, evacuated tubes with 2 ml 6 *M* HCl at 120° for six hours. Phenol (2-3 mg) was added to increase the yields of tyrosine. The 3-nitro-tyrosine was stable to acid hydrolysis under these conditions.

Table I. The reaction of insulin with TNM

reaction time *min.*	*moles/mole of insulin*	
	tyrosine	3-NO_2-tyrosine
0	4.00	0.00
5	2.03	1.05
10	1.50	1.47
25	0.77	1.98
75	0.21	2.39
100	0.10	2.50

RESULTS

A time study of the reaction of insulin with TNM appears in Table I. After 100 minutes reaction time, of the four tyrosine residues present in insulin, 0.1 residue remained unmodified, 2.5 residues were nitrated and 1.4 residues were unaccounted for by amino acid analysis.

The gel chromatography elution profile of the nitrated insulin in 7 *M* urea on Sephadex G-50 is shown in Figure 1 along with the elution volumes of several calibration substances. Approximately 33% of the material elutes at the same volume (30 ml) as unreacted insulin. The material eluting in the shoulder at 24 ml is thought to be a dimer since it elutes at the same volume as ribonuclease (mol.wt. 13,7000). The first peak to elute in Figure 1, at 18.5 ml, is attributed to trimers and/or larger aggregates.

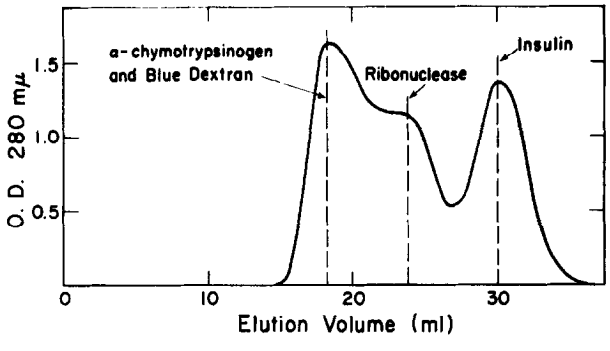

Figure 1. The elution profile of nitrated insulin on a 1.1x60 cm column of Sephadex G-50M equilibrated with 0.05 *M* Tris, 0.01 *M* NaCl, 7 *M* urea buffer, at pH 7.5. The sample volume was 0.5 ml and fractions were 1.2 ml. Flow rate was 4.8 ml/hr.

Table II. The reaction of glycyl-L-tyrosine with TNM by method A

reaction time *min.*	*moles/mole of glycine*	
	tyrosine	3-NO_2-tyrosine
0	0.97	0.00
10	0.20	0.17
30	0.04	0.21
100	0.02	0.24

A time study of the reaction of glycyl-L-tyrosine with TNM by method A is shown in Table II. After 100 minutes 0.02 residue of tyrosine remained unmodified, 0.24 residue was nitrated and the remaining 0.74 residue was not detected by amino acid analysis after hydrolysis of the reaction mixture. The elution profile of the nitrated glycyl-L-tyrosine on Sephadex G-10 is shown in Figure 2. The amino acid analysis of the hydrolysate of the peak eluting at the void volume (24 ml) indicated the presence of glycine and ammonia but no tyrosine or 3-nitro-tyrosine. The hydrolysate of the material in the last peak (60 ml) contained equal amounts of glycine and 3-nitro-tyrosine, indicating it to be glycyl-3-nitro-L-tyrosine. The nitrated glycyl-L-tyrosine obtained by method B (nitration at low pH), however, gave a single peak on the Sephadex G-10 column at 62 ml and yielded equal amounts of glycine and 3-nitro-tyrosine on acid hydrolysis.

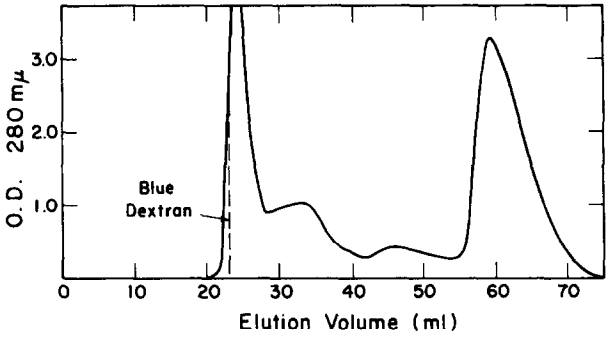

Figure 2. The elution profile of nitrated glycyl-L-tyrosine on a 1.1x60 cm Sephadex G-10 column equilibrated with water. Sample volume was 0.5 ml and fractions were 1.33 ml. Flow rate was 7.5 ml/hr.

DISCUSSION

The results presented here can be interpreted as the formation of intermolecular crosslinks accompanying the nitration of tyrosine residues with TNM. At present it appears that the crosslinking occurs between tyrosine residues since other residues are not affected by the reaction. Furthermore, the crosslinking in insulin is not caused by a special property of the insulin molecule since the reaction also occurs on nitration of glycyl-L-tyrosine with TNM. Therefore, it is probably a general phenomenon that should be investigated whenever TNM is used as a modifying reagent. The occurrence of crosslinking is not surprising when one considers the results reported by Bruice *et al.* on the nitration of various phenols with TNM (10). In most cases their yields of nitrated phenols were only 20-30%. The nitration of p-cresol with TNM led to a 30% yield of Pummerer's ketone which is obtained via the free radical coupling of two molecules of p-cresol. Unidentifiable tars, apparently polymeric, were also obtained from most phenols tested.

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