A Convenient Spectrophotometric Procedure for the Determination of Amino-Terminal Tyrosine Residues*

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ABSTRACT: The conversion of NH_2-terminal tyrosine residues with 4 equiv of *N*-bromosuccinimide (NBS) in acetic acid buffer, or with excess NBS in formic acid, into the chromophore of 5,7-dibromo-6-hydroxyindole-2-carboxamide (λ_{max} 315 mμ) permits the rapid assay of NH_2-terminal tyrosine in model peptides and tryptic fragments from glucagon, globin, and chymotrypsinogen, in which the sequence -Arg-Tyr or Lys-Tyr- occurs with more than random frequency. After tryptic digestion glucagon showed one, globin two, and chymotrypsinogen A three NH_2-terminal tyrosine residues per mole by this method.

Among several methods for the identification and estimation of the NH_2-terminal residue in proteins (Fraenkel-Conrat *et al.*, 1955; Bailey, 1962), the most useful ones involve reagents such as fluorodinitrobenzene, phenyl isothiocyanate, and 1-dimethylaminonaphthalene-4-sulfonyl (dansyl)[1] chloride (Gray and Hartley, 1963). The quantitative determination of tyrosine by these methods is often complicated by competitive reaction with the phenolic hydroxyl group (*cf.* Fraenkel-Conrat *et al.*, 1955).

Although the reaction of acylamino acids, peptides, and proteins with *N*-bromosuccinimide (NBS) has been widely studied (Witkop, 1961; Spande and Witkop, 1967), it has been reported that unprotected amino acids are oxidized with NBS in aqueous solution at pH 3.4–4.8 to CO_2, N_2, and aldehydes (Konigsberg *et al.*, 1960). Amino acid residues in a peptide chain do not react with NBS, unless there is a reactive side chain, as in the case of tryptophan, tyrosine, or histidine. The reaction of NBS with these residues has been used for nonenzymatic cleavage of C-peptide bonds (Wilson and Cohen, 1963; Patchornik *et al.*, 1958). The disappearance of tyrosine or tryptophan can be conveniently followed by ultraviolet spectroscopy.

We have recently reported (Wilchek *et al.*, 1967) that NBS at an acid pH oxidizes tyrosine derivatives which possess a free α-amino group to 6-hydroxy 2-substituted indoles with a characteristic ultraviolet absorption at 315 mμ which on basification is shifted to 355 mμ. In the present studies we have applied this type of oxidation to the rapid, quantitative, and specific determination of NH_2-terminal tyrosine residues in peptides and proteins. Since there is apparently no known instance of an NH_2-terminal tyrosine residue in naturally occurring proteins (Dayhoff *et al.*, 1965), we have attempted to apply the method to the detection of fragments resulting from the tryptic cleavage of Lys-Tyr or Arg-Tyr sequences in proteins. We examined glucagon, the globin component of sperm whale myoglobin and chymotrypsinogen, in which one out of the two, two out of three, and three out of the four tyrosines, respectively, follow a basic residue. From a cursory examination of known protein sequences, it appears that tyrosine immediately precedes or follows an arginine or lysine residue much more often than would be predicted statistically. Examples are the bovine trypsin inhibitor and bovine pancreatic ribonuclease. This observation may reflect a real preference by tyrosine for an arginine or lysine neighbor, and is suggestive of the possibility that even such a short sequence of a protein is stabilized by an internal hydrogen bond.

Experimental Section

Materials and Methods. L-Tyrosyl-L-alanine, L-tyrosyl-L-phenylalanine, and L-tyrosyl-L-tryptophan were obtained from Yeda, Rehovoth, Israel. Glucagon was purchased from Sigma Chemicals, St. Louis, Mo. Myoglobin was obtained from Mann Laboratories, New York, N. Y., and chymotrypsinogen A (chromatographically homogeneous) from Worthington Biochemicals, Freehold, N. J. The NBS used was from Eastman (practical grade). All other chemicals were of reagent grade.

Synthesis of Model Peptides. The following peptides were synthesized according to published procedures: *N*-carbobenzyloxyglycylglycine benzyl ester and glycylglycylglycine benzyl ester hydrobromide (Ben-Ishai, 1954). The protected tyrosyl peptides were synthesized from carbobenzyloxy-L-tyrosine (Bergmann and Zervas, 1932) and $(glycyl)_{2-4}$ benzyl ester hydrobromide by the *N,N'*-dicyclohexylcarbodiimide method (Sheehan and Hess, 1955). Carbobenzyloxyglycylglycylglycylglycine benzyl ester was prepared from carbobenzyloxyglycylglycine and glycylglycine benzyl ester hydro-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: dansyl, 1-dimethylaminonaphthalene-4-sulfonyl; NBS, *N*-bromosuccinimide.

TABLE I: Analytical Data on the Model Peptides Containing L-Tyrosine.

Peptide	Yield (%)	Mp (°C)	Formula	Calcd (%) C	Calcd (%) H	Calcd (%) N	Found (%) C	Found (%) H	Found (%) N
Z-L-Tyr-Gly-Gly-OBl[a]	78[b]	128–130	$C_{28}H_{29}N_3O_7$	64.73	5.63	8.09	64.48	5.51	8.29
Z-L-Tyr-Gly-Gly-Gly-OBl	73[b]	162–164	$C_{30}H_{32}N_4O_8$	62.49	5.59	9.72	62.24	5.75	9.99
Z-L-Tyr-Gly-Gly-Gly-Gly-OBl	65[b]	189–191	$C_{32}H_{35}N_5O_9$	60.65	5.57	11.05	60.82	5.73	11.31
Z-Gly-Gly-Gly-Gly-OBl	71[c]	183–186	$C_{23}H_{26}N_4O_7$	58.71	5.57	11.91	58.92	5.32	11.73
L-Tyr-Gly-Gly	91[d]		$C_{13}H_{17}N_3O_5$	52.87	5.80	14.23	53.02	5.63	14.01
L-Tyr-Gly-Gly-Gly	85[d]		$C_{15}H_{20}N_4O_6$	51.13	5.72	15.90	51.22	5.81	15.89
L-Tyr-Gly-Gly-Gly-Gly	80[d]		$C_{17}H_{23}N_5O_7$	49.87	5.66	17.11	49.57	5.43	16.84

[a] Abbreviations used: Z = $C_6H_5CH_2OCO$; Bl = $C_6H_5CH_2$. Recrystallized from: [b] ethyl alcohol, [c] dimethylformamide–ether, and [d] water–ethyl alcohol.

bromide by the same method. The carbobenzyloxy group was removed by treatment with hydrobromic acid in acetic acid for 15 min at room temperature.

The free peptides were obtained by catalytic hydrogenation of the protected peptides in the presence of palladium on charcoal. All peptides were chromatographically homogeneous in at least two solvent systems (thin-layer chromatography 1-butanol–acetic acid–water (25:6:25) and 1-butanol–pyridine–acetic acid–water (30:20:6:24)). The analytical data of the synthetic peptides are listed in Table I.

Tryptic Digestion of Proteins. A solution of 15 mg of glucagon in 5 ml of 0.05 M phosphate buffer (pH 7.8) was digested with 150 μl of a trypsin solution (1 mg/ml) in a procedure slightly modified from that of Bromer *et al.* (1957). An identical solution of glucagon served as reference solution. Myoglobin was converted into globin by acid precipitation as described by Edmundson and Hirs (1961), then denatured by boiling for a few minutes (96°). A slurry was prepared in 1.0 M phosphate (pH 8) and a portion was treated with trypsin, again following Edmundson's procedure. After 10 min complete solution occurred. The untreated portion, when diluted (1:1) with 0.2 N formic acid, as described below, became a solution and was used as reference. Chymotrypsinogen was reduced following the procedure of Pechère *et al.* (1958). The following, somewhat shortened dialysis schedule was used (500 ml of buffer, 5°): 3 hr, 0.02 M HCl adjusted to pH 9 with NH_4OH; 6 hr, 0.01 M HCl–0.01 M EDTA adjusted to pH 8.5 with NH_4OH; 6 hr, 0.005 M HCl–0.005 M EDTA (pH 8.0) (NH_3) and two 1-day dialyses against water adjusted to pH 8 with NH_4OH. The resulting suspension was divided into two 8-ml portions and 2 ml of 0.2 M phosphate buffer (pH 8) was added to each one. To one portion 100 μl of a trypsin solution (1 mg/ml) was added. After 2 hr, a clear solution was obtained. The untreated solution, when diluted (1:1) with 0.2 N formic acid, also became clear and was used as reference for the spectrophotometric procedure.

Spectrophotometric Method. Ultraviolet absorption spectra were recorded on a Cary 11 double-beam self-recording spectrophotometer with 1-cm cells; 2 mM stock solutions of the model peptides, Tyr-$(Gly)_{2-4}$, Tyr-Ala, Tyr-Phe, Tyr-$(Gly)_2$-Tyr, Tyr-Tyr-OCH_3, and Tyr-Gly-Phe were prepared in water with the addition of a few drops of 1.0 N HCl to dissolve the peptides containing phenylalanine. To 2.0 ml of each solution was added 2.0 ml of 0.2 M HOAc or 0.2 M acetate buffer (pH 5.0 or 6.0) and 0.6 (3 equiv) or 0.8 ml (4 equiv) of a 20 mM freshly prepared aqueous NBS solution with rapid stirring. After standing at room temperature for 20–24 hr, the ultraviolet spectrum of each solution was recorded, and the OD_{315} was noted and corrected to a volume of 4.0 ml. The OD_{275} of the stock solutions was also measured and corrected for the dilution to 4.0 ml. In addition, three 10-ml samples containing 4.0 ml of the stock solution of Tyr$(Gly)_3$, 4.0 ml of 0.2 M HOAc, and 1.2 (3 equiv), 1.6 (4 equiv), and 2.0 ml (5 equiv) of 20 mM NBS were prepared. After 20 hr, the absorbance at 315 mμ was determined. An aliquot of 1.0 ml was diluted to 2.0 ml with 1.0 N NaOH and the absorbance at 340–345 mμ was measured. Similarly 3.0 ml of a stock solution of Tyr-Ala, 4.0 ml of 0.2 M formic acid, and either 1.2 (4 equiv) or 3.0 ml (10 equiv) of 20 mM NBS were mixed and heated briefly on a steam bath for 10 min before measurement of OD_{315}. The following conditions were used for the NBS addition to the protein samples.

GLUCAGON. A 0.1 M formic acid solution (2.0 ml) containing 0.25 μmole/ml of digested glucagon (OD_{275} 1.55; 1.5 mg/2 ml) was placed in the sample cell and oxidized with 325 μl of 20 mM NBS (6.5 μmoles), added in several portions with rapid stirring (magnetic flea). A similar solution containing undigested glucagon was placed in the reference cell and oxidized with the same quantity of NBS. Both cells were heated for 10 min on the steam bath, then cooled, and the absorption spectrum of sample *vs.* reference was determined. An absorbance at 315 mμ of 0.98 (corrected to 2.0 ml) was measured.

GLOBIN. Formic acid solution (2.0 ml of 0.1 M) containing 0.10 μmole/ml of digested globin (10 min or 24 hr of tryptic digestion) (OD_{278} 1.71) was treated with 350 μl of 20 mM NBS (7 μmoles). A reference solution containing undigested globin was treated

TABLE II

	pH 3		pH 5	pH 6
Tyrosyl Peptides + NBS in Acetic Acid				
Concentration of buffer (M)	0.087	0.083	0.087	0.083
Equivalents of NBS/Tyr	3	4	3	4
OD_{315}/OD_{275}, Tyr-Gly-Gly	2.41	3.16	2.46	3.45
Tyr-Gly-Gly-Gly	2.45	3.28	2.55	3.67
Tyr-$(Gly)_4$	2.35	3.40	2.47	3.69
Tyr-Phe	Turbid	Turbid	2.18	3.21
Tyr-Ala	2.06	2.84	2.09	3.34
Tyr-Gly-Phe	Turbid	Turbid	2.28	3.34
Tyr-$(Gly)_3$ + NBS in 0.080 M HOAc				
Equivalents of NBS/Tyr	3	4	5	
OD_{315}/OD_{275}	2.40	3.14	2.43	
Oxidation Product from Tyr-$(Gly)_3$ in 0.5 M NaOH, then 0.3 M HCl				
OD_{340}/OD_{275}	2.81	3.96	3.52	
OD_{315}/OD_{275}	2.46	3.50	3.41 (br)	
Tyr-Ala + NBS in 0.08 M Formic Acid				
OD_{315}/OD_{275}, equivalent of NBS/Tyr	4	10		
5-min heating	2.92	3.01		
10-min heating	3.12	3.27		
Tyr-Ala Oxidation Product in 0.5 M NaOH, then 0.3 M HCl				
OD_{340}/OD_{275} (OH^-)	3.86			
OD_{315}/OD_{275} (H^+)	2.90			

in a similar fashion. After 10-min heating on the steam bath, an OD_{315} of 0.84 was obtained (24-hr digestion).

S-SULFOCHYMOTRYPSINOGEN A was oxidized with equivalent amounts of NBS and both were heated briefly on the steam bath. Additional 250- and 100-μl portions of NBS added had no further effect. The OD_{315} was 0.79 after correction for dilution.

Results

In Table II the results of addition of 3 and 4 equiv of NBS per mole of tyrosine to the synthetic peptides are tabulated. The results of addition of 3 equiv at pH 5, and 4 equiv at pH 6, in acetic acid buffers, and 4 and 10 equiv in formic acid (to Tyr-Ala alone) are included. The data are expressed as a ratio of the 315-mμ absorbance (corrected for any dilution) obtained after 20–24 hr at room temperature (or heating as indicated) to the initial tyrosyl absorbance at 275 mμ. Table III contains the results on NBS oxidation of the tryptic digests of glucagon, globin, and 5-sulfochymotrypsinogen A expressed as molar extinction at 315 mμ. A graphical representation of molar extinction at 315 mμ *vs.* the number of NH_2-terminal tyrosines released on digestion is shown in Figure 1.

TABLE III

Trypsin-Digested Protein + NBS in 0.1 M Formic Acid	ϵ_{315}	ϵ_{315} 4200	ϵ_{340}	ϵ_{340} 5300
Glucagon	3,900	0.93		
Globin				
10-min digest	4,300	1.02		
24-hr digest	8,400	2.0	10,700	2.02
S-Sulfochymotrypsinogen	12,300	2.9		

Discussion

The ratio OD_{315}/OD_{275} reaches a maximum value in acetic acid buffer at 4 equiv of NBS/equiv of tyrosine peptide, or extinctions of 4300–4700, assuming an extinction of 1370 for the tyrosine chromophore in acid medium (Wetlaufer, 1962). This corresponds to a conversion of approximately 25% from tyrosine into the 6-hydroxyindole chromophore which has an extinction of ~17,000 at 315 mμ. This 25% conversion is reproducible and did not depend on the value of the peptides or proteins used in this study. Very little difference was noted between oxidations with 3 equiv at pH 3 or 5, though with 4 equiv at pH 6, slightly increased ratios were observed (maximum extinction at

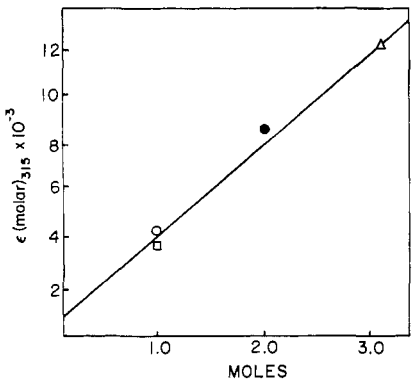


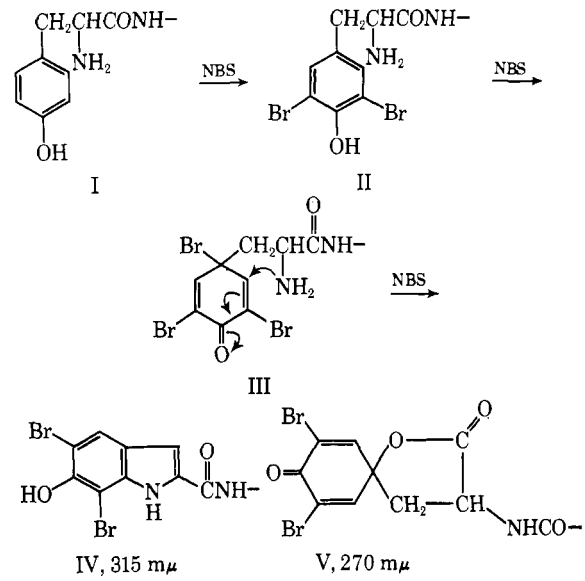
FIGURE 1: Moles of N-terminal tyrosine per mole of protein digest (□, glucagon; ○, globin (10 min); ●, globin (24 hr); △, chymotrypsinogen A).

315 mμ, 5050). With 5 or more equiv of NBS at pH 3, the ratio again decreased due to destruction of the 315-mμ chromophore by NBS. Since addition of exactly 4 equiv to an NH_2-terminal tyrosine residue in protein fragments would be complicated by competition for NBS by other residues present, *e.g.*, tryptophan, histidine, cystine, methionine, and interior tyrosine, it would be convenient to add a sufficient excess of NBS to offset this consumption without affecting the 320-mμ chromophore. The simple expedient of conducting the oxidations with formic acid buffer in place of acetic acid, permitted the use of a large excess of NBS. Formic acid is known to be oxidized by NBS and consequently protects the hydroxyindole from destruction, yet does not interfere with its formation. With 4 and 10 equiv of NBS on Tyr-Ala in 0.08 M formic acid, the 315-mμ absorption could be developed rapidly with heating to approximately the same extinctions as observed with 4 equiv in acetic acid and a 20–24-hr reaction time at room temperature.

On basification, the absorption at 315 mμ, whether developed in acetic or formic acid, shifts to 340–350 mμ with an approximately 20% increase in extinction. The exact wavelength depends upon whether 3 or 4 equiv of NBS was used. When either 3 or 4 equiv was added, this shift with base is reversible; with 5 equiv of NBS, it is no longer reversible. The resulting absorption after reacidification differs from the starting absorption by a much greater extinction and a broader absorption peak.

The use of formic acid permitted the addition of a large excess of NBS to proteins. The use of undigested protein in the reference cell, to which equal amounts of NBS were added, cancelled the effects of NBS on interior residues which might produce some absorption at 315 mμ. With trypsin-digested glucagon, globin, and *S*-sulfochymotrypsinogen A and excess NBS, distinct peaks at 315–320 mμ were observed, corresponding in intensity to one, two, and three NH_2-terminal residues, respectively, of tyrosine per mole of protein. This absorption could be shifted with base to approximately the same region as with the oxidized peptides. In the case of globin, one NH_2-terminal tyrosine residue was detected 10 min after the digestion with trypsin began,

SCHEME I



the point at which the suspension cleared. Two residues were detected at the end of the digestion (24 hr), when the digest had again become turbid.

The presence of a single tyrosine or tryptophan in an interior position of a protein chain probably would not affect the production of the 315-mμ chromophore from NH_2-terminal tyrosine, for the two model peptides, Tyr-Gly-Gly-Tyr and Tyr-Trp behaved with NBS in the same manner as NH_2-terminal tyrosine peptides lacking the additional tyrosine or tryptophan. If many of either or both residues are present in a protein, it is anticipated that this spectrophotometric procedure for NH_2-terminal tyrosine, after some enzymatic cleavage of the X–Tyr bond (X = a peptide bond susceptible to enzymatic cleavage), might become subject to large errors, even when corrected with a protein blank, due to differences in oxidizability between the digested and undigested protein. A proper control for an authentic NH_2-terminal tyrosyl protein might use the same protein with its NH_2-terminal protected by a suitable blocking group.

The reaction pathway from tyrosine I (Scheme I) to the production of the 315-mμ chromophore IV is likely to involve the intermediates II and III. The NBS oxidations of tyrosine in proteins thus fall into two basic types, depending upon whether the tyrosine is NH_2 terminal or within the peptide chain. The former gives rise to a chromophore at 315 mμ, the 2-substituted 6-hydroxyindole IV, while the latter forms the bromodienonelactone V, with absorption at 270 mμ. This chemical behavior is in close analogy to the action of tyrosinase upon tyrosine-containing proteins where a "dopachrome" pattern and a "dopaquinone" pattern arises, when tyrosine is NH_2 terminal or COOH terminal, respectively (Yasunobu *et al.*, 1959). The enzymatic conversion of tyrosine to a 5,6-dihydroxyindole chromophore approaches 75% yield and could per-

haps be elaborated into an assay more sensitive, but less convenient, than the oxidation by NBS.

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Thermal Denaturation of Myoglobin. I. Kinetic Resolution of Reaction Mechanism*

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ABSTRACT: The thermal denaturation of sperm whale myoglobin was investigated kinetically at pH 6.85 and ionic strength 0.096 in sodium phosphate buffer. Progress curves of the absorbance changes at 290, 409, and 540 mμ were obtained for temperatures between 40 and 90°. Three reaction phases in the denaturation process were resolved experimentally. The first phase was a rapid equilibrium. In the second phase, the reaction rate showed a very sharp dependence on temperature at 80°. This effect was reflected in the Arrhenius plot as a deviation from linearity. The limiting slope of the Arrhenius plot at the higher temperatures gave a value of $\Delta H^{\ddagger} = +109$ kcal/mole. Assuming $\Delta F^{\ddagger} = +30$ kcal/mole, the value $\Delta S^{\ddagger} = +224$ eu was calculated. The third phase was the formation of precipitate. An interpretation of mechanism is suggested, involving a conformational disturbance in the region of the heme group in the first phase, unfolding of the helical regions in the molecule in the second phase, followed by a sequence of polymerization steps, leading to precipitation in the third phase.

The concepts relating to the denaturation of proteins have, in the last decades, been subject to considerable refinement. In view of what is known today about the structure of globular proteins, it is of interest to ask the question: Does the denaturation process take place in a single transition or by means of a series of discrete kinetic steps, which are experimentally demonstrable?

A globular protein, such as myoglobin, consists of a number of helical and nonhelical regions, which are far from being identical in composition and which differ in their interactions with the rest of the molecule. It might be expected that the stability of these regions, for example, toward heat, will not be the same, that is to say different regions of the molecule undergo thermal transitions in different ways at different rates. On the other hand, considering the complexity of a molecule like myoglobin, it is conceivable that a strong cooperative effect exists in the molecule which stabilizes the native configuration and that any single disturbance of this configuration leads to a total loss of the cooperative effect. Such an assumption would be correct, if the denaturation process is a single-stage transition.

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