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Staphylococcal Enterotoxin C. I. Phenolic Hydroxyl Ionization*

Concordia R. Borja

ABSTRACT: The ionization of the phenolic groups of enterotoxin C produced by *Staphylococcus aureus* strain 137 has been studied by spectrophotometric titration at 295 mμ in the range between pH 7.5 and 13.0. Of the 21 tyrosyl residues per mole of protein, 5 residues are deduced to be "free," located on the surface of the molecule, freely exposed to the solvent and hence easily accessible to OH^-. The ionization of these five "free" tyrosyl groups is reversible with $pK_{app} = 10.02$ and with no time dependence. The remaining 16 tyrosyl residues are postulated to be embedded in the interior ("buried") and capable of ionizing only after unfolding of the protein molecule. The buried tyrosyl groups ionize at pH values higher than 11.0 and the ionization process is not reversible and is time dependent. Six of the buried tyrosyl residues ionize between pH 11.0 and 12.0 with a pK_{app} of 11.5 and ten ionize above pH 12.0. Treatment of enterotoxin C with 5 M guanidine hydrochloride results in the normalization of all the 21 tyrosyl groups with a pK_{app} of 9.9.

The existence of two types of tyrosyl residues, free and buried, has also been demonstrated by studying the reaction of enterotoxin C with N-acetylimidazole, tetranitromethane, and tyrosinase. Five tyrosyl residues per mole of protein react with *N*-acetylimidazole at pH 7.5, five to six residues react with tetranitromethane at pH 8.0, and five groups are oxidized by tyrosinase at pH 6.5.

Enterotoxin C elaborated by *Staphylococcus aureus* strain 137 is a protein with a molecular weight of 34,100 (Borja and Bergdoll, 1967), and consists of a single polypeptide chain cross-linked by one disulfide bridge (Huang *et al.*, 1967). Amino acid analysis of enterotoxin C revealed 21 tyrosyl residues per molecule (Huang *et al.*, 1967).

Since the pioneering work of Crammer and Neuberger (1943) on the ionization of the phenolic hydroxyl groups of tyrosine, insulin, and egg albumin, many investigators have adapted the spectrophotometric method to follow the dissociation of hydrogen ions from the phenolic groups of both native and modified proteins. Spectrophotometric titrations have revealed at least two types of tyrosyl residues in many proteins (Beaven and Holiday, 1952; Wetlaufer, 1962), namely, residues which instantaneously ionize in a manner similar to the free tyrosine molecule ("free" or "exposed") and residues which are either hydrogen bonded to specific acceptor groups or embedded in the protein interior and capable of ionizing only slowly or after denaturation ("buried").

The states of tyrosyl residues in proteins have been the object of many investigations employing spectrophotometric titration, iodination, solvent perturbation techniques, and use of specific reagents as *N*-acetylimidazole (Riordan *et al.*, 1965), tetranitromethane (Sokolovsky *et al.*, 1966), and cyanuric fluoride (Kurihara *et al.*, 1963). Tyrosinase has also been used by several investigators (Sizer, 1953; Yasunobu *et al.*, 1959; Frieden *et al.*, 1959; Lissitzky *et al.*, 1960; Cory and Frieden, 1967), to determine the accessibility of the tyrosyl groups on the surface of the protein and to gain insight as to the relationship of these tyrosyl groups to the ac-

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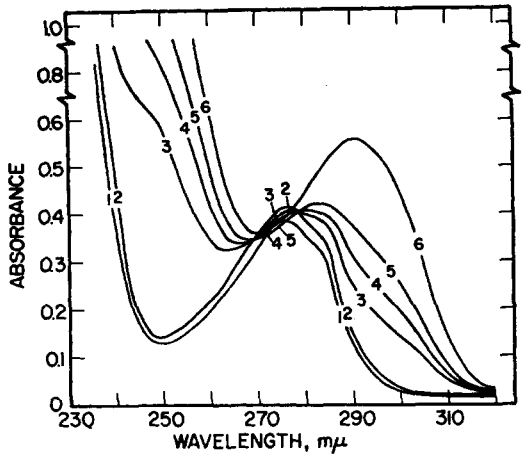

FIGURE 1: Ultraviolet absorption spectra of enterotoxin C in 0.15 M KCl at 25° and at different pH values taken about 20 sec after preparation of alkaline solutions. Curve 1, pH 3.0; curve 2, pH 7.35; curve 3, pH 10.85; curve 4, pH 11.5; curve 5, pH 12.0; and curve 6, pH 13.0. The concentration of the protein in curves 1–5 was 1.0×10^{-5} M and that in curve 6 was 0.95×10^{-5} M.

tive site. In this work, the technique of spectrophotometric titration and use of specific chemical reagents (*N*-acetylimidazole and tetranitromethane) were employed to study the types of tyrosyl residues in enterotoxin C. In order to correlate the spectrophotometric data to the reactivity of the tyrosyl residues, reaction of enterotoxin C with tyrosinase has also been investigated and included in this communication.

Experimental Section

Materials. Purified enterotoxin C used in this study was prepared by employing the purification technique as described in a previous paper (Borja and Bergdoll, 1967). The concentration of the enterotoxin solutions in 0.15 M KCl was determined by measuring the absorbance at 277 mμ, using the extinction value ($E^{1\%}_{1\,cm}$) of 12.1, and the molecular weight was taken to be 34,100 (Borja and Bergdoll, 1967).

Solutions of HCl and KOH (carbonate free) used in the spectrophotometric titrations were prepared so that they were 0.15 M in KCl. Guanidine hydrochloride (Matheson Coleman and Bell) was purified according to the method of Nozaki and Tanford (1967). Tyrosinase was purchased from Worthington Biochemical Corp.

Spectrophotometric Observation and Titration. Absorption measurements were made on a Beckman Model DB-G spectrophotometer using 1-cm quartz cells with stoppers to avoid absorption of carbon dioxide. The temperature in the cell compartment was maintained at around 25° by allowing the circulation of water from a constant temperature bath through channels in the sample compartment. Enterotoxin concentrations used for the titrations were in the range of $0.95–2.5 \times 10^{-5}$ M. A solution of the protein in 0.15 M KCl was titrated with 1 or 2 M KOH and 1 M HCl to the desired pH by using a syringe type buret of 1-ml capacity (Radiometer, type SBU1a) connected to a titrator (Radiometer, type TTT11) and titrigraph (Radiometer, SBR2c) which recorded automatically the volume of titrant used. Titrations were carried out in a titration assembly (Radiometer, type TTA31), consisting of a titration vessel (0.5–8.0-ml capacity) enclosed in a special jacket through which constant-temperature water (25 ± 0.1° or better) was circulated and provided with a four-hole cover to accommodate pH electrodes, buret, and nitrogen inlet tubes. Stirring was carried out using a Teflon-coated magnet and a magnetic stirrer mounted with the titration assembly. Measurements of pH were made with a Radiometer pH Meter 26 with connections to the titrator and equipped with a pH scale reading from 0 to 14 and also provided with an expanded scale for greater accuracy. Electrodes, Radiometer, type G2222C (glass) and type K4112 (calomel), were used. Absorbance measurements and recordings of absorption spectra on the Beckman Model 93506 potentiometric recorder were made 20 sec after reaching the desired pH, and subsequent measurements and spectra recordings were continued for 1 hr or more. The number of tyrosyl residues ionizing immediately after attainment of the desired pH was determined by extrapolating the values to zero time from measurements made at intervals beginning about 20 sec after adjustment of the solutions to the pH desired. Enterotoxin solutions in 0.15 M KCl treated with 5 M guanidine hydrochloride were allowed to stand for about 5 hr at 25° before titration. Since the absorbance of guanidine hydrochloride solutions is very high, precautions and absorbance measurements were followed as described by Cha and Scheraga (1960). Spectrophotometric titrations of enterotoxin C in 8 M urea–0.15 M KCl were carried out after allowing the solution to stand for about 45 hr at 5°. Before titration the temperature of the protein solutions was brought to 25°.

The number of ionizable tyrosyl residues was obtained by employing the method of Inada (1961) and using a molecular extinction of 2300 for the ionized tyrosine. Absorbance values at 295 mμ for neutral solutions of enterotoxin C were subtracted from the absorbance readings at 295 mμ given by solutions at different pH values to obtain the difference in absorbance.

Reaction of Tyrosyl Residues of Enterotoxin C with N-Acetylimidazole. The reactivity of the tyrosyl residues of enterotoxin C with *N*-acetylimidazole was studied according to the method of Riordan *et al.* (1965). The enterotoxin (3–4 mg/ml) was incubated with the reagent in 3 ml of 0.01 M Tris buffer (pH 7.5). In the absence of denaturing agents, a 150-fold molar excess of *N*-acetylimidazole was sufficient to acetylate the "free" tyrosyl groups of enterotoxin C and a much greater excess (360-fold molar excess) was required in the presence of urea or guanidine hydrochloride. The reaction was allowed to proceed for 1 hr at room temperature, after which the reaction mixture was passed through a 2.2 × 64 cm column of Sephadex G-15 (Pharmacia Fine Chemicals) to terminate the acetylation. Enterotoxin C was also acetylated with acetylimidazole in the presence of 8 M urea and 5 M guanidine hydrochloride. The decrease in absorbance at 278 mμ of 1160/mole on O acetylation of *N*-acetyltyrosine (Riordan *et al.*, 1965) has been used to determine the number of acetylated tyrosyl residues.

Reaction of Enterotoxin C with Tetranitromethane. Nitration of enterotoxin C was carried out as reported by Sokolovsky *et al.* (1966). Enterotoxin C (10 mg) in 3 ml of 0.05 M Tris buffer at pH 8.0 was treated with 5 μl of tetranitromethane. The reaction was allowed to proceed overnight at room temperature, after which the reaction mixture was passed through a Sephadex G-10 (Pharmacia Fine Chemicals) column (2.2 × 60 cm) in water to remove the nitroformate. The number of moles of nitrotyrosine formed per mole of protein was determined from the spectral characteristics of 3-nitrotyrosine; that has an absorption maximum at 428 mμ and ε 4100 at pH 8.0 (Sokolovsky *et al.*, 1966).

Oxidation of Enterotoxin C by Tyrosinase. Oxidation of the tyrosyl groups of enterotoxin C was measured at 280 mμ at 25° in a Beckman DB-G spectrophotometer. The reaction mixture contained sufficient enterotoxin C to be 10^{-4} M in tyrosine and tyrosinase (0.1 mg/6 ml of reaction mixture) in 0.5 M sodium phosphate buffer (pH 6.5). A control containing only the protein was run simultaneously. At various times throughout the reaction period, aliquots of the control and tyrosinase-treated sample were removed and absorbance measurements were taken at 280 mμ. The increase in extinction coefficient (Δε) at 280 mμ for the oxidation of a tyrosyl group (non N terminal) is 4.0 ± 0.1 × 10^3 as reported by Cory and Frieden (1967). This value was used to determine the number of tyrosyl groups in enterotoxin C oxidized by tyrosinase.

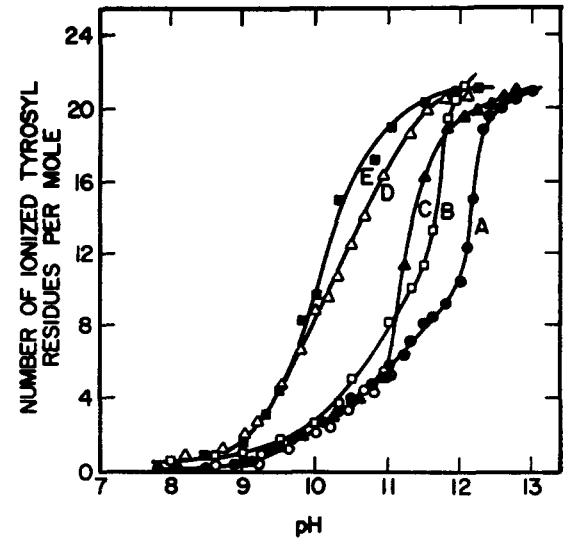

FIGURE 2: Spectrophotometric titration curves of enterotoxin C at 25° (0.95–2.5 × 10^{-5} M). (A) (●) Forward titration obtained about 20 sec after preparation of alkaline sample solutions in 0.15 M KCl; (B) (□) forward titration in 8 M urea–0.15 M KCl; samples were preincubated for about 45 hr at 5° before titration; (C) (▲) forward titration in 0.15 M KCl and curve was obtained after equilibration of the change at each pH (alkaline samples were allowed to stand for about 5 hr); (D) (△) back-titration after exposure of enterotoxin C (in 0.15 M KCl) solutions to pH 13.0 for about 3 hr; (E) (■) forward titration in 5 M guanidine hydrochloride–0.15 M KCl; samples were preincubated for about 5 hr at 25° before titration; (○) back-titration from pH 11.0.

Results

Ultraviolet Absorption Spectra. Recordings of the absorption spectrum of enterotoxin C at various pH values are shown in Figure 1. Curve 2 in Figure 1 is the absorption spectrum of an enterotoxin C solution in 0.15 M KCl, which is characterized by a broad band with a minimum at 249 mμ, a maximum at 277 mμ, and a little hump at 281–285 mμ. Although not indicated in Figure 1, enterotoxin C solutions at pH values ranging from 5.5 to 7.8 in 0.15 M KCl gave spectra identical with that shown in Figure 1 (curve 2). Isosbestic points at 260 mμ and 278.5 mμ denote the presence of a reversible ionic equilibrium due to the dissociation of the phenolic groups of tyrosine. Above pH 12.0 (representative example is curve 6 of Figure 1 at pH 13.0), the curves break away from the isosbestic points. The absorption spectrum of enterotoxin C solution in 0.15 M KCl (pH 3.0) is shown in curve 1 of Figure 1, which is similar in shape to that at pH 7.35 (curve 2). At pH 3.0, however, the absorption maximum has undergone a hypsochromic shift of 2 mμ and absorbance has been reduced about 6%. The absorption maximum of enterotoxin C at pH 3.0 is at 275 mμ, which is that for tyrosine itself as reported in the literature (Shugar, 1952). The pattern of the spectral shift at various alkaline pH values is shown in Figure 1 (curves 3–6). At pH 13.0, the absorption spectrum of enterotoxin C is similar to that of tyrosine after ionization of the phenolic groups (Shugar, 1952; Inada, 1961). Inada (1961) reported for a completely ionized solution of tyrosine at pH 13.0 a negative peak at 273 mμ and a positive peak at 295 mμ with a change in molar extinction (Δε) of 2305. For comparison, enterotoxin C in strongly alkaline solution has a positive peak at 291 mμ with a minimum at 269 mμ (curve 6, Figure 1) and a change in molar extinction of 48,860 at 295 mμ for 21 tyrosyl residues or about 2325 per tyrosyl group. The difference in the molar extinction of tyrosine before and after ionization of the phenolic group was also measured by Tanford and Roberts (1952) and a value of 2300 was reported. Using a Δε value of 2300, the number of ionized phenolic groups per mole of enterotoxin C, which was calculated from the data at 295 mμ shown in curve 6 of Figure 1, is 21.4.

Titration Curves. Curves A and C in Figure 2 represent the ionization of the phenolic groups of enterotoxin C about 20 sec and 5 hr or more, respectively, after the preparation of the alkaline samples. Time dependence of ionization was observed above pH 11.0, but below this pH, the process was completely reversible and not time dependent. Analysis of the data as shown in curve A of Figure 2, by the method of Tachibana and Murachi (1966), gave an apparent pK of 10.02 for five phenolic groups ionizing between pH 8.5 and 11.0 (first stage of ionization). Six phenolic groups dissociated between pH 11.0 and 12.0, which is considered to be the second stage of the ionization, and above pH 12.0 ten groups ionized, which may represent the third stage of ionization.

The results of the spectrophotometric titration of solutions of enterotoxin C preincubated with 8 M urea–0.15 M KCl at 5° for about 45 hr are shown in curve B of

Figure 2. Eight residues dissociated between pH 8.0 and 11.0, and at pH 12.0 all 21 phenolic groups ionized instantaneously. When solutions of enterotoxin C were titrated spectrophotometrically in 5 M guanidine hydrochloride–0.15 M KCl, the ionization was found to be instantaneous at any pH value and the absorbance at 295 mμ was not time dependent even after 5 hr of standing. All of the 21 phenolic groups ionized in a normal way, with an apparent pK of 9.9 (curve E, Figure 2). The data of the reverse titration from pH 13.0 shown in Figure 2, curve D (samples were kept at pH 13.0 in 0.15 M KCl for about 3 hr before back-titration), are fairly close to those of the forward titration in the presence of 5 M guanidine hydrochloride (curve E, Figure 2).

Phenolic Hydroxyl Ionization in Enterotoxin C with Changes in pH and Incubation Time. The changes in the ionization of phenolic groups in enterotoxin C associated with a change in pH and with time were also studied. Changes in the ionization of the tyrosyl residues were noticeable above pH 11.0. At pH 11.2, the number of ionizing groups changed from 6 (immediate) to 11 groups (maximum value obtained after about 4 hr), and at pH 11.5, the change was from 8 (immediate) to a maximum of 16 groups. The ionization changes became more rapid at pH 11.8 (9 to a maximum of 19 groups ionized), and at pH 12.0, within 1.5 hr, the number of ionizing phenolic groups changed from 11 to 19. Alteration in the dissociation of the phenolic groups with time was found to be very pronounced above pH 12.0; there was an increase of ionizing groups from 15 to 20 in a period of about 10 min. To reach the maximum of 21 ionized phenolic groups, a pH of 12.8 had to be attained. At pH 12.8 and above, the ionization became instantaneous.

Reaction of Enterotoxin C with N-Acetylimidazole and Tetranitromethane. Five tyrosyl residues were acetylated with a 150-fold molar excess of *N*-acetylimidazole in 0.01 M Tris buffer (pH 7.5) and five to six tyrosyl groups were nitrated with tetranitromethane in 0.05 M Tris buffer (pH 8.0). In 8 M urea, eight to ten tyrosyl residues reacted with *N*-acetylimidazole (360-fold molar excess) and in 5 M guanidine hydrochloride, all 21 phenolic hydroxyl groups were acetylated (360-fold molar excess of reagent).

Oxidation of Enterotoxin C with Tyrosinase. From the change in extinction coefficient at 280 mμ, 5 tyrosyl residues of the 21 present in enterotoxin C were oxidized by tyrosinase.

Discussion

The contributions of tryptophan and cystine to absorption at 295 mμ have been considered to be negligible (Tanford and Roberts, 1952; Ley and Arends, 1932). Since enterotoxin C contains only two tryptophanyl and one cystinyl amino acid residues (Huang *et al.*, 1967), determination of the number of phenolic groups ionizing at different pH values has been made possible without significant interference. A value of 21 tyrosyl residues per mole of enterotoxin obtained from the spectrophotometric data at 295 mμ and pH 13.0 is in good agreement with the result of amino acid analysis, *i.e.*, 21 tyrosines (Huang *et al.*, 1967).

The dependence of the reactivity of tyrosyl residues upon protein conformation is apparent from the present studies. Thus, three stages of ionization may be distinguishable in a forward titration of enterotoxin C solutions with alkali. In stage 1, from pH 8.5 to 11.0, only the ionization of five tyrosyl residues per mole of protein occurs reversibly and without time dependence. These five residues are readily acetylated at pH 7.5 with *N*-acetylimidazole and also oxidized by tyrosinase at pH 6.5. It can be inferred that the five tyrosyl residues are free and are located on the surface of the enterotoxin molecule in direct contact with the solvent and are thus easily accessible to OH^-. The second stage from pH 11.0 to 12.0 represents a region where a time-dependent ionization of six residues occurs with a pK_{app} of approximately 11.5. In stage 3, at pH values higher than 12, a rapid ionization of ten tyrosyl residues ensues. In the presence of 5 M guanidine hydrochloride, all 21 tyrosyl residues are exposed and behave in a normal way on the basis of spectrophotometric titration. Additional evidence is provided in this study in which all of the 21 tyrosyl groups are acetylated when the enterotoxin is exposed to guanidine hydrochloride. These observations are indicative of the disruption of the internal structure of native enterotoxin C. It is worthy of note at this point to mention the unfolding of enterotoxin C brought about by denaturing agents (urea and guanidine hydrochloride) as revealed by viscosity studies in our laboratory (Borja and Bergdoll, 1969).

Two types of tyrosyl residues could be distinguished in enterotoxin C as revealed by their susceptibility to ionization caused by alkali and by the effects of modifying agents like *N*-acetylimidazole, tetranitromethane, and tyrosinase. Of the 21 tyrosyl groups, 5 of them most likely belong to the free type and the remaining 16 residues are postulated to be embedded in the interior (buried).

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Staphylococcal Enterotoxin C. II. Some Physical, Immunological, and Toxic Properties*

Concordia R. Borja and Merlin S. Bergdoll

ABSTRACT: The enterotoxin C molecule produced by strain 137 undergoes considerable unfolding upon treatment with 5 M guanidine hydrochloride as indicated by a large change in intrinsic viscosity from 3.4 ml/g for the native toxin in 0.05 M sodium phosphate buffer (pH 6.8) to a value of 22.1 ml/g for the expanded configuration. The effect of 8 M urea on the molecular domain is not as dramatic as that produced by 5 M guanidine hydrochloride. On removal of guanidine or urea from enterotoxin C solutions, the resulting viscosity data resemble those given by the untreated enterotoxin. Guanidine-treated and urea-treated enterotoxin C give identical or nearly identical precipitin reactions as that exhibited by the native toxin and also evoke emesis at concentrations identical with that of the untreated toxin. Acetylation of five tyrosyl groups of enterotoxin C does not affect the immunological and toxic properties, which suggests that the "free" tyrosines are not required for serological and emetic activities. Acetylation of all the 21 tyrosyl residues results in an almost total loss of precipitating capacity and ability to induce vomiting in monkeys. The effective average number of determinants per molecule of enterotoxin C which can bind antibody molecules simultaneously was found to be three.

The staphylococcal enterotoxins, namely A, B, and C, which have been identified and purified, are protein in nature. They have also been found to elicit the formation of antibodies when injected to rabbits. Thus, the antigenic property of these toxins has provided a convenient means to identify the type of enterotoxin produced by different strains of *Staphylococcus aureus* under study and has also simplified the technique involved in the detection of new enterotoxins. Of the three enterotoxins that have been purified, only studies on the serological and emetic activities of enterotoxin B have been reported (Dalidowicz *et al.*, 1966), relating these properties of the native toxin to those of the modified (reduced and alkylated) and denatured (use of guanidine hydrochloride) toxin.

The present communication deals with the progressive effect of increasing concentration of alkali on the serological and toxic activities of enterotoxin C. It was also of interest in our laboratory to investigate the changes on the immunological and toxic properties of enterotoxin C that might ensue on treatment of the toxin with denaturing agents (guanidine hydrochloride and urea), a specific chemical modifying agent (*N*-acetylimidazole), and on oxidation with tyrosinase, and to relate these changes to those given by native enterotoxin C. The results are included in this paper.

An attempt has also been made to determine the effective number of determinants per molecule of enterotoxin C which can simultaneously bind with the antibody molecules.

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