Comparative Kinetic Stabilities of Staphylococcal Enterotoxin Types A, B, and C

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Staphylococcal enterotoxin types A and C, were observed by viscosimetry and near-ultraviolet difference spectroscopy to unfold at concentrations of aqueous guanidine hydrochloride greater than 1 M. Apparent rate constants of unfolding calculated from spectral curves differed markedly for the two enterotoxins. Rate constants for the unfolding of enterotoxin A in 2 or 3 M guanidine hydrochloride solution were 30- to 40-fold larger in value than those observed for enterotoxin C. In contrast, rate constants for the unfolding of enterotoxin C in 4 or 5 M guanidine hydrochloride solution were only 4- to 5-fold larger than those previously reported for enterotoxin type B (Warren, J. R., Spero, L., and Metzger, J. F. (1974) Biochemistry 13, 1678-1683). In addition, the types B and C, enterotoxins unfolded at nearly identical rates in 6 M guanidine hydrochloride and 8 M urea solution. Enterotoxin A unfolded about 50-fold faster in 8 M urea than enterotoxin B and C. Therefore, unfolding of enterotoxin A by guanidine hydrochloride or urea appears to have a considerably lower activation energy than unfolding of enterotoxin B or C, by the denaturants. It is suggested that the observed differences in kinetic stability reflect a significant dissimilarity of the native structure of enterotoxin A to the native structures of enterotoxin B and C. Enterotoxin A is known to demonstrate greater biological activity than enterotoxin B. Consequently, the dissimilarity of enterotoxin A structure indicated by the isothermal denaturation data is probably of functional importance.

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EXPERIMENTAL PROCEDURES

Materials—Enterotoxin A was prepared from a fermentation supernatant of *Staphylococcus aureus* strain 137-H-2. Enterotoxin B from a supernatant of *Staphylococcus aureus* strain 10-275, and enterotoxin C, from a supernatant of *Staphylococcus aureus* strain 137-H-2. The enterotoxins were purified to molecular homogeneity by previously published cation exchange chromatography techniques (9, 10). Ultraumat grade guanidine hydrochloride and urea were purchased from SchwarzlMann. Deionized water was used.

Difference Spectroscopy—UV Ultraviolet difference spectroscopy was performed utilizing a Cary model 15 spectrophotometer. For each denaturation experiment, an aliquot of a stock solution of enterotoxin in 0.15 M KCl was diluted by precise volumetric techniques with guanidine hydrochloride or urea solution and a second aliquot from the same solution was diluted to an identical extent with 0.15 M KCl. Enterotoxin in guanidine hydrochloride or urea was positioned in the sample chamber of the spectrophotometer and enterotoxin in 0.15 M KCl in the reference chamber, and the difference spectrum was recorded from 325 to 270 nm with appropriate KCl and guanidine hydrochloride or urea blanks in the sample.

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1 Enterotoxin types C, and C, are cross-reacting variants which differ in isoelectric pH. Enterotoxin C is the more alkaline variant (5).
and reference chambers. All experiments were performed at ambient temperature (22-24°C). Protein concentrations were calculated from an extinction coefficient (ε₆₀₀) of 14.6 for enterotoxin A (10), of 14.0 for enterotoxin B (9), and of 12.1 for enterotoxin C (11). All values for the difference in molar absorbance at 287 nm (Δε₆₀₀) were reproducible to within 10% or less.

Viscosimetry—Viscosities were determined in Cannon-Ubbelohde semi-microviscosimeters (Cannon Instrument Co.) having water flow times of around 250 s. The viscosimeters were immersed in a 24 ± 0.05°C circulating water bath. Flow times were measured on solutions cleared of particulates by filtration across 5-µm Millipore discs. Flow times of protein solutions were rechecked after 24 h to assure constant values. Results are reported as the reduced viscosity, ηᵥ, calculated from the flow times of enterotoxin-containing (t₀) and enterotoxin-free solutions (t) by the expression (t - t₀)/t₀ + (1 - t₀/t). Results were expressed as ηᵥ values relative to water.

**RESULTS**

Equilibrium Stabilities of Enterotoxins—The ultraviolet difference spectrum of enterotoxin A exposed to guanidine hydrochloride up to a concentration of 3 M or of enterotoxin C₁ exposed to guanidine hydrochloride up to 6 M was initially positive with maxima at 284 to 286 and 276 to 279 nm. However, at concentrations of guanidine hydrochloride greater than 1 M, the initial positive spectrum of each toxin disappeared with time and was eventually replaced by a negative difference spectrum demonstrating well defined major and minor minima at 287 and 280 nm, respectively. The spectral blue shift of enterotoxin A and enterotoxin C₁ between 1 and 2 M guanidine hydrochloride can be appreciated in the inset of Fig. 1. The negative difference spectrum of enterotoxin A or C₁ at guanidine hydrochloride concentrations above 1 M was identical with the previously reported negative difference spectrum of guanidine hydrochloride-denatured enterotoxin B (5). Also, a steep increase in the viscosity of enterotoxin A or C₁ was observed between 1 and 2 M guanidine hydrochloride (Fig. 1). Both difference spectroscopy and viscosimetry indicate, therefore, a cooperative loss of native structure for both enterotoxin A and enterotoxin C₁ over this concentration range of guanidine. As shown in Fig. 1, the viscosity of enterotoxin B demonstrated no increase until the guanidine hydrochloride concentration exceeded 2 M. Also, the ultraviolet denaturation blue shift of enterotoxin B occurs from 2 to 4 M guanidine hydrochloride (8). Thus, both native enterotoxin A and C₁ demonstrated somewhat less equilibrium stability toward guanidine hydrochloride denaturation than enterotoxin B.

Kinetic Stabilities of Enterotoxins—To obtain precise information on possible differences in conformational stabilities of the three enterotoxin variants, detailed kinetic analysis of enterotoxin unfolding in guanidine hydrochloride or urea solution was accomplished. The unfolding rates of the enterotoxins in guanidine solution varied widely. For example, unfolding of enterotoxin B in 3 M guanidine hydrochloride as followed by change in Δε₆₀₀ was not complete until about 8000 min of elapsed time (Fig. 2). In sharp contrast, unfolding of enterotoxin A reached equilibrium within 50 min. Enterotoxin C₁ required approximately 3000 min for unfolding in 3 M guanidine. Semilog plots were linear over 90 to 95% of the unfolding curves of enterotoxin A in 1.5, 2, and 3 M guanidine hydrochloride (Fig. 3) and of enterotoxin C₁ in 2, 3, 4, 5 and 6 M guanidine hydrochloride (Fig. 4). The apparent rate constants of unfolding were obtained from the slopes of the straight line semilog plots and are reported in Table I. The previously reported rate constants of unfolding for enterotoxin B in guanidine hydrochloride solution (8) are included in Table II.
Table I. The rate constants of unfolding were determined by measuring the decrease in molar absorbance at 287 nm of enterotoxin at the indicated guanidine hydrochloride concentrations. Values for enterotoxin B are from Ref. 8.

<table>
<thead>
<tr>
<th>Guanidine hydrochloride (M)</th>
<th>Rate of enterotoxin unfolding (×10^4 min^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A</td>
<td>Type B</td>
</tr>
<tr>
<td>1.5</td>
<td>19.2</td>
</tr>
<tr>
<td>2.0</td>
<td>72.2</td>
</tr>
<tr>
<td>3.0</td>
<td>321.1</td>
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<tr>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>4.0</td>
<td>4.7</td>
</tr>
<tr>
<td>5.0</td>
<td>8.5</td>
</tr>
<tr>
<td>6.0</td>
<td>81.2</td>
</tr>
</tbody>
</table>

The rate constants of unfolding were determined at the same concentration of the three enterotoxins (1 × 10^{-4} M), and thus the values are directly comparable. Such a comparison reveals that enterotoxin A unfolding was 40-fold faster in 2 or 3 M guanidine hydrochloride solution than enterotoxin C, unfolding. However, the unfolding of enterotoxin C, in 4 or 5 M guanidine hydrochloride was only 4- to 5-fold faster than the unfolding of enterotoxin B; the unfolding rates of these two toxins were comparable in 6 M guanidine. Likewise, enterotoxin C, unfolded with an apparent rate constant of 2.1 × 10^{-4} min^{-1} in 8 M urea. This value is very close to that previously reported for enterotoxin B in 8 M urea (2.0 × 10^{-4} min^{-1}) (8), whereas the apparent rate constant of enterotoxin A unfolding in 8 M urea (118.1 × 10^{-4} min^{-1}) was 50-fold greater than the unfolding rate constants of enterotoxin B or C, in the urea solution. Therefore, a large difference in kinetic stability toward isothermal denaturation by guanidine hydrochloride or urea appears to exist between enterotoxin B (the most stable) and enterotoxin C, (the least stable). Despite the difference in the equilibrium stabilities of enterotoxin B and enterotoxin C, these two enterotoxin variants are similar in kinetic stability toward isothermal denaturation.

**DISCUSSION**

Unfolding of enterotoxin B by 8 M urea followed by reduction and alkylation of the protein's single intramolecular disulfide bond and then extensive dialysis against neutral salt solution results in a folded enterotoxin B derivative lacking the kinetic stability toward isothermal denaturation observed for untreated native enterotoxin B (12). This interference with stable refolding of enterotoxin B by "bulky" carboxyamidomethyl or carboxymethyl groups covalently positioned at half-cystines 92 and 112 indicates that the 92—112 disulfide bond is located in a region of toxin structure critical for native conformation. Carboxyamidomethyl enterotoxin B unfolded in 2 M guanidine hydrochloride solution with an apparent rate constant of 44.1 × 10^{-4} min^{-1} (12). The unfolding behavior of the carboxyamidomethyl derivative of enterotoxin B (12) is thus very similar to that reported in this paper for native enterotoxin A (Fig. 1, Table I). The important question arises as to whether or not native conformations vicinal to the disulfide bond could explain the marked variation in kinetic stabilities of the enterotoxin variants (Table I). Analysis of the primary amino acid sequence of enterotoxin B by the technique of Chou and Fasman (13) suggests that the 92—112 disulfide bond forms a covalent cross-link between a β sheet at residues 89 to 94 and a β sheet at 111 to 118 (14). The primary structure of enterotoxin A in the immediate vicinity of its single disulfide bond is now available (15) and Chou-Fasman analysis (13) can be applied to this region. The 6 amino acid residues contiguous to half-cystine 112 at positions 113 to 118 of enterotoxin B have been conserved in enterotoxin A (Met-Tyr-Gly-Gly-Val-Thr). The β breaker Glu-119 of enterotoxin B is substituted for in enterotoxin A by the β former leucine; also, the β former threonine at position 111 of enterotoxin B is separated from the NH₂-terminal side half-cystine of enterotoxin A by the weak β former alanine. Thus, the COOH-terminal side half-cystine of enterotoxin A closely resembles half-cystine 112 of enterotoxin B by being incorporated into a peptide sequence having a high probability for β sheet formation. Likewise, the 5 amino acid residues just proximal to the NH₂-terminal side half-cystine of enterotoxin A (Tyr-Thr-Gly-Gly-Gln) are β formers, with the exception of glycine which is a β indifferent residue. Including the threonine immediately distal to the NH₂-terminal side half-cystine of enterotoxin A, the calculated value for the β sheet conformational parameter of this stretch of amino acid residues is 1.20 (a value ≥ 1.05 indicates a high probability of β sheet formation for a given sequence of 5 or more amino acid residues (13)). This value is close to our previously reported value of 1.28 for β sheet 89 to 94 of enterotoxin B. It appears, therefore, that the disulfide bonds of both enterotoxin types A and B serve to covalently lock two β sheets in a stable, antiparallel configuration. Consequently there are no obvious differences apparent for probable secondary structures in the immediate vicinity of the intramolecular disulfide bonds which might account for the difference in kinetic stability. However, Chou-Fasman analysis (12) of the enterotoxin B sequence also indicates a high probability for the incorporation of the 92—112 disulfide bond in a large core of repeating antiparallel β sheet from residues 81 to 148 (14). It is possible that deletion of strong β formers or substitution by amino acid residues with β breaker properties (or both) more distal from the disulfide than those presently analyzed might prohibit formation of a stable repeating antiparallel β sheet around the intramolecular disulfide bond of enterotoxin A. The specific differences in amino acid sequence which could result in an absence of stable secondary structures in enterotoxin A can be determined only when the complete sequence of this variant becomes known.

Unfolding kinetics in guanidine hydrochloride solution have been previously utilized in this laboratory to quantify the effect of peptide bond "nicking" and disulfide bond reduction on the conformation of enterotoxin B (12), and to demonstrate complementation between the 1 to 97 and 98 to 239 peptide chains of enterotoxin B (16). The present report demonstrates that the staphylococcal enterotoxins, although expressing qualitatively identical toxic and mitogenic activities (1, 4), vary greatly in kinetic stability. Although the near- and far-ultraviolet circular dichroism spectra of enterotoxin types B and C, are essentially identical (14, 17), a measurable difference in the kinetic stability of these two variants was found (Table I). Also, other studies have shown that tryptic cleavage of two peptide bonds in enterotoxin C, induced only minor changes in the near- and far-ultraviolet circular dichroic spectra, but doubled the protein's unfolding rate in 4 M guanidine (17). Thus, variation in kinetic stability appears to approach antigenic reactivity (1) as a sensitive indicator of structural differences between natural and derivatized variants of enterotoxin.
Comment can be made on the degree of structural homology between the enterotoxin variants. Significant quantitative differences in the biological activity of enterotoxin types A and B have been described. Enterotoxin A has been shown to demonstrate greater immunosuppressive activity than enterotoxin B. A dose of 0.1 μg/ml of enterotoxin A was found sufficient to suppress the in vitro humoral antibody responses of C57BL/6 spleen cells to sheep erythrocytes by greater than 90%; comparable inhibition was not obtained by enterotoxin B until toxin concentrations of 3 to 6 μg/ml (2). Also, the intravenous 50% effective dose of the A variant for enterotoxicity in rhesus monkeys is 0.03 μg/kg (0.017 to 0.065, 95% confidence limits) (10), compared to a 50% effective dose of 0.10 μg/kg (0.05 to 0.20, 95% confidence limits) for the B variant (9). The quantitative difference in biological activity of enterotoxins A and B corresponds to the large difference in kinetic stability for the two enterotoxins (Table I). Rate constants for the unfolding of enterotoxin types B and C are much closer in value than those reported for enterotoxin A (Table I). Biological data statistically equivalent to data reported for enterotoxins A and B are not available for enterotoxin C. However, enterotoxin types B and C closely resemble each other by the presence of peptide bonds highly susceptible to trypsin hydrolysis (18). Such labile peptide bonds are absent in the enterotoxin A molecule (18). Also, Spero and his colleagues (17) have recently reported that the NH₄-terminal 6500-dalton fragment and its adjacent 4000-dalton fragment obtained from enterotoxin C, by double cleavage with trypsin are very similar in amino acid composition to the equivalent peptide segments at positions 1 to 54 and 55 to 97 of enterotoxin B, respectively. Available biological and chemical data are compatible, therefore, with the kinetic data of Table I in suggesting a high degree of structural homology between enterotoxin types B and C, and significantly less structural homology of these two enterotoxins with enterotoxin A. Since enterotoxin A is apparently a more active toxic and immunosuppressive agent than enterotoxin B (2, 9, 10), it will be important to specifically identify those structural differences which exist between the enterotoxin A and B molecules.

REFERENCES
Comparative kinetic stabilities of staphylococcal enterotoxin types A, B, and C1.
J R Warren