Isothermal Denaturation of Aqueous Staphylococcal Enterotoxin B by Guanidine Hydrochloride, Urea, and Acid pH†

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ABSTRACT: The denaturation of staphylococcal enterotoxin B at 23 ± 1° by aqueous guanidine hydrochloride, urea, or dilute HCl was studied by measuring change in the intrinsic viscosity and near-ultraviolet difference spectrum of the protein. Prolonged exposure (hours to days, depending on denaturant concentration) of enterotoxin B to guanidine up to 6 M or urea up to 9 M was required for unfolding to reach equilibrium. Refolding of denatured toxin to native protein after dilution of denaturant was complete within minutes to a few hours. Thus, the native conformation of enterotoxin B is favored by a large activation energy of denaturation and a comparatively small activation energy of renaturation. These isothermal denaturation experiments indicate a very stable core of structure in native enterotoxin B. However, toxin in 0.15 M KCl was rapidly denatured following addition of HCl to a pH of below 3.5. Below pH 2.3 slow association of acid-denatured toxin into a 7.6S aggregate was detected. The lability of enterotoxin to acid pH suggests stabilization of structure by clusters of acid and basic groups on the native protein's surface.

Certain strains of *Staphylococcus aureus* elaborate water-soluble exoproteins which are capable of inducing an acute gastroenteritis in man (Casman *et al.*, 1963). One of these exoproteins, staphylococcal enterotoxin B, can be obtained in high purity from the culture supernatants of appropriate staphylococcal strains (Schantz *et al.*, 1965). SEB[1] is also a nonspecific mitogen, stimulating lymphocytes *in vitro* to undergo blastoid transformation with increased mitotic activity (Peavy *et al.*, 1970) and to release macrophage migration-inhibition factor (Kaplan, 1972). SEB, a globular protein with a molecular weight of 28,500, is composed of one peptide chain containing 239 amino acid residues (Huang and Bergdoll, 1970; Schantz *et al.*, 1965). The single intramolecular disulfide bond of SEB spans a stretch of 21 amino acids between residues 92 and 112; 32 of the 34 aromatic residues in the toxin molecule are located outside of the closed disulfide loop. The basic and acidic residues are distributed along the entire length of the SEB chain. Our laboratory is presently investigating structural features of the native or modified SEB molecule requisite for *in vivo* enterotoxic or immunogenic (Warren *et al.*, 1973) and *in vitro* mitogenic activity. An important part of this effort has been a study of the stability of SEB under various solvent conditions. Detailed information on the denaturation of other protein enterotoxins or mitogens is presently not available in the literature. Characteristics of the isothermal denaturation of a protein give important insights into structural features of the protein in its native state (Tanford, 1968). The native conformation of SEB in 0.15 M KCl was found to be stable toward disruption by Gdn·HCl and urea in that unfolding of the protein in aqueous solutions of these denaturants showed a large time dependency. However, the protein was relatively susceptible to acid denaturation, drastic alteration in SEB structure having occurred below pH 3.5. The implications of our isothermal denaturation data for the structure of native SEB and its biologic activity are discussed.

Experimental Section

Materials. SEB was obtained from bacterial cultures of *Staphylococcus aureus* strain 10-275 grown for 18 hr in an aerated medium containing 4% N-Z Amine A from Sheffield and 1% yeast extract. Strain 10-275 is a high SEB producer derived from the wild-type strain S-6 (Casman *et al.*, 1963) by cloning with specific anti-SEB antibody. The procedure of Schantz *et al.* (1965) was used to isolate and purify SEB from culture supernatants except that initial elution of toxin from Amberlite CG-50 resin was achieved at pH 6.4. SEB prepared by this technique exhibits a high degree of molecular homogeneity as determined by sedimentation velocity and equilibrium ultracentrifugation (Wagman *et al.*, 1965), discontinuous electrophoresis in polyacrylamide gel (Schantz *et al.*, 1965), and the presence of one N-terminal residue, glutamic acid, and one C-terminal residue, lysine (Spero *et al.*, 1965). Crystalline lysozyme, ovalbumin, and bovine serum albumin were utilized as supplied commercially. Ultra Pure grade guanidine hydrochloride and urea were purchased from Schwarz/Mann. Inorganic compounds were analytical reagent grade products. Deionized water was used in preparing all solutions.

Methods. Ultraviolet difference spectroscopy was performed with a Cary Model 15 spectrophotometer using techniques described in detail elsewhere (Wetlaufer, 1962). For denaturation experiments, stock solutions of SEB in 0.15 M KCl were diluted by precise volumetric techniques. SEB in Gdn·HCl, urea, or HCl–KCl solution was positioned in the sample chamber of the Cary 15 and SEB in 0.15 M KCl in the reference chamber, and the difference spectrum was recorded from 325 to 270 nm with appropriate KCl and denaturant blanks in the sample and reference chambers. Renaturation experiments were done in identical fashion, except that denatured SEB in 6 M Gdn·HCl or 9 M urea instead of native SEB in 0.15 M KCl was present in the reference chamber. All experiments were done at 23 ± 1°. SEB concentrations were calcu-

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[1] Abbreviations used are: SEB, staphylococcal enterotoxin B; Gdn·HCl, guanidine hydrochloride.

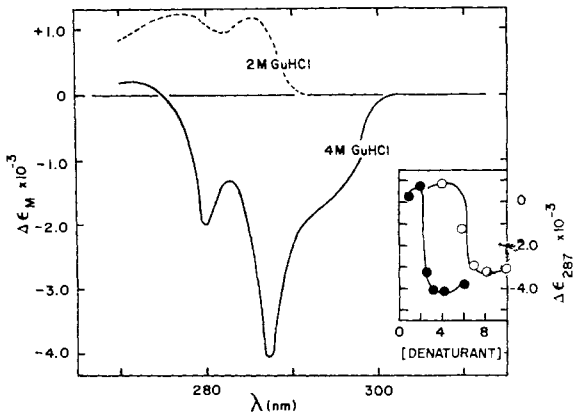

FIGURE 1: The denaturation blue-shift induced in the near ultraviolet difference spectrum of aqueous SEB at 23 ± 1° by increasing the concentration of guanidine hydrochloride from 2 to 4 M. A similar blue-shift was obtained by increasing aqueous urea concentration from 4 to 8 M. Inset: Values observed for $\Delta\epsilon_M$ at λ 287 nm by SEB at the indicated guanidine hydrochloride (●) or urea (○) concentrations. The $\Delta\epsilon_{287}$ reported at Gdn·HCl concentrations above 2 M and urea above 4 M was determined at equilibrium, $\Delta\epsilon_{287}$ at 2 M Gdn·HCl and 4 M urea within minutes of adding SEB to solution (see text). All experiments were performed with the sample cell containing SEB in denaturant (plus 0.15 M KCl) and the reference cell SEB in 0.15 M KCl. Solutions were unbuffered with a measured pH of 6.0–6.9 for SEB–Gdn·HCl and of 7.3–8.0 for SEB–urea. The protein concentration of the SEB–denaturant solutions was $1-2 \times 10^{-5}$ M.

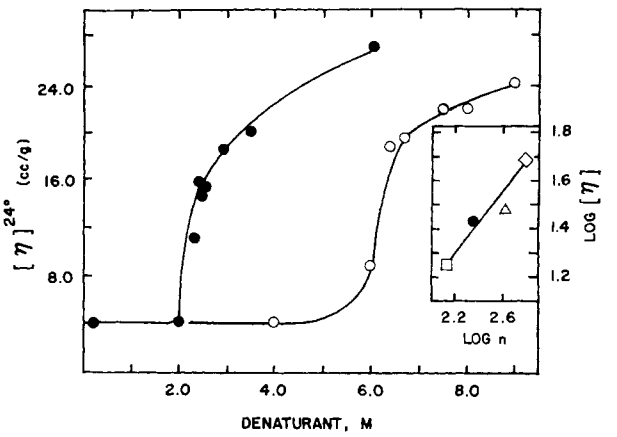

FIGURE 2: The intrinsic viscosity of aqueous SEB at 24 ± 0.05° as a function of guanidine hydrochloride (●) or urea (○) concentration. The supporting electrolyte was 0.15 M KCl. Values given for $[\eta]^{24}$ represent equilibrium values at Gdn·HCl concentrations over 2 M and urea above 4 M. The intrinsic viscosity of SEB in 2 M Gdn·HCl or 4 M urea was constant during incubation in these solvents for 7 days. Inset: The intrinsic viscosity of SEB in 6 M Gdn·HCl with or without 0.4 M β-mercaptoethanol (●) and of lysozyme (□), ovalbumin (△), and bovine serum albumin (◇) in 6 M Gdn·HCl plus 0.4 M β-mercaptoethanol as a function of the number of amino acids (n) in each protein.

lated from an extinction coefficient ($E^{1\%}_{277}$) of 14.0 (Schantz *et al.*, 1965). All values reported for $\Delta\epsilon_{287}$ in this paper were reproducible to within 10% or less.

Viscosities were determined with Cannon-Ubbelohde semimicro viscometers (Cannon Instrument Co.) having water flow times of around 250 sec. The viscometers were immersed in a circulating water bath maintained at 24 ± 0.05° and flow times were measured on solutions cleared of particulates by filtration over 5-μm Millipore discs. Flow times of protein solutions were rechecked after a minimum of 16 hr to assure constancy of values. Values for intrinsic viscosities were obtained by extrapolating the reduced viscosity, $\eta_{red} = \eta_{sp}/c = (\eta - \eta_0/\eta_0 c)$, measured at three or four protein concentrations from 0.5 to 3.5% (g/v) to zero protein concentration (Tanford *et al.*, 1955). Reduced viscosities of SEB denatured by guanidine or urea then renatured in 0.15 M KCl were measured at an SEB concentration of 1.5%, of SEB in acidified 0.15 M KCl at a protein concentration of 0.5%.

Sedimentation velocity experiments were done at 52,000 rpm and 20° with a Spinco Model E analytical ultracentrifuge.

Results

Effect of Gdn·HCl and Urea on the Ultraviolet Absorbancy and Viscosity of SEB. Upon initial exposure of SEB to aqueous Gdn·HCl up to a concentration of 6 M or aqueous urea up to 9 M a positive difference spectrum was observed in the near-ultraviolet region with maxima at 284–286 and 276–279 nm. With passage of time, the positive spectrum of SEB exposed to Gdn·HCl at concentrations greater than 2 M or to urea above 4 M was progressively replaced by a negative difference spectrum which eventually showed well-defined minima at 287 and 280 nm and a shoulder at 292–297 nm (Figure 1). A gradual diminution in the intensity of the positive spectrum of SEB in 2 M Gdn·HCl or 4 M urea was also observed over a period of a week without significant alteration of the relative maxima at 285 and 278 nm or increase in the intrinsic viscosity of the protein (Figure 2). This nonspecific spectral change in dilute denaturant solution must reflect a small conformational alteration and/or slow hydrolysis. The negative difference spectrum seen at higher concentrations of guanidine and urea is distinctive for a denatured protein (Wetlaufer, 1962); the minima at 287 and 280 nm can be attributed to exposure of buried tyrosine residues and the 292–297-nm shoulder to exposure of a buried tryptophan to external solvent upon SEB unfolding. The tyrosine bands should be sensitive indicators of important conformational events since the SEB molecule contains 21 tyrosine residues distributed along the entire length of its peptide backbone (Huang and Bergdoll, 1970). Changes in the 292–297-nm shoulder could be generated by structure rearrangements localized to the single tryptophan of SEB; thus, this region of the difference spectrum has not been further characterized. The increased or decreased molar absorbancy obtained at equilibrium at 287 nm was dependent upon the concentration of Gdn·HCl or urea (inset, Figure 1). This spectral blue shift was accompanied by a large increase in intrinsic viscosity of the protein (Figure 2). The equilibrium extent of SEB unfolding

$$N \underset{}{\overset{K}{\rightleftharpoons}} U$$

during this transition was calculated by the expression

$$K = ([\eta] - [\eta]_N)/([\eta]_U - [\eta])$$

where $[\eta]$ is the intrinsic viscosity at the indicated guanidine or urea concentration, $[\eta]_N$ the viscosity of native SEB in 0.15 M KCl (4.0 cm^3/g), and $[\eta]_U$ the viscosity of SEB in 6 M Gdn·HCl containing 0.4 M β-mercaptoethanol (27.0 cm^3/g). The equilibrium extent of unfolding showed a large dependence upon denaturant concentration for values of K between 0.4 and 2.3 in Gdn·HCl and 0.2 and 2.0 in urea. Assuming that the value of $[\eta]_U$ is independent of denaturant concentration, K is proportional to the 6th power of guanidine and 11.5th power of urea. Therefore both difference spectroscopy and viscometry indicate a cooperative loss of native structure of SEB between 2 and 4 M Gdn·HCl or 4 and 8 M urea. The value of

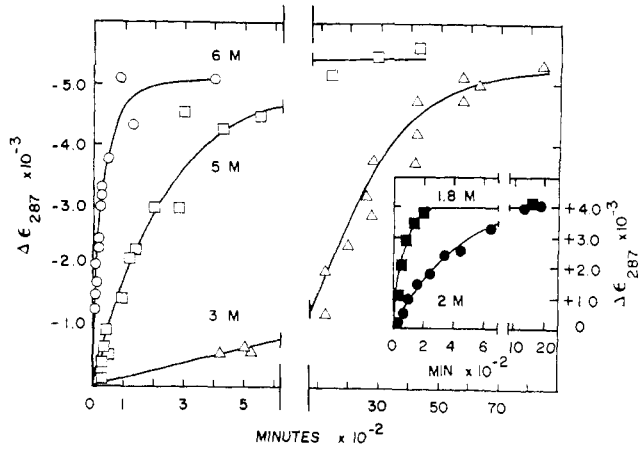

FIGURE 3: The decrease in molar absorbancy at 287 nm during SEB unfolding as a function of time in 3 M (△), 5 M (□), and 6 M (○) Gdn·HCl solution. Sample solution was SEB in denaturant plus 0.15 M KCl, the reference solution native SEB in 0.15 M KCl. Inset: The increase in molar absorbancy at 287 nm with time during SEB refolding in 1.8 M (■) and 2 M (●) Gdn·HCl. SEB previously denatured in 6 M Gdn·HCl was diluted with 0.15 M KCl then placed in sample compartment. Reference solution was completely unfolded SEB in 6 M Gdn·HCl. SEB concentration in the kinetic experiments was *ca.* 10^{-5} M. In all unfolding and refolding experiments, the difference spectrum initially observed within 180 sec of preparing sample SEB–denaturant solutions was used to calculate $\Delta\epsilon_{287}$ for subsequent scans.

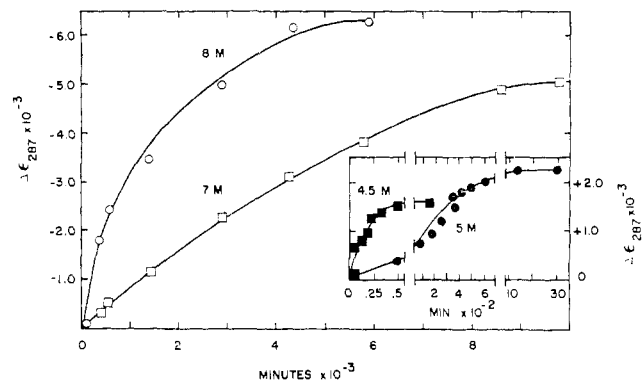

FIGURE 4: The decrease in molar absorbancy at 287 nm during SEB unfolding as a function of time in 7 M (□) and 8 M (○) urea. Inset: The increase in molar absorbancy at 287 nm with time during SEB refolding in 4.5 M (■) and 5 M (●) urea. SEB concentration in the urea kinetic experiments was *ca.* 10^{-5} M protein.

the viscosity for SEB showed a further increase between 3.5 and 6.0 M Gdn·HCl or 8.0 and 9.0 M urea (Figure 2). However, the intrinsic viscosity of SEB in 6 M Gdn·HCl was not increased by the addition of 0.4 M β-mercaptoethanol. Furthermore, when compared to the viscosity of reduced and completely unfolded lysozyme, ovalbumin, and bovine serum albumin, the intrinsic viscosity of oxidized or reduced SEB in 6 M Gdn·HCl was a strict function of the number of amino acid residues in the SEB molecule (inset, Figure 2). The failure of mercaptoethanol to increase the intrinsic viscosity of SEB in 6 M Gdn·HCl could be attributed to residual loop structure rendering the disulfide bond inaccessible to reduction by the mercaptan. It is more likely, however, that SEB was completely devoid of noncovalent structure in 6 M Gdn·HCl (Tanford *et al.*, 1967) with the drag caused by the closed loop having offset that obtained by reduction and full extension of randomly coiled SEB in this guanidine solution.

In further experiments, SEB was unfolded in 6 M Gdn·HCl or 9 M urea and then diluted to 2 M Gdn·HCl or 4 M urea with an appropriate volume of 0.15 M KCl. When the SEB–2 M Gdn·HCl or SEB–4 M urea solution was placed in the sample chamber of the Cary 15 and scanned in the near-ultraviolet against SEB–6 M Gdn·HCl or SEB–9 M urea in the reference chamber, a positive difference spectrum developed with elapsed time which showed features identical with those of the negative spectrum in Figure 1, *e.g.*, 287 and 280 nm maxima and a 292–297-nm shoulder. The observed equilibrium values of +4750 for the total change in the molar absorbancy at 287 nm of SEB diluted to 2 M guanidine and +4000 of SEB diluted to 4 M urea compared closely to the absolute difference in molar absorbancy between SEB in 2 and 6 M Gdn·HCl or 4 and 9 M urea *vs.* SEB in 0.15 M KCl (inset, Figure 1). Therefore, the blue shift induced in the ultraviolet spectrum of SEB was completely reversed by dilution of guanidine or urea to nondenaturing concentrations. Also, the reduced viscosity of SEB unfolded in 6 M guanidine for 24 hr or 9 M urea for 72 hr and then dialyzed against 0.15 M KCl for 48 hr was found to be 4.0 ± 0.2 cm^3/g (compare to the curve of Figure 2), confirming the reversibility of SEB unfolding by these denaturants.

Time Dependence of SEB Unfolding and Refolding in Aqueous Gdn·HCl and Urea. The decreasing molar absorbancy at 287 nm of SEB in aqueous 6 M Gdn·HCl reached equilibrium within 100 min but not for about 3.5 days in 3 M Gdn·HCl (Figure 3). The unfolding of SEB in 8 M urea continued until the third day of exposure to this solvent, in 7 M urea until about the sixth day (Figure 4). Semilog plots of the difference between the decreased molar absorbancy at time t and the final decrease at equilibrium ($\Delta\epsilon_t - \Delta\epsilon_\infty$) against elapsed time were linear for 80–90% of the total observed absorbancy change in both the Gdn·HCl (representative plots shown in Figure 5) and urea solutions. Values were obtained for the apparent rate constant of unfolding from the slope of the straight line plots[2] and are reported in Table I. A similar treatment of the decrease in absorbancy at 280 nm also revealed a linear relationship with time and the calculated rate constants were close in value to those determined at 287 nm, *e.g.*, 100, 7.2, 3.7, and 2.6 × 10^{-4} min^{-1} at 6, 5, 4, and 3.5 M Gdn·HCl and 2.4 or 1.0 × 10^{-4} min^{-1} at 8 or 7 M urea. The kinetics of renaturation of SEB unfolded in 6 M Gdn·HCl or 9 M urea were characterized by measurement of increase in molar absorbancy at 287 or 280 nm with time following dilution with 0.15 M KCl to 1.5, 1.8, or 2 M Gdn·HCl and 3, 4.5, or 5 M urea. Again, a linear relationship was maintained[2] (representative plots shown in the inset of Figure 5) for the observed increased absorbancy with time (insets, Figures 3 and 4) and values for the apparent rate of refolding calculated from $\Delta\epsilon_{287}$ (Table I) corresponded closely to those derived from $\Delta\epsilon_{280}$, *e.g.*, 9.6 and 110 × 10^{-4} min^{-1} at 2 and 1.8 M Gdn·HCl, 10.8 and 107 × 10^{-4} min^{-1} at 5 and 4.5 M urea. Salient features of SEB refolding include: (1) the magnitude of the increase in refolding rate after dilution of guanidine from 2 to 1.8 M was about equal to that of the increase in unfolding rate after raising guanidine concentration from 5 to 6

[2] The apparent first-order kinetics of SEB unfolding and refolding imply absence of stable intermediates with an absorbancy significantly different from native or completely denatured SEB. Whether the N ⇄ U transition follows true first-order kinetics for SEB cannot be decided without further studies of change in another property of the protein or the effect of large variation in protein concentration. Nevertheless, the apparent first-order kinetics obtained by difference spectroscopy allow easy calculation of rate constants and are thus useful for this report.

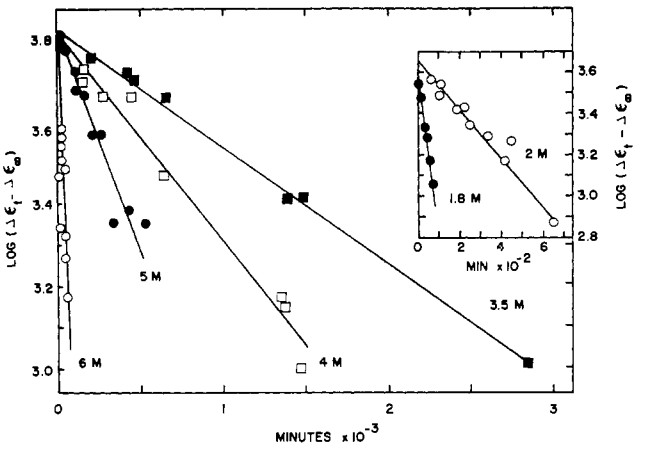

FIGURE 5: Apparent first-order kinetic plots for the unfolding of SEB at the indicated Gdn·HCl concentrations. $\Delta\epsilon_t - \Delta\epsilon_\infty$ represents the difference between the decrease in molar absorbancy at 287 nm and time t and the final decrease observed at equilibrium ($t = \infty$). Inset: Kinetic plots for the refolding of SEB previously denatured in 6 M Gdn·HCl. Absolute values of $\Delta\epsilon_t - \Delta\epsilon_\infty$ were used in calculations for refolding kinetics.

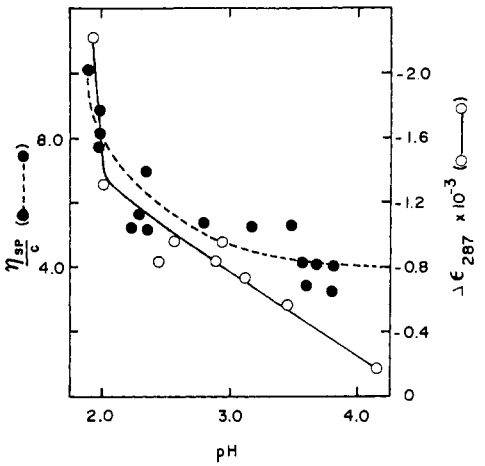

FIGURE 6: The effect of pH upon the reduced viscosity (●) and molar absorbancy at 287 nm (○) of aqueous SEB at ionic strength 0.15. In viscosity experiments, 9 volumes of 3–20 mM HCl was added to 1 volume of 5% SEB; in difference spectroscopy experiments, 0.01–0.1 volumes of 200 mM HCl or 0.05–0.2 volumes of 10 mM HCl was added to 1 volume of 0.30–0.33 × 10^{-4} M SEB. The supporting electrolyte was 0.15 M KCl. Values of pH are measured values of acidified SEB solutions following viscometry or spectroscopy.

M (Table I); (2) the more than tenfold increase in SEB refolding rate following decrease in urea from 5 to 4.5 M was not matched by a comparable increase in unfolding rate from 7 to 8 M urea (Table I); (3) refolding of SEB in 1.5 M Gdn·HCl or 3 M urea was complete within 180 sec of dilution.

Denaturation of SEB by Acid pH. The addition of HCl to SEB in 0.15 M KCl to a pH of 4.0 had no effect on the hydrodynamic or spectral properties of the protein. However, from pH 3.5 to 2.2 there was a small increase in reduced viscosity to about 6.0 cm^3/g followed by a steep increase at pH values lower than 2.2 (Figure 6). A parallel decrease in the molar absorbancy at 287 nm accompanied the increase in reduced viscosity (Figure 6). At a pH of 3.0 or below the acid difference spectrum of SEB developed a very sharp minimum at 287 nm, at a pH of less than 2.0 well-defined minima at both 287 and 280 nm. At pH 2.3 or above the observed changes in viscosity or absorbancy developed within minutes of addition of acid and then remained constant. But below pH 2.3 the initial increase in viscosity or decrease in absorbancy was followed over a period of several days by a slow increase in viscosity and red shifting of the ultraviolet difference spectrum. Sedimentation velocity centrifugation of SEB incubated at pH 2.2 for 24 hr revealed slow-sedimenting material with an $s_{20,w}$ of 2.2 S and fast-sedimenting material with an $s_{20,w}$ of 7.8 S. Centrifugation of SEB in HCl–KCl (pH 3.5–4.0) showed a single symmetrical peak with an $s_{20,w}$ of 2.7 ± 0.1 S, a value identical with that reported for native SEB (Wagman *et al.*, 1965); of SEB maintained at pH 1.8 for 24 hr, a single peak with an $s_{20,w}$ of 7.4 S. Treatment of SEB with acid therefore resulted in expansion of the monomeric protein, beginning at pH 3.5 where the protein carries about 60% of its maximum positive charge (Chu, 1968), with aggregation of acid-denatured SEB at a pH less than 2.2.

TABLE I: The Apparent Rate of Unfolding and Refolding of SEB in Aqueous Guanidine Hydrochloride and Urea.[a]

Denaturant	Concn (M)	Rate of Unfolding ($\times 10^4$ min^{-1})	Rate of Refolding ($\times 10^4$ min^{-1})
Gdn·HCl	6.0	81.2	
	5.0	8.5	
	4.0	4.7	
	3.5	2.5	
	2.0		11.6
	1.8		66.2
Urea	8.0	2.0	
	7.0	1.2	
	5.0		7.3
	4.5		104.0

[a] Apparent rate constants determined by measuring the decrease or increase in molar absorbancy at 287 nm and 23 ± 1° of SEB in 0.15 M KCl and the indicated amount of denaturant.

Discussion

The concentration range of Gdn·HCl or urea over which SEB was denatured is typical for globular proteins (Tanford, 1968). However, the unfolding of SEB was much slower than generally observed (Simpson and Kauzmann, 1953; Kauzmann and Simpson, 1953; Nelson and Hummel, 1962; Tanford *et al.*, 1966; Pace and Tanford, 1968; Warren and Gordon, 1971). For example, rate constants for unfolding of hen egg-white lysozyme in Gdn·HCl at 25° (Hamaguchi and Kurono, 1963) range from 770 × 10^{-4} min^{-1} in 3.8 M Gdn·HCl to 3090 × 10^{-4} in 6 M Gdn·HCl. The rate of SEB unfolding in Gdn·HCl at 24° was one to two orders of magnitude slower (Table I). Since lysozyme assumes a very stable native conformation in aqueous solution (Steiner, 1964; Ogasahara and Hamaguchi, 1967; Warren and Gordon, 1970), this comparison reveals the unusually stable conformation of native SEB. The relatively rapid refolding of denatured SEB confirms that the native structure of this protein is strongly favored. Even though a quantitative estimate cannot be directly made from our isothermal denaturation data, the kinetic stability of native SEB must be due to a very large activation energy of unfolding and a small activation energy of refolding. This suggests a highly unfavorable free energy

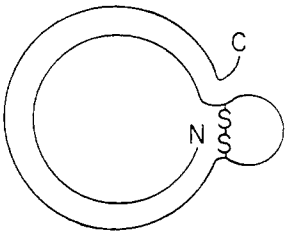

FIGURE 7: A schematic representation of the placement of the intramolecular disulfide bond in the SEB molecule, based on the published primary structure of the protein (Huang and Bergdoll, 1970).

of solvent interaction for critically activated SEB during unfolding (Tanford, 1970). SEB neither contains an unusually large content of aliphatic and aromatic residues nor shows long stretches of contiguous nonpolar amino acids along its peptide backbone (Huang and Bergdoll, 1970) to explain such an unfavorable interaction with solvent. A possible reason for the kinetic stability is apparent, however, from the schematic model shown in Figure 7. The SEB molecule contains a single disulfide bridge between half-cystine-92 and -112 defining a minor stretch of 21 residues. This arrangement would permit extensive interaction unhindered by constraints of disulfide cross-links of the N-terminal 91 and C-terminal 127 residues and assumption by this major loop of a conformation with a very low free energy. The fraction of tyrosine residues in native SEB accessible to solvent, *e.g.*, 24–29% (Chu, 1968), is small compared to the solvent accessibility of tyrosines reported for other proteins (Williams *et al.*, 1965; Laskowski, 1966). Presence of the 292–297-nm shoulder in the negative spectrum of denatured SEB (Figure 1) also indicates that Trp-197 is largely buried in native SEB. Since 20 of the 21 tyrosines and the tryptophan are in the major loop, it appears that the aromatics are located in a very compact "core" of structure having minimal contact with external solvent. In this setting, activation of SEB for denaturation would necessitate increased solvent contact with disruption of this "core" of structure and have a large positive configurational free energy. The ability of aliphatic or aromatic side chains to form ordered structures stable toward Gdn·HCl and urea when incorporated into polymers permitting extensive side chain–side chain interaction has been demonstrated (Auer and Doty, 1966).

The lability of SEB toward acid pH suggests another important structural feature of the native protein. SEB is a basic protein (Schantz *et al.*, 1965) and the expansion occurring at low pH could be attributed to a large net positive charge on the protein. But it is established that net protein charge has little to do, apart from association–dissociation reactions, with protein conformation at different pH values. The effect of low pH on a protein is best understood in terms of the behavior of specific acid groups titrated over the pH range of denaturation (Tanford, 1968). The SEB hydrogen ion titration curve (Chu, 1968) shows that in 0.16 M KCl the histidines titrate normally. Acid expansion of SEB must be due to protonation of carboxylate groups critically involved in maintaining native SEB conformation. Recently Chu and Crary (1969) reported that ten carboxyls per SEB molecule are nonreactive at pH 4.75 toward glycine methyl ester except in the presence of 6 M Gdn·HCl. Potentiometric titration of SEB in dilute KCl solution has shown anomalous titration below pH 3.0 of about ten carboxylate groups (Chu, 1968) coinciding with the acid denaturation transition of the protein. Thus, interaction of ten carboxylates in SEB with glycine ester or protons is strongly influenced by the protein's conformation. Since charged carboxylic acid side chains have little hydrophobic character (Tanford, 1970), it is improbable that all ten carboxylates are buried in the SEB interior. A significant number of these carboxylates are probably positioned on the protein surface but in close proximity to cationic regions formed by clusters of basic groups. Nonreactivity of these carboxylates to the cationic glycine ester would be due to repulsive forces between the vicinal cationic groups and the ester. More importantly, protonation of these critical carboxylates would result in mutual repulsion between the neighboring cationic groups with considerable local electrostatic stress placed on the native molecule. A more flexible conformation with "destruction" of the cationic regions and reduction of the local positive electrostatic free energy would thus be favored during titration of the critical carboxylates.

Could clustered basic groups or the unusual stability of the SEB molecule promote activity *in vivo?* Both serologic and emetic activity of SEB are markedly diminished after acetylation or succinylation of lysyl ϵ-amino groups (Chu *et al.*, 1969); yet full biologic activity is retained after guanidination of 31–32 of the 33 SEB lysines (Spero *et al.*, 1971). Biologic activity thus depends upon interaction of SEB cationic groups with anionic regions on specific antibody or intestine receptor sites for toxin. A cooperative interaction of discrete cation clusters on the SEB surface with specific immunoglobulin on lymphoid cells or toxin receptor sites on gastrointestinal cells would enhance the immunogenicity and enterotoxicity of the protein. The role of basic residues in the mitogenic activity of SEB is presently unknown. Also, the relationship of the unusual conformational stability of SEB to biologic activity is not clear. Naturally occurring apoferritin (Listowsky *et al.*, 1972), human growth hormone (Aloj and Edelhoch, 1972), and Kunitz soybean trypsin inhibitor (Fish and Leach, 1973) are examples of proteins from unrelated biologic sources showing a high degree of conformational stability. Thus, a very stable native form is not unique to this staphylococcal enterotoxin and may be totally unrelated to *in vivo* expression of its biologic specificity. To better define the relationship between stability and enterotoxicity, our laboratory is now investigating the structural stability of other antigenic variants of the staphylococcal enterotoxins.

Acknowledgments

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References

Aloj, S., and Edelhoch, H. (1972), *J. Biol. Chem. 247*, 1146.
Auer, H. E., and Doty, P. (1966), *Biochemistry 5*, 1716.
Casman, E. P., Bergdoll, M. S., and Robinson, J. (1963), *J. Bacteriol. 85*, 715.
Chu, F. S. (1968), *J. Biol. Chem. 243*, 4342.
Chu, F. S., and Crary, E. (1969), *Biochim. Biophys. Acta 194*, 287.
Chu, F. S., Crary, E., and Bergdoll, M. S. (1969), *Biochemistry 8*, 2890.
Fish, W. W., and Leach, S. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 496.
Hamaguchi, K., and Kurono, A. (1963), *J. Biochem. (Tokyo) 54*, 111.

Huang, I.-Y., and Bergdoll, M. S. (1970), *J. Biol. Chem. 245*, 3518.
Kaplan, J. (1972), *Cell. Immunol. 3*, 245.
Kauzmann, W., and Simpson, R. B. (1953), *J. Amer. Chem. Soc. 75*, 5154.
Laskowski, M., Jr. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 20.
Listowsky, I., Blauer, G., Englard, S., and Betheil, J. J. (1972), *Biochemistry 11*, 2176.
Nelson, C. A., and Hummel, J. P. (1962), *J. Biol. Chem. 237*, 1567.
Ogasahara, K., and Hamaguchi, K. (1967), *J. Biochem. (Tokyo) 61*, 199.
Pace, N. C., and Tanford, C. (1968), *Biochemistry 7*, 198.
Peavy, D. L., Adler, W. H., and Smith, R. T. (1970), *J. Immunol. 105*, 1453.
Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A., and Bergdoll, M. S. (1965), *Biochemistry 4*, 1011.
Simpson, R. B., and Kauzmann, W. (1953), *J. Amer. Chem. Soc. 75*, 5139.
Spero, L., Jacoby, H. M., Dalidowicz, J. E., and Silverman, S. J. (1971), *Biochim. Biophys. Acta 251*, 345.
Spero, L., Stefanye, D., Brecher, P. I., Jacoby, H. M., Dalidowicz, J. E., and Schantz, E. J. (1965), *Biochemistry 4*, 1024.
Steiner, R. F. (1964), *Biochim. Biophys. Acta 79*, 51.
Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Tanford, C., Buzzell, J. G., Rands, D. G., and Swanson, S. A. (1955), *J. Amer. Chem. Soc. 77*, 6421.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Tanford, C., Pain, R. H., and Otchin, N. S. (1966), *J. Mol. Biol. 15*, 489.
Wagman, J., Edwards, R. C., and Schantz, E. J. (1965), *Biochemistry 4*, 1017.
Warren, J. R., and Gordon, J. A. (1970), *J. Biol. Chem. 245*, 4097.
Warren, J. R., and Gordon, J. A. (1971), *Biochim. Biophys. Acta 229*, 216.
Warren, J. R., Spero, L., and Metzger, J. F. (1973), *J. Immunol. 111*, 885.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.

On the Tetramer–Dimer Equilibrium of Carbon Monoxyhemoglobin in 2 M Sodium Chloride†

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ABSTRACT: The state of aggregation of human carbon monoxyhemoglobin in solution at neutral pH has been studied by light scattering at concentrations ranging from about 0.7 to 12 g/l. As in our earlier work, the scattering behavior at low ionic strength (0.1 M) indicates only intact tetrameric hemoglobin, and implies that the tetramer–dimer dissociation constant can be no more than about 10^{-6} mol/l. In contrast, data on systems in 2 M NaCl, with or without phosphate or Tris buffer, demand an upward revision of our previous estimate of dissociation—to dissociation constants on the order of 10^{-5} mol/l., but still smaller than values reported in a number of investigations. Stripping the hemoglobin of bound organic phosphate (diphosphoglycerate) has no measurable effect on the dissociation.

It appears well established that human adult hemoglobin (hemoglobin A) in the deoxy (Hb)[1] form does not dissociate appreciably into dimers, even in the presence of 2 M NaCl at neutral pH; *i.e.*, the tetramer–dimer dissociation constant is less than 10^{-6} mol/l. (Norén *et al.*, 1971; Kellett, 1971; Thomas and Edelstein, 1972). However, there remains disagreement on the dissociation of carbon monoxyhemoglobin, particularly at high ionic strength (Antonini and Brunori, 1970; Edelstein *et al.*, 1970; Norén *et al.*, 1971; Kellett, 1971).

In a previous light-scattering study of the effect of 2 M NaCl on the apparent molecular weight of Hb and HbCO, we found no evidence for dissociation at the level of discrimination afforded by our experiments (Norén *et al.*, 1971). Using the same light-scattering technique, we have now supplemented our earlier results with some measurements on HbCO stripped of organic phosphate since it has been suggested that 2,3-diphosphoglycerate bound to hemoglobin might affect the dissociation. We have also somewhat extended the hemoglobin concentration range studied in both directions: 0.7–12 g/l. rather than 1.7–7 g/l.

Experimental Section

Materials. Fresh whole blood was obtained from the local blood bank. The cells were washed several times with saline and the hemoglobin was converted to HbCO by treatment

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[1] Abbreviations used are: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carbon monoxyhemoglobin; DPG, 2,3-diphospho-D-glyceric acid; IHP, inositolhexaphosphoric acid; Bis-Tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol.