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Hydrogen-1 Nuclear Magnetic Resonance Investigation of *Clostridium pasteurianum* Rubredoxin: Previously Unobserved Signals†

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ABSTRACT: Previously unobserved signals were located in the 470-MHz ^{1}H NMR spectra of oxidized and reduced rubredoxin (Rd) from *Clostridium pasteurianum*. When the protein was oxidized, some of the resonances broadened beyond detection. Longitudinal relaxation (T_1) measurements identified a number of these peaks as arising from residues close to the paramagnetic iron; these resonances exhibited short T_1 values attributable to the dominant electron–nuclear dipolar relaxation mechanism. The chemical shifts of these peaks were not strongly dependent on the oxidation state of the protein, although relative ratios of line widths of several peaks in the spectra of oxidized and reduced Rd suggested localized conformational changes of the protein as a result of oxidation. Furthermore, spectra of the oxidized protein collected in the range 8–60 °C revealed no appreciable changes in the chemical shifts of these peaks with temperature. These results seem to point out a negligible dipolar contribution, due to either magnetic anisotropy or zero field splitting, to the observed shifts in the spectrum of oxidized Rd. Resonances were assigned to tyrosine-11 or phenylalanine-49 (but not to either specifically) on the basis of their T_1 values and the X-ray diffraction data of the protein molecule [Watenpaugh, K. D., Sieker, L. C., Herriott, J. R., & Jensen, L. H. (1973) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. B29*, 943–956; and a further refinement deposited with the Protein Data Bank]. An upfield-shifted peak at about −1.1 ppm in the spectra of both oxidized and reduced Rd was assigned to a methyl group. Broad, rapidly relaxing peaks in the 8–10 ppm spectral region of oxidized Rd and even broader peaks in the 0 to −4.5 ppm spectral region of reduced Rd were identified with the ligated cysteinyl α- and β-hydrogens, respectively, as suggested by an analysis of their chemical shifts, line widths, and longitudinal relaxation properties.

Our interest in high-potential iron–sulfur proteins (HiPIPs)[1] and ferredoxins prompted us to investigate rubredoxin (Rd) as a potential model for assigning the hyperfine-shifted ^{1}H NMR peaks in spectra of the more complicated iron–sulfur proteins. For example, in contrast to the HiPIPs, which have 4Fe-4S clusters (Carter, 1977), Rd has no acid-labile sulfur and only one iron atom, which is nearly tetrahedrally coordinated to the sulfur of four cysteinyl ligands (Cammack et al., 1977). Various physical techniques, including X-ray diffraction (Herriott et al., 1970; Watenpaugh et al., 1973, 1979), ESR (Atherton et al., 1966), Mössbauer (Phillips et al., 1970), NMR (Phillips et al., 1970), and laser Raman

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[1] Abbreviations: HiPIPs, high-potential iron–sulfur proteins; 4Fe-4S, cluster containing four iron atoms and four inorganic sulfur (acid-labile) atoms; Rd, rubredoxin; ESR, electron spin resonance; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

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(Long et al., 1971; Yachandra et al., 1983) spectroscopies, have been applied to the Rd from *Clostridium pasteurianum* in order to elucidate structural details of the iron–sulfur complex. However, in the one previous ^{1}H NMR study, which was carried out at 220 MHz, Phillips et al. (1970) did not locate the expected hyperfine-shifted resonances of the iron ligands. These signals are of interest because they generally are sensitive indicators of the electronic structure of the active site (Phillips, 1973; La Mar, 1979).

We report herein on a 470-MHz ^{1}H NMR study of the oxidized and reduced forms of *C. pasteurianum* rubredoxin. The spectra contain previously unobserved peaks that we assign, along with others, to hydrogens of the cysteinyl ligands. Resonances due to certain aromatic ring hydrogens that are close to the iron have been identified and partially assigned.

Materials and Methods

Protein. The Rd from *C. pasteurianum* was isolated and partially purified as described by Armstrong et al. (1980). The final purification by ammonium sulfate chromatography was as described by Lovenberg (1972). The final $A_{ox}^{280}/A_{ox}^{490}$ was 2.4.

NMR Spectroscopy. A typical NMR sample consisted of about 2 mg of the lyophilized protein dissolved in 0.5 mL of ^2H_2O containing 0.1 M phosphate buffer. The pH of the sample was adjusted to the desired value by addition of 0.1 M KO^{2}H or 0.1 M ^{2}HCl from a micrometer syringe with rapid stirring. Reduction of the protein was carried out by addition of 2 equiv of solid sodium dithionite to the solution. ^{1}H NMR spectra were obtained with a Nicolet Magnetics 11.3-T spectrometer operating in the quadrature mode over a bandwith of ±6000 Hz and using 16 384 data points. A 90° pulse (8 μs) was employed, and the repetition rate was about 1 s^{-1}. The residual solvent signal was suppressed by a presaturation pulse from the decoupler, which was turned off during acquisition of the NMR signal. About 2000 transients were collected for each spectrum to achieve an adequate signal-to-noise ratio.

For the measurement of spin–lattice relaxation times (T_1), the conventional 180°–τ–90° pulse sequence (Vold et al., 1968) was used with the 180° pulse replaced by a composite 90_x–240_y–90_x pulse (Freeman et al., 1980). The recycling time was about 2 s, which is greater than 5 times the longest T_1 of the signals of interest. Chemical shifts were measured relative to the residual HO^{2}H signal, which was assumed to be 4.76 ppm downfield from sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) at 25 °C; no magnetic susceptibility correction to the reference chemical shift was made. The chemical shifts reported are relative to DSS. Line widths were determined by using the subroutine NTCCAP available on the Nicolet 1180 data system. Ring current shift calculatins were carried out by using the FORTRAN subroutine of Perkins (1982) and the physical model of Johnson & Bovey (1958).

X-ray Coordinates. Energy-refined coordinates for rubredoxin were obtained from the Protein Data Bank (Bernstein et al., 1977).

Results and Discussion

Figure 1 displays the 470-MHz ^{1}H NMR spectra of (A) oxidized and (B) reduced Rd in 99.9% ^2H_2O, pH 6.3, 25 °C. Increasing the spectral width to 25 000 Hz on a 200-MHz instrument did not reveal any additional resonances. The peaks in the two spectra were labeled alphabetically from low field to high field. Those from the oxidized protein are primed; those from the reduced protein are unprimed. We do not imply by this nomenclature that peaks with the same letter designation in the two spectra arise from the same atom in the oxidized and reduced forms of the protein. The spectra reported here contain several features not reported earlier (Phillips et al., 1970): (i) resonances a′–c′ in the spectrum of oxidized Rd (Figure 1A) were not detected, perhaps because of the lower sensitivity of the 220-MHz instrument used; (ii) resonances a–c in the spectrum of reduced Rd (Figure 1B) were not observed, although the reported sweep width was from 40 to −27.5 ppm.

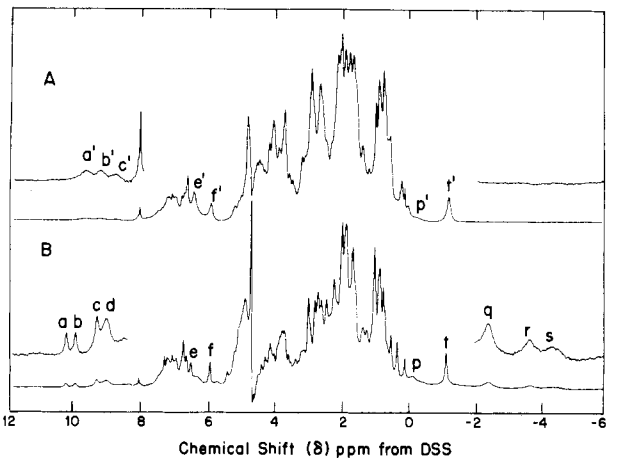

FIGURE 1: The 470-MHz ^{1}H NMR spectra of (A) oxidized and (B) reduced *C. pasteurianum* rubredoxin in ^2H_2O, 25 °C, pH 6.3. The protein was exchanged extensively with ^2H_2O. See text for assignments.

When Rd was oxidized (Figure 1A), all the resonances, in general, became broader, and counterparts of resonances d, q, r, and s were not observed. The broadening results from the increased paramagnetism of the iron in the oxidized state. The line widths of a–c in the spectrum of reduced Rd are in the 45–50 Hz range; resonance d appears broader and more intense, suggesting overlap with another resonance. The line widths of resonances a′–c′ in the spectrum of oxidized Rd are around 200–220 Hz. Therefore, the ratio of line widths in the oxidized and reduced forms is estimated to be ~4.5. Resonance q has a line width of ~180 Hz, and using the above value for the ratio of line widths in the two states of the protein, we estimated the line width of this resonance in the oxidized state to be ~850 Hz. A computer simulation of the signal obtained by keeping the area constant, but changing the line width by a factor of 4.5, indicated that the expected signal in oxidized Rd from the group giving rise to peak q in reduced Rd will be broadened beyond detection (data not shown). It is not surprising that counterparts of peaks r and s, which are even broader in reduced Rd (Figure 1B), are not seen in the spectrum of oxidized Rd (Figure 1A). Thus, the absence of these peaks in the spectrum of oxidized Rd can be explained as the result of line broadening, and one does not need to postulate that they shift greatly on oxidation of the protein.

The longitudinal relaxation behavior of selected resonances in the spectrum of oxidized Rd is illustrated in Figure 2. Signals a′–c′, e′, f′, p′, and t′ relax much more efficiently than others because of the influence of the paramagnetic iron. Since an electron–nuclear dipolar mechanism dominates the spin–lattice relaxation of these signals, irrespective of whether they are contact- or pseudocontact-shifted, useful relative distance information can be obtained via (La Mar, 1979)

$$T_1^i/T_1^j = (d_i/d_j)^6 \quad (1)$$

where T_1 is the measured longitudinal relaxation time and d is the internuclear distance between the hydrogen and the paramagnetic iron. Good estimates of T_1 values were obtained

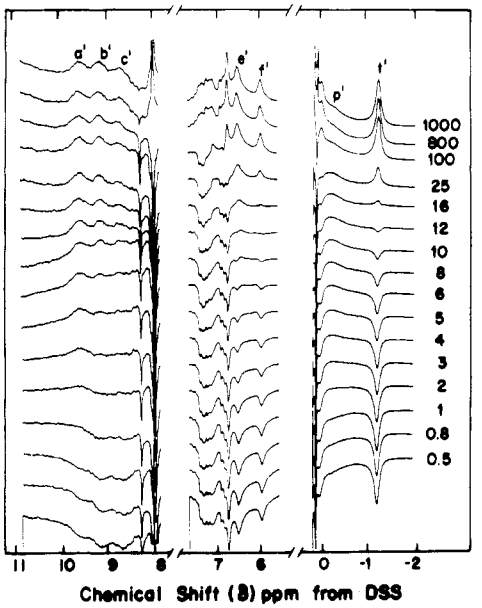

FIGURE 2: The 470-MHz ^{1}H NMR relaxation study of oxidized *C. pasteurianum* rubredoxin. Spectra, obtained with the conventional 180°–τ–90° pulse sequence, illustrate the longitudinal relaxation behavior of various paramagnetically perturbed resonances. The τ values (ms) are given at the end of each trace.

from the null points by using the equation $T_1 = \tau_{null}/\ln 2$ (Farrar & Becker, 1971). Clearly, estimation of absolute distances requires definitive assignment of at least one peak with known distance from the iron.

The observed shifts of the paramagnetically influenced peaks in spectra of reduced and oxidized Rd may have a dipolar and/or contact-shift contribution. The high-spin iron in both the oxidized and reduced states in tetraphenylporphyrin complexes has bees shown to possess negligible magnetic anisotropy by solution NMR measurements (La Mar et al., 1973; Goff & La Mar, 1979). However, ESR studies of *Pseudomonas oleovorans* Rd (Peisach et al., 1971) have indicated that the Fe^{III} ($S = {}^5/_2$) is in a nearly completely rhombic environment ($E/D = 0.28$). Similarly, Mössbauer spectroscopic studies of model compounds of the active site of Rd by Coucouvanis et al. (1981) have demonstrated that the high-spin Fe^{II} ($S = 2$) in reduced Rd exhibits magnetic anisotropy. These results would seem to raise the question as to whether the iron might exhibit magnetic anisotropy in reduced and oxidized Rd in solution at room temperature. In such an instance, the resulting dipolar shift would be proportional to the g tensor magnetic anisotropy and a geometric term, $(3\cos^2\theta - 1)/d^3$, where θ is the angle between the unique magnetic axis and the vector joining the hydrogen and the iron atom and d is the magnitude of the vector. As a result, even a slight difference in conformation between the structures of the oxidized and reduced protein would be expected to result in significant differences in the chemical shift. Small changes throughout the diamagnetic region of the spectra (Figure 1) of oxidized and reduced Rd suggest that there are localized conformational changes (vide infra). However, one can find peaks from groups close to the iron (based on their rapid relaxation rate) that have very similar chemical shifts in the oxidized and reduced states: e.g., peaks e/e', f/f', and t/t'. This suggests that these resonances probably incur a negligible dipolar shift contribution due to magnetic anisotropy in reduced and oxidized Rd. Nevertheless, the high-spin Fe^{III} systems, a dipolar shift can arise from the presence of large zero field splitting (ZFS), which lifts the electronic degeneracy (La Mar et al., 1973). In this case, the dipolar shift is given as

$$(\Delta H/H_0)^{dip} = [B(3\cos^2\theta - 1)/d^3](D/T^2) \qquad (2)$$

where D is the ZFS parameter and $B = 28g^2\beta^2/(9k^2)$. Spectra of oxidized Rd collected as a function of temperature in the range 8–60 °C (data not shown) revealed that the faster relaxing resonances e', f', and t' did not exhibit any appreciable changes in their chemical shifts with temperature as found earlier (Phillips et al., 1970). We also observed that the broad downfield-shifted resonances a'–c' showed little changes in chemical shift as the temperature was varied. If there were a major dipolar contribution due to ZFS to the observed shift of any of the above mentioned peaks, then one would have expected to find a strong dependence of the chemical shift on temperature as dictated by the term T^{-2} in eq 2. In the case of *C. pasteurianum* Rd the iron–sulfur bonds of the complex are of nearly equal lengths (ranging from 2.24 to 2.33 Å), and the S–Fe–S bond angles deviate from perfect tetrahedral symmetry by ±5° (Watenpaugh et al., 1979). Whether this lowering of symmetry would lead to any sizable ZFS is not obvious. However, the NMR results appear to indicate that the observed paramagnetically influenced signals in the spectrum of oxidized Rd (Figure 1A) experience a negligibly small dipolar shift contribution arising from such effects.

Resonances e', f', and t' have similar relaxation rates (τ_{null} = 12–13 ms, Figure 2). This dictates that groups responsible for these signals are approximately equidistant from the iron. Morgan et al. (1978) have determined, on the basis of an analysis of the X-ray data, that the side chains of tyrosine-11 and phenylalanine-49 are within 4.2 Å from the sulfur of the cysteinyl ligands of Rd and suggested that the aromatic groups can interact with the iron complex by a π–π mode.

A computer-assisted examination of the hydrogen-attached X-ray diffraction coordinates of *C. pasteurianum* Rd [Watenpaugh et al. (1979) and a further refinement deposited with the Protein Data Bank] reveals the aromatic side chains closest to the iron are those of Tyr-11 and Phe-49. Three of the four ring hydrogens of Tyr-11 and four of the five ring hydrogens of Phe-49 are located between 4.7 and 7.0 Å from the iron. The H_{ϵ^1} of Tyr-11 is more distant at 8.22 Å, and the H_{δ^2} of Phe-49 is at 7.56 Å. Therefore, it is not unreasonable to assign resonances e' and f' in the 5.5–6.5 ppm region (Figure 1) to the ring hydrogens of Tyr-11 and/or Phe-49. Relative peak intensity measurements[2] indicate the peak t' corresponds to three hydrogen atoms. Furthermore, this resonance remained a single Lorenzian peak in spectra obtained between 8 and 60 °C. Thus, it seems likely that peak t' arises from a methyl group, which is close both to the iron *and* to an aromatic ring so as to experience paramagnetic dipolar relaxation and a ring current shift contribution.

A computer-assisted search[3] for methyl groups within 7 Å of the iron yielded the side chains of valine-8 and valine-44. Further constraining that this methyl group must also be close to an aromatic ring ruled out valine-8. The γ_2-methyl carbon of valine-44 is 4.50 Å from the iron and a mean distance of 5.85 Å from the ring of phenylalanine-49. However, the calculated ring current shift of the valine-44 C_{γ^2}-H_3 hydrogens

[2] Intensities were measured by cutting out and weighing peaks. Overlap of peaks and base-line distortions limited the accuracy of these measurements. The following ratios of peaks were obtained: peak t to peaks a + b, ~1.64; peak t to peak q, ~1.16; peak t to peaks r + s, ~0.76. Normalizing the intensity of peak t to three protons led to a + b = 1.8 protons, q = 2.6 protons, and r + s = 3.9 protons.

[3] We thank an anonymous referee for informing us of the availability of a refined set of the X-ray coordinates of the rubredoxin molecule and for providing us with a table of distances of various groups from the iron atom. Our results derived from the proton-attached coordinates of the protein, as obtained from the Protein Data Bank, matched his.

was 0.05 ppm, i.e., too small and in the wrong direction to explain the chemical shift of peak t′. As a further complication, the ring current calculations predicted that the resonance from the C_{γ^2}-H_3 of isoleucine-33 (12.9 Å from the iron) should be at −1.3 ppm and the resonance from the C_δ-H_3 of the same residue (12.2 Å from the iron) should be at −2.5 ppm. Groups that far from the iron would be expected to have much more normal T_1 values and line widths much less sensitive to the oxidation state of the protein than the high-field peaks appearing in Figure 1. Further evidence will be required to assign t′ to a specific methyl group.

The assignment of peaks e′ and f′ in the 5.5–6.5 ppm region to the ring hydrogens of Tyr-11 and/or Phe-49 allows us to estimate via eq 1 the upper limit of distances of groups a′–c′ (8–10 ppm) from the iron. The distance from the iron to the most remote of the ring hydrogens of Tyr-11 and Phe-49 is 8.2 Å. By associating this distance with the relaxation rate of resonances e′ and f′ (τ_{null} = 12 ms), we calculate that peaks a′–c′ (τ_{null} ~2.5 ms) arise from groups that are within ~6.3 Å of the iron. This in turn leads to the conclusion that the broader upfield-shifted signals p–s in the 0 to −4.5 ppm spectral region of reduced Rd belong to residues that are still closer to the iron.

In the simplest case, where NMR signals are observed from a paramagnetic system that is magnetically isotropic, the longitudinal and transverse relaxation rates are given as (Swift, 1973)

$$T_{1N}^{-1} = T_{2N}^{-1} = KS(S + 1)\mu^2 T_{1e} d^{-6} \qquad (3)$$

where K contains the appropriate constants, S is the spin, μ is the magnetic susceptibility, T_{1e} is the electron relaxation time, and d is the distance between the nucleus (hydrogen) and the paramagnetic center (iron). In the course of our experiment, the reduced protein became oxidized slowly, and hence, reliable spin–lattice relaxation times were not obtained with this sample; our analysis is based on line widths. The inverse sixth power of the ratio of line widths of q and b yields ~1.24, which is the ratio of the distances from the iron of the groups associated with these peaks. From this ratio and the estimated distance of 6.3 Å for peak b, we determined that the maximum distance from the iron to the group responsible for peak q′ is ~5.0 Å.

A computer-assisted examination of the hydrogen-attached coordinates of *C. pasteurianum* Rd [Watenpaugh et al. (1979) and further refinement deposited with the Protein Data Bank] identifies the following hydrogen atoms as being within 5.0 Å of the iron [distances (Å) are given within parentheses]: one H_β of valine-8 (3.96), two H_β's of tyrosine-11 (4.18 and 3.52), one H_β of leucine-41 (4.04), the γ_2-methyl group of valine-44 (4.36, assuming averaging of the three methyl hydrogen distances by rapid rotation), and all the eight β-hydrogens of the four cysteinyl ligands (3.13–4.20)—a total of 15 hydrogen atoms, eight of which belong to the iron ligands and seven to other residues not linked to the iron. Peaks q–s in the −2 to −4.5 spectral region of reduced Rd (Figure 1B) were found to follow a $1/T$ dependence in the range 5–65 °C (Phillips et al., 1970), thus establishing that these signals possess a hyperfine-shift contribution. Relative intensity measurements[2] of the upfield-shifted peaks reveal the following: p (one or more hydrogens, base line unclear), q (2.6), r + s (3.9, taken together). These four peaks can account for eight hydrogens: peaks r and s probably correspond to two hydrogens each, peak q corresponds to two or three hydrogens, and peak p corresponds to two or one hydrogen.

From a detailed solution NMR study of five-coordinate, high-spin Fe^{II} complexes, with presumably large ZFS as evidenced by their narrow linewidths, Goff & La Mar (1977) established that high-spin ferrous iron exhibits a very small magnetic anisotropy and that the resulting hyperfine shifts of the iron ligands are predominantly contact in origin. The predominant hyperfine-shift mechanism for ligands to the high-spin Fe^{II} of Rd, which exists in a more nearly symmetrical environment (tetrahedral) than the iron of these complexes (distorted square pyramidal), should also be contact. Thus, the Rd peaks that exhibit the largest hyperfine shifts must arise from iron ligands. Two classes of hyperfine-shifted peaks are observed (Figure 1B): peaks a–d that are sharper and shifted downfield and peaks p–s that are broader and shifted upfield. These are assigned to the iron-liganded cysteine residues, with the broader peaks arising from β-hydrogens and the sharper peaks arising from α-hydrogens. Relative intensity measurements[2] appear to support these assignments. Furthermore, the inverse sixth power of the ratio of line widths of peaks q and b yields ~1.24, which seems to be in reasonable agreement with the distance ratio of 1.19 obtained for the cysteinyl α- and β-hydrogens from the crystal structure data (the ratios fall into two sets; one is around 1.18 and the other around 1.5).[3]

The observed sign reversal of the chemical shifts of the α- and β-hydrogens is consistent with π-spin transfer being the contact shift mechanism (Horrocks, 1973). Since the magnitude of the contact shift involving π-orbitals depends upon the extent of angular overlap of the p orbitals (La Mar, 1973), a spread of chemical shifts may be expected for the β-hydrogens of the ligated cysteines because of their fixed geometry.

The oxidation-induced increases in line width (Figure 1) vary for different assigned resonances. Peaks e/e′, f/f′, and t/t′ do not broaden to the same extent as those assigned to the cysteinyl ligands: the oxidized/reduced line width ratios are ~1.8 (e/e′, f/f′, and t/t′) vs. ~4.5 (cysteinyl α-hydrogens). As dictated by eq 3, such a difference can be explained only by invoking differential changes in the distance parameter, d. This suggests that a localized conformational change accompanies the redox reaction.

The small magnitude of the contact shift (upper limit 7 ppm) presumably associated with the ligated cysteines in Rd is in sharp contrast to the hyperfine-shift pattern observed in HiPIP (Krishnamoorthi et al., 1986), whose β-hydrogens of the iron ligands resonate much farther downfield (~100 ppm).

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Resolution of Highly Purified Toxic-Shock Syndrome Toxin 1 into Two Distinct Proteins by Isoelectric Focusing†

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ABSTRACT: Highly purified toxic-shock syndrome toxin 1 (TSST-1) was prepared by differential precipitation with ethanol and resolubilization in water followed by successive electrofocusing in pH gradients of 3-10, 6-8, and 6.5-7.5. TSST-1, thus isolated, migrated as two distinct protein bands with isoelectric points of 7.08 (TSST-1a) and 7.22 (TSST-1b). When tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, both toxins migrated as homogeneous bands with molecular weights of 22 000. The gel bands were visualized by silver staining. The two toxins have nearly identical amino acid compositions and are immunologically identical as shown by Ouchterlony reactivity against TSST-1 hyperimmune serum. TSST-1a and TSST-1b have the same biological activities as TSST-1: the capacity to induce fever, enhancement of host susceptibility to lethal endotoxin shock, nonspecific T lymphocyte mitogenicity, and suppression of immunoglobulin M synthesis against sheep erythrocytes. These two proteins have been isolated from several different TSS-associated *Staphylococcus aureus* strains. The data suggest that the differences in isoelectric point result either from the presence of a cofactor or from alternative conformations. Since only two bands appear, microheterogeneity as a result of deamination or acetylation is unlikely.

Toxic-shock syndrome (TSS) is a multisystem illness characterized by acute onset of high fever, hypotension or dizziness, rash, desquamation of skin upon recovery, and variable multisystem involvement (Todd et al., 1978; Davis et al., 1980; Shands et al., 1980; Tofte & Williams, 1981). *Staphylococcus aureus* producing TSST-1 have been isolated from nearly 100% of menstrual-associated TSS patients (Schlievert et al., 1981; Bergdoll et al., 1981). TSST-1 has been cited by several investigators as a major toxin most likely responsible for the symptoms of TSS (Schlievert et al., 1981; Bergdoll et al., 1981;

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