Molecular Properties of Lysostaphin, a Bacteriolytic Agent Specific for Staphylococcus aureus*

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SUMMARY

Lysostaphin is a zinc-metalloenzyme which has a molecular weight of 25,000 when determined by sedimentation equilibrium in dilute aqueous salt solutions. Combination of the sedimentation coefficient, $s_{20,w} = 2.32$, and diffusion coefficient, $D_{20,w} = 7.83$, gave a native molecular weight of 25,800. Sedimentation equilibrium of the protein in 6 M guanidine hydrochloride, with and without 2-mercaptoethanol, gave molecular weights of 25,800 and 27,800, respectively. The molecular weight of the fully dissociated protein was also determined to be 24,500 by the technique of gel filtration in 6 M guanidine hydrochloride and 25,500 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In each case, only a single protein species was shown to be present. These data indicate that the lysostaphin molecule is a single polypeptide chain.

The frictional coefficient ratio for lysostaphin of 1.39 is higher than values obtained for most globular proteins. The optical rotatory dispersion spectrum was also different from that invariably observed with globular proteins and revealed no evidence of helical structure in the molecule. The amino acid composition of lysostaphin was unusual in that half-cystine was found to be absent.

Lysostaphin is a zinc-containing enzyme which has a specific lytic action against microorganisms of the genus Staphylococcus. It was originally isolated by Schindler and Schuhardt (1) from cell-free filtrates of the gram-positive coccus, Staphylococcus staphylolyticus. Its lytic activity was shown in tests with over 50 strains of Staphylococcus aureus, and shown to be independent of phage type, resistance to other antibiotics, and the condition of the cell wall or degree of capsulation of the bacterium.

Preliminary studies on the physical characterization of purified lysostaphin have been reported by these investigators (2).

Because of the increasing importance of staphylococcal infections in man, and the emergence of many antibiotic-resistant strains of S. aureus, the development of new antibacterial agents is important. Since lysostaphin has a high degree of antistaphylococcal activity, it could prove to have potential in the treatment of resistant staphylococcal infections. We report here some of the properties of lysostaphin, including molecular weight, amino acid composition, and gross conformational structure. Information about these parameters is necessary if further insight is to be obtained into the nature and specificity of this antibacterial enzyme.

EXPERIMENTAL PROCEDURE

Materials

A sample of lysostaphin purified from cell-free filtrates of S. staphylolyticus was kindly supplied by Dr. P. A. Tavormina.* This sample was judged to be physically homogenous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by ultracentrifugal analysis.

Ultrapure guanidine hydrochloride was purchased from Heico Chemicals (Delaware Water Gap, Pennsylvania) and used without further purification. Iodoacetic acid was recrystallized from n-hexane. All other chemicals were reagent grade and were used as supplied.

Methods

Electrophoresis—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method of Shapiro, Viñuela, and Maizel (3) as extended by Weber and Osborn (4).

Amino Acid Composition—Amino acid analyses were performed by the accelerated system of Hubbard (5) on a Spinco model 120B automatic amino acid analyzer. Duplicate samples were hydrolyzed by 6 N HCl under vacuum for 24, 48, and 72 hours at 110° (6). Tryptophan was estimated by reaction of lysostaphin with N-bromosuccinimide (7) in 6 M guanidine hydrochloride. Cystine and cysteine were determined as cysteic acid by the performic acid oxidation method of Moore (8).

Sedimentation and Diffusion Coefficients—All experiments were performed with a Spinco model E analytical ultracentrifuge. Sedimentation and diffusion coefficients were determined at 25° in 0.1 M NaCl (pH 6.5) and 0.2 M phosphate, 0.1 M citric acid buffer (pH 4.0) under a concentration range of 2 to 10 mg per ml.

* This work was supported by Grant AI07617 from the United States Public Health Service.

1 Lysostaphin contains 1 molecule of zinc per mole of protein. Dr. P. A. Tavormina, personal communication.
Sedimentation velocity experiments were performed, with schlieren optics, at rotor speeds of 59,780 rpm. Sedimentation coefficients were calculated from the standard equation by Schachman (9) and corrected to the density and viscosity of water at 20° (10). Diffusion coefficients were determined at 12,410 rpm with a double sector, capillary type, synthetic boundary cell, and the schlieren optical system. After boundary formation, pictures were taken at 8-min intervals and analyzed in the usual manner (11). Diffusion coefficients were calculated by the standard equation (12) and corrected to the density and viscosity of water at 20° (13).

**Sedimentation Equilibrium**—These studies were performed with a Spineo model E analytical ultracentrifuge, equipped with Rayleigh interference optics. The method used was essentially that of Yphantis (14) except that occasionally solution column heights longer than 3 mm were used (5 to 7 mm). Low initial protein concentrations (0.1 to 0.3 mg per ml) and relatively high rotor speeds were used to ensure that the concentration of the solute at the upper meniscus was effectively zero. The Rayleigh patterns observed were photographed on Kodak type II-G spectroscopic plates. All experiments were performed at 25° with a double sector cell and sapphire cell windows. Fluorocarbon FC-43 was used as a base fluid to give a transparent cell bottom. Equilibrium was established by ascertaining that no further change in fringe displacement occurred with time. Observed fringe displacements were corrected for the effect of cell window distortion by running a water blank immediately after each experiment without disassembling the cell. The molecular weight of lysostaphin was determined in 0.2 M phosphate, 0.1 M citrate buffer at pH 4.0 and in 6 M guanidine hydrochloride in the absence and presence of a reducing agent (0.1 M 2-mercaptoethanol). Interference patterns were measured with a Nikon two-dimensional comparator. The natural logarithm of the blank corrected fringe displacements, lnf, was plotted against the distance from the axis of rotation, squared, r², and the slope of the line calculated by the best linear fit of the data obtained by the method of least squares. The weight average molecular weight, Mₐ, was calculated as described by Yphantis (14):

\[
Mₐ = \frac{2RT}{\omega^2(1-\psi)} \frac{d\ln f}{d^2}
\]

where R is the gas constant expressed as 8.314 × 10⁵ ergs deg⁻¹ mol⁻¹, ω is the angular velocity of the rotor, ρ is the density of the solvent in grams per cc, and ψ is the partial specific volume of the protein in milliliters per g. The density of the phosphate-citrate buffer was determined to be 1.011 g per cc at 25°. The density of 6 M guanidine hydrochloride was 1.148 g per cc at 25°.

**Gel Filtration**—The method used for the estimation of the molecular weight of lysostaphin by gel filtration in 6 M guanidine hydrochloride was that of Fish, Mann, and Tanford (15). Blue dextran was used to measure the void volume and DNP-alanine to measure the interstitial volume. Two independent runs were performed on reduced and carboxymethylated (16) samples of lysostaphin.

**Optical Rotatory Dispersion**—The optical rotatory dispersion spectrum of lysostaphin was measured with a Cary model 60 spectropolarimeter over the wave length range of 220 to 360 mp. The lyophilized protein was dissolved in 0.2 M phosphate, 0.1 M 2-mercaptoethanol.

### Table I

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount formed after hydrolysis for 24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
<th>Calculated no. of residues per molecule at mol wt 25,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>15.96</td>
<td>15.53</td>
<td>15.83</td>
<td>16</td>
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<tr>
<td>Histidine</td>
<td>8.46</td>
<td>8.92</td>
<td>8.82</td>
<td>9</td>
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<tr>
<td>Arginine</td>
<td>6.52</td>
<td>5.35</td>
<td>5.64</td>
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<td>Aspartic</td>
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<td>23.87</td>
<td>23.53</td>
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<tr>
<td>Threonine</td>
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<td>21.78</td>
<td>22.11</td>
<td>22</td>
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<td>Serine</td>
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<td>17.48</td>
<td>15.62</td>
<td>21</td>
</tr>
<tr>
<td>Glutamic</td>
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<td>15.17</td>
<td>15.68</td>
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<td>Proline</td>
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<td>19.28</td>
<td>13</td>
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<td>Glycine</td>
<td>34.14</td>
<td>31.67</td>
<td>33.94</td>
<td>33</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.89</td>
<td>10.80</td>
<td>12.40</td>
<td>12</td>
</tr>
<tr>
<td>Valine</td>
<td>13.89</td>
<td>14.79</td>
<td>14.93</td>
<td>15</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.78</td>
<td>6.99</td>
<td>6.92</td>
<td>7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10.50</td>
<td>11.70</td>
<td>11.94</td>
<td>12</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.61</td>
<td>11.32</td>
<td>11.17</td>
<td>11</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>14.52</td>
<td>15.53</td>
<td>15.59</td>
<td>15</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.40</td>
<td>7.39</td>
<td>6.66</td>
<td>7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: a The molecular weight of 22,000 does not include the a residues of trypophan as they were determined independently. 

b This value was extrapolated to zero time hydrolysis.

c Measured separately by direct analysis as described in the text.

d Phosphatase (0.2 M), 0.1 M citrate, pH 4.0.
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ing 0.1 mM EDTA. All solutions were then filtered, and the concentration of the protein solution was determined by analysis of the fringe displacement produced in a synthetic boundary cell in the ultracentrifuge. A 1-mm cell was used for measurements above 240 rpm and a 1-cm cell was used for measurements below 240 rpm. The mean residue rotation, $[\eta]$, was evaluated by use of the following equation:

$$[\eta] = \frac{M_0 [\alpha]}{100}$$

where $M_0$ is the mean residue mass and $[\alpha]$ is the specific rotation defined as:

$$[\alpha] = \frac{\alpha}{dc}$$

where $\alpha$ is the observed angle of rotation at wave length $\lambda$, $d$ is the length of the light path in decimeters, and $c$ is the concentration of solute in grams per ml.

RESULTS

**Amino Acid Analysis**—The amino acid composition of lysostaphin is shown in Table I. The values given for each amino acid, unless otherwise indicated, are the averages obtained from analyzing duplicate samples at each time interval. The calculated number of residues per molecule was based upon the average of each time interval and rounded to the nearest integer. The values for valine, isoleucine, and leucine are those obtained after 72-hour hydrolysis and the value for serine was estimated by extrapolation to zero hydrolysis time. Tryptophan was estimated by N-bromosuccinimide (7) and the value given is the average of four separate determinations. Cystine and cysteine were not detected either by direct hydrolysis or after performic acid oxidation to cysteic acid.

**Sedimentation Velocity and Diffusion**—Lysostaphin sedimented as a single peak in the ultracentrifuge in dilute salt solutions. Both the sedimentation ($s_{20,w}$) and diffusion coefficients ($D_{20,w}$) were unaffected by changes in the solvent composition and were independent of protein concentration. The average value of the $s_{20,w}$ obtained from all determinations was found to be 2.32 S ± 0.07 and the average $D_{20,w}$ was found to be 7.83 $D$ ± 1.31.

**Molecular Weight**—An estimation of the native molecular weight of lysostaphin was calculated from the sedimentation and diffusion coefficients (17). Values of 2.32 S and 7.83 $D$ were used and a native molecular weight of 25,900 was obtained.

All molecular weight data obtained by sedimentation equilibrium under various experimental conditions are shown in Table II. Values obtained for lysostaphin in dilute aqueous salt solutions were calculated by a partial specific volume, $\beta$, of 0.725 ml per g derived from the amino acid composition (18). This method has been shown to give fairly good results for proteins that are single polypeptide chains in the native state (19). In solutions of concentrated guanidine hydrochloride an apparent specific volume, $\phi'$, 0.01 ml per g below the $\beta$ used in the dilute salt solution, was used to obtain the molecular weight of the dissociated protein. All plots illustrating the relationship between the natural logarithm of the fringe displacement (log, $j$) and the square of the radial distance from the center of rotation ($r^2$) were linear, indicating a lack of observable molecular weight heterogeneity. Fig. 1, A, B and C, shows typical graphical examples of the data obtained for lysostaphin in the phosphate-citrate buffer and 6 M guanidine hydrochloride and 6 M guanidine hydrochloride in the presence of 0.1 M 2-mercaptoethanol, respectively. These data indicate that the measurements of the molecular weight of lysostaphin by sedimentation equilibrium were relatively independent of protein concentration, rotor speed, or solvent conditions.

The molecular weight of lysostaphin was also estimated by gel filtration in concentrated aqueous solutions of guanidine hydrochloride (15) and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (2, 4). Two independent gel filtration experiments showed the presence of only one protein component, and identical molecular weights of 24,500 were obtained. The electrophoretic method revealed only a single migrating band and a molecular weight of 25,500 was obtained. Table III summarizes all molecular weight data.

The frictional coefficient ($f$) of a macromolecule can be calculated from the molecular weight and sedimentation coefficient with the relationship:

$$f = \frac{M(1 - \beta_p)}{Ns_{20,w}} = 5.20 \times 10^{-4} \text{ g sec}^{-1}$$

**FIG. 1.** Sedimentation equilibrium of lysostaphin. A, 0.02% lysostaphin in 0.2 M phosphate-0.1 M citrate, pH 4.0, at 29,307 rpm with a column height of 5.8 mm; B, 0.02% lysostaphin in 6 M guanidine hydrochloride, pH 5.9, at 36,014 rpm with a column height of 6.4 mm; C, 0.014% lysostaphin in 6 M guanidine hydrochloride-0.1 M 2-mercaptoethanol, at 36,894 rpm and a column height of 6.4 mm. Ordinates, natural logarithm of the fringe displacement; abscissas, squares of the radial distance. $r_{20,w}$ is bottom of the cell.
TABLE III
Molecular weight of lysostaphin

<table>
<thead>
<tr>
<th>Method</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation equilibrium</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>25,000 ± 1,000</td>
</tr>
<tr>
<td>6-0 guanidine hydrochloride</td>
<td>27,500 ± 300</td>
</tr>
<tr>
<td>6-0 guanidine hydrochloride + 0.1-0 m2-mercaptoethanol</td>
<td>25,500 ± 500</td>
</tr>
<tr>
<td>Sedimentation and diffusion</td>
<td>25,500 ± 1,000</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>24,500 ± 1,000</td>
</tr>
<tr>
<td>Polyacrylamide electrophoresis</td>
<td>25,500 ± 2,000</td>
</tr>
</tbody>
</table>

* This is the average value obtained from four independent experiments in 0.2-0 phosphate-0.1-0 citrate, pH 4.0. Calculated with $\bar{\rho} = 0.725$ ml per g.

* This is the average value obtained from three independent experiments. Calculated with $\bar{\rho}' = 0.715$ ml per g.

* This is the average value obtained from four independent experiments. Calculated with $\bar{\rho}' = 0.715$ ml per g.

Optical Rotatory Dispersion—The optical rotatory dispersion of lysostaphin in the ultraviolet region of the spectrum is shown in Fig. 2. Identical dispersion profiles were obtained in the presence and absence of 0.1-0 EDTA. A value of mean residue molar mass, $M_0$, equal to 107 was calculated from the amino acid composition. There are troughs at 240 and 297 m$\mu$ with corresponding mean residue $[\psi]$ values of $-380$ and $-190$, respectively. Peaks were observed at 231 and 257 m$\mu$ which had respective $[\psi]$ values of $-140$ and $-160$.

**DISCUSSION**

Lysostaphin migrated as a single component on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and as a single peak in sedimentation velocity experiment, suggesting that the sample was homogeneous. This was confirmed by sedimentation equilibrium experiments which indicated that the experimentally observed molecular weight of lysostaphin in the same solvent was not significantly altered by changes in protein concentration or rotor speed. The linearity exhibited by graphic analysis of the relationship between fringe displacement and radial distance in the cell is also consistent with sample homogeneity.

The observed optical rotatory dispersion spectrum of lysostaphin is unusual. Studies by Jirgensons (21) reveal that proteins with a moderate to high a-helix content exhibit a characteristic negative Cotton effect with a sharp minimum at 233 m$\mu$ and a strong positive specific rotation between 189 and 202 m$\mu$. Although the optical rotatory dispersion of lysostaphin was not examined below 220 m$\mu$, the minimum at 233 m$\mu$ was not observed. Instead, a strong positive rotation at 231 m$\mu$ was detected. The negative Cotton effect with a minimum at 297 m$\mu$ is also uncharacteristic of helical structure. Other studies by Jirgensons (22) on the optical rotatory dispersion of nonhelical proteins in the far ultraviolet spectral zone were also dissimilar to lysostaphin. Studies by Fasman and Potter (23) on a synthetic polypeptide with $\beta$-H type of structure (poly-S-benzyl-L-cysteine) describe an optical rotatory dispersion which exhibits a negative specific rotation at 240 m$\mu$, similar to that observed with lysostaphin. However, without a more detailed investigation of the actual conformation of lysostaphin, it cannot be concluded that this protein bears any structural similarity to the synthetic peptide. The optical rotatory dispersion spectrum of lysostaphin was unchanged when 0.1-0 EDTA was added to the solvent. This suggests that, with these experimental conditions, the zinc moiety may still be bound to the protein or that its removal causes no detectable conformational change in the molecule.

The frictional coefficient ratio, $f/f_{\text{min}}$, is dependent on two factors, namely effective solvation and asymmetry (24). For the hypothetical unhydrated sphere from which $f_{\text{min}}$ was calculated, $f/f_{\text{min}} = 1$. Typically, globular proteins exhibit a ratio in the range of 1.10 to 1.25 (24), indicating that both hydration and deviation from spherical shape are small. In this regard the $f/f_{\text{min}}$ ratio of 1.39 obtained for lysostaphin suggests that it does not behave as a typically globular protein. The data indicate that lysostaphin is either more asymmetrical or possesses a large amount of hydration.

The molecular weight data obtained by sedimentation equilibrium and shown in Table II indicate that lysostaphin consists of a single polypeptide chain. The elevated values in solutions of 6-0 guanidine hydrochloride could possibly be a result of preferential interactions between the guanidine hydrochloride and the protein molecule. Hade and Tanford (25) have shown that some unfolded proteins in this solvent do exhibit a tendency to bind
guanidine hydrochloride preferentially, yielding molecular weight estimations which are as much as 5 to 10% too large. Cassada and Eisenberg (26) have indicated that this problem of preferential interaction with solvent components can be partially overcome by treating the 6 M guanidine hydrochloride solution as a single diffusible component. If the experimentally derived data are treated as a two-component system, the thermodynamic value, \( v \), is replaced by an apparent specific volume, \( \phi' \), which represents the combined effects of partial specific volume and preferential interactions. Several investigators (27-29) have experimentally evaluated \( \phi' \) for some proteins in solutions of concentrated guanidine hydrochloride and obtained values 0.01 to 0.02 ml per g less than the partial specific volume, \( \bar{v} \), obtained in dilute aqueous salt solutions. All sedimentation equilibrium data were recalculated with a value for \( \phi' \) of 0.715 ml per g. These values are also presented in Table II and show better agreement with the native molecular weight. Because of this uncertainty in the exact molecular weight of the fully dissociated protein molecule, two other independent methods of molecular weight determinations were used, namely, gel filtration in 6 M guanidine hydrochloride and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Under the conditions used in gel filtration, the protein is dissociated into its polypeptide chains, which behave as linear random coils (30). The molecular weight of such a randomly coiled protein is then a function of the number of amino acids in the polypeptide chain and, since all molecules being compared will have the same gross conformation, there will be a direct relationship between molecular weight and elution volume. The molecular weights obtained by this method were extremely reproducible and gave a value of 24,500, which is close to the value obtained for the molecular weight of native lysostaphin, by sedimentation equilibrium. Like gel filtration, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate can be used to determine polypeptide chain molecular weight and detect elements of subunit structure without a prior knowledge of of any thermodynamic parameters, such as partial specific volume. Again, molecular weights obtained by this method are very reproducible and a value of 25,500 was obtained for lysostaphin. Despite the uncertainties about preferential binding and conformational structure in dissociating solvents, the data presented indicate that lysostaphin has a molecular weight of 25,000 and consists of a single polypeptide chain.

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REFERENCES
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