Spectrophotometric Titration of Tyrosine Residues in Human Lysozyme*

(Received for publication, October 19, 1970)

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SUMMARY

The ionization of tyrosine residues in human lysozyme was investigated by measurement of spectral changes at pH values between 8.74 and 12.75. Below pH 11.70, at ionic strength 0.10, the titration curve is fully reversible. An average of 4 tyrosine eq per mole are ionized at this pH. At pH values higher than 11.70, the spectra are time-dependent. Reversal from any pH above 11.7, after 12 to 30 hours, leads to precipitation. Analysis of the results suggests that of the 6 tyrosine residues in the molecule, 3 are readily accessible and titrate as equivalent groups with pK values. When, on the average, more than one of these residues are ionized, a time-dependent exposure of the buried tyrosine residue occurs. A comparison of these results with the ionization behavior of the 3 tyrosine residues in hen egg white lysozyme is presented and discussed in terms of differences in the sequences of the two enzymes.

In addition to its widespread occurrence in diverse normal tissues, human lysozyme is present in large quantities in the urine of individuals with monocytic and monomyelocytic leukemia (1). The enzyme, which appears to be identical with that obtained from normal human tissues and secretions, is similar in many ways to hen egg white lysozyme, but there are important differences, as well. Both proteins are small with sedimentation coefficients close to 1.80 S; substrate specificities are similar, although against the cell wall of Micrococcus lysodeikticus the human enzyme is between 2 and 12 times as active (1). Inhibition and nuclear magnetic resonance studies also point to common structural and functional features at the active site (2, 3). Recently, Canfield has demonstrated extensive sequence homology in the two proteins (4); the number and pairing of cystine residues are the same; the catalytically important glutamate and aspartate residues (residues 35 and 52, respectively, in the egg white enzyme) occur in the same position (Asp 52 becomes residue 53 in the human sequence because of an insertion); valine 100 and a number of other active site residues are identical in both. While there is still some uncertainty in assignments, it appears that approximately 70 residues occupy the same positions. Finally, both proteins are basic, with isoelectric points near pH 11.

Among the notable differences thus far discerned it may be mentioned that only six kinds of amino acids occur to the same extent and that the proteins exhibit no immunological cross-reactivity (1, 4, 5).

Because of the functional similarities, the extensive sequence homology, including disulfide pairing, and the hydrodynamic and acid-base similarities, it is not unreasonable to expect that the two proteins may have quite similar secondary and tertiary structures. Crystallographic data on human lysozyme have been reported quite recently (6), but those results are of a preliminary nature. In this paper, we report the titration properties of the 6 tyrosine residues (4) of human lysozyme.

EXPERIMENTAL PROCEDURE

Three samples of human lysozyme isolated from the urine of patients with monocytic or monomyelocytic leukemia were used in this work. Two of the samples from the urine of an individual with monocytic leukaemia were provided by Dr. Robert E. Canfield of the College of Physicians and Surgeons, Columbia University. The protein had been purified by ion exchange chromatography on carboxymethylcellulose. In a subsequent step, the protein was desalted on Sephadex G-25, equilibrated with 1% acetic acid, and then lyophilized. The third sample was provided by Dr. Elliott F. Osserman of the College of Physicians and Surgeons, Columbia University. The lysozyme had been isolated from the urine of a patient with monomyelocytic leukaemia by bentonite adsorption and elution with 5% aqueous pyridine. Rates are 2 to 4 times as great with the human enzyme. The lysozyme assay method of Osserman and Lawlor (1) can be used over a broader range of concentrations and indicates that human lysozyme is 8 to 12 times as active against the cell walls of M. lysodeikcticus as hen egg white lysozyme.

* This research work was supported in part by Grant GB 7128 from the National Science Foundation and by Grant GM 10576 from the National Institutes of Health.

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1 Results of comparisons of enzymatic activity of human and hen egg lysozyme depend on the method of assay. The ratio of activities is not as broad using any one assay method as suggested by the range stated, i.e. human lysozyme is 2 to 12 times as active as hen egg white lysozyme. Any of several methods which measure the rate of decrease of turbidity of a suspension of killed M. lysodeikcticus cells by observing optical density changes at a non-absorbing spectral wave length in the visible indicates that such
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Fig. 1. Spectra of human lysozyme at pH 4.90 and at various pH values between 8.74 and 12.75. The three most alkaline spectra are time-dependent, as explained in the text. Spectra were recorded at 5 to 10 min after adjusting to pH values indicated.

Fig. 2. Ionization of tyrosyl residues of human lysozyme as measured by increase in molar extinction coefficient at 290.5 nm; 25°C; 0.10 ionic strength. X—X, ε290.5 from spectra measured at 5 to 10 min after adjusting the pH; O—O, ε290.5 from spectra measured at 12 to 30 hours after adjusting to pH as described in text.

RESULTS AND DISCUSSION

Fig. 1 shows the spectra of human lysozyme at pH 4.90 and through a pH range of 8.74 to 12.75. All of the solutions were measured within 5 to 10 min after preparation. The three most alkaline spectra are not at equilibrium. Above pH 11.7, the spectra are time-dependent. Between pH 11.7 and 12, the spectra change over a 12-hour interval and then remain constant for the next 18 hours, at least. Above pH 12, the optical density values at 280 nm and higher increase for several hours and then fall, as precipitation and settling of precipitate in the cuvettes occur. Additional spectra were measured but not included to avoid crowding of the figure. Excluding the two most alkaline curves, which change with time through the measurement, there are two isosbestic points in this spectral interval at 280 and 271.5 nm with molar extinction coefficients of 34,700 and 32,150, respectively.

In Fig. 2 are shown the molar extinction coefficients at 290.5 nm as a function of pH. Changes at 295 nm are not shown in the figure because the spectra are too steep at that wave length to allow secure definition of the curve with small enough pH intervals. However, the shape of the titration at 295 nm closely parallels that at 290.5 nm. As stated above, the values above pH 11.7 are time-dependent. The open circles in the figure are values representing the highest extinction coefficients achieved in a 12- to 30-hour period with solutions in which no precipitate was visible and for which optical densities at wave lengths longer than 350 nm were the same as at 5 to 10 min. Depending on pH, the three most alkaline solutions shown became cloudy at different times during that time period and the optical densities rose, then fell. The remaining three open circles, at pH values 12.08, 11.88, and 11.69, represent readings at 12 to 30 hours, during which time no changes were discerned. The solutions remained clear and readings at long wave lengths indicated no appreciable scatter.

Reversal from any pH above 11.7, after 30 hours, to lower pH resulted in precipitation even with those solutions that remained clear for 30 hours.

The reversible portion of the curve in Fig. 2 comprises two parts. The first, from the acid value at pH 4.90 to a break at pH 10.77, involves a Δε290.5 of 6,000; the second, from pH 10.77 to pH 11.69, involves a Δε290.5 of 2,025. Thus, three times as...
many residues titrate in the first interval. Because of the appearance of turbidity, on standing, at the highest pH values and the consequent failure to achieve a clearly marked plateau at these values, the calculation of average number of groups titrated at any lower pH value is not absolutely straightforward. The procedure we adopted was this. The maximum value of $\Delta \alpha_{685}$ observed, using the spectra given by the most alkaline solutions ($\text{pH} > 12.7$) before turbidity and scattering were evident, was $12,200 \pm 200$. Assuming this represented the titration of all 6 residues, $\Delta \alpha_{685}$ per residue is $2,083 \pm 38$. At 295 nm, the maximum change observed was $16,000 \pm 400$ or $2,667 \pm 67$ per residue. The best matching of average number of tyrosine residues titrated in the reversible range occurred by assigning a value of $\Delta \alpha_{685} = 2,680$ per residue and $\Delta \alpha_{690} = 2,000$ per residue. The former value is within 1% of that observed for the tyrosine titrations of ribonuclease (10) and hen egg lysozyme (11), and accordingly the latter value at 290.5 was used to further analyze the titration curve. Thus, 4 of the 6 tyrosine eq, on the average, are reversibly titrated per mole of human lysozyme.

To extract intrinsic constants from the data requires knowledge of the net charge ($\alpha$) and electrostatic interaction constant ($\omega$) over the pH range of the tyrosine titration. From preliminary acid-base binding studies between pH 2 and 11 at ionic strength 0.10, we have estimated values of $\alpha$ between pH 8.74 and 11.70, assuming no ions other than hydrogen and hydroxyl are bound in this pH range. These values are given in Table I; the isoionic point, $z = 0$, from these data is 10.80. A plot of $[\text{pH} - \log (\alpha_n) / (n - r)]]$ versus $z$, using $n = 3$, gives a curve with a straight line segment over the pH range 8.74 to approximately 10.3. For this straight line segment, the best value of $\omega$ from the slope is 0.098, but values as high as 0.11 are possible. This may be compared with a computed value of 0.0945. The value of the intrinsic constant at $z = 0$ is 9.76 with an upper limit of 9.84. From the midpoint for three groups at pH 9.45, using the computed value of $\omega$, $\text{pK}_{\text{in}}$, is 9.81. A corresponding plot of the data with $n = 4$ also gives a straight line segment, extending in fact over a wider pH range, but $\omega$ from the slope is an unreasonably high value of 0.19.

Between pH 10.77 and 11.70, the curve is too steep to be accounted for as due to the reversible ionization of a single residue. A satisfactory, although neither necessarily correct nor unique, construction of the entire reversible portion of the titration between pH 8.7 and 11.70 can be made by assuming two sets of groups. Three residues titrate with an intrinsic $\text{pK}$ of 9.81 and two with an intrinsic $\text{pK}$ of 11.56. There is some overlap of the two sets at all pH values above 9.2 but not enough to obscure the break at pH 10.75 to 10.80. When on the average, then, more than 1 of the 2 residues with abnormally high $\text{pK}$ values are ionized, irreversible unfolding occurs and the remaining buried residue is exposed. From these data, however, we cannot distinguish between the fourth and fifth ionizing groups on a molecule, although they quite probably have somewhat different intrinsic dissociation constants.

We may conclude then that 3 residues are freely exposed to titration. They behave as if they are equivalent with intrinsic $\text{pK}$ values near 9.81. (Further subdivision of the three is, of course, possible but not warranted from these results.) One residue is buried and is titratable only after unfolding. The remaining two both have anomalously high $\text{pK}$ values. We cannot decide, from among a number of alternatives, what the state of these residues individually must be before the nontitratable tyrosine becomes accessible, except that, on the average, one of these residues can be reversibly titrated.

It is interesting to compare these results with those obtained with hen egg white lysozyme, which contains 3 tyrosine residues. A number of investigators have measured the tyrosine titration (11-15), and some degree of anomaly has been noted by all. According to Tojo et al. (11), the 3 tyrosine residues titrate with apparent $\text{pK}$ values of 9.95, 11.6, and 12.6 at ionic strength 0.2. Above pH 12.3, the ionization is time-dependent and results in the exposure of the 3rd residue. One residue is thus freely accessible, a second has an anomalously high $\text{pK}$, and the third is partly or totally inaccessible.

Human and hen egg lysozyme have 2 tyrosine residues located in identical positions in the sequence, residues 20 and 53 (54 in human lysozyme). The 3rd residue in hen egg lysozyme, tyrosine 23, is substituted by an isoleucine residue in the human enzyme. In addition to residues 20 and 54, the human enzyme has tyrosine residues at positions 38, 45, 63 (Trp 62 in hen egg lysozyme), and 119 (4). One might reasonably infer that residues 45 and 119, which substitute for charged arginine and aspartate residues, respectively, are two of the reversibly titrated residues in the human enzyme. Tyrosine 38 occupies a position which is filled by phenylalanine in the hen egg enzyme. The latter residue appears to be at least partly interior (16) and thus tyrosine 38 may be one of the groups that titrates with an anomalously high $\text{pK}$. These suppositions are, of course, meaningful only if the two enzymes have closely similar structures, at least in the vicinity of these tyrosine residues.3 The behavior of tyrosine 63, which is in the active site, should be more apparent.

<table>
<thead>
<tr>
<th>pH</th>
<th>Average number of ionized tyrosine residues ($\gamma$)</th>
<th>Estimated net charge ($\alpha$)</th>
<th>Estimated net charge ($\alpha$)</th>
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<tr>
<td>8.74</td>
<td>0.67</td>
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<td>1.11</td>
<td>5.3</td>
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<td>10.45</td>
<td>2.72</td>
<td>1.5</td>
<td>11.70</td>
</tr>
</tbody>
</table>

$\gamma = \Delta \alpha_{685}$ at the pH values cited.
from titration studies in the presence of inhibitors. Such studies are in progress.

Acknowledgments—We are most grateful to Dr. Robert E. Canfield and to Dr. Elliott Osserman for providing the lysozyme used in these studies and for invaluable advice and discussions.

REFERENCES

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