Infrared Titration of Lysozyme Carboxyls

SERGE N. TIMASHEFF

Pioneering Research Laboratory, Eastern Marketing and Nutrition Research Division, United State

Infrared Titration of Lysozyme Carboxyls

SERGE N. TIMASHEFF

Pioneering Research Laboratory, Eastern Marketing and Nutrition Research Division, United States Department of Agriculture, Agricultural Research Service, and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154[1]

AND

JOHN A. RUPLEY

Department of Chemistry, University of Arizona, Tucson, Arizona 85721[2]

The dissociation of carboxylic groups in lysozyme was measured as a function of pH by differential infrared absorption spectroscopy. Analysis of both the 1707 cm^{-1} (COOH) and the 1565 cm^{-1} (COO^-) bands showed that of the 10 ionizable groups, two have highly abnormal pK values, 2.0 and 6.5, while the others vary between 3.5 and 5.5. Assignments to specific residues in the amino acid sequence are made in terms of known features of their microenvironment.

Previous acid–base titration studies of lysozyme have been interpreted in terms of the presence of several carboxylic groups with abnormal pK values, one ionizing at a pH close to 6.5 and two or more remaining unprotonated even at below pH 2 (1–3). Potentiometric titrations measure only the uptake of protons and cannot distinguish between the titration of various types of ionizable groups when there is an overlap in the pH zones of their ionizations. Furthermore, in the case at hand, the titration of even a normal carboxyl group would overlap with the zone of ionization of the single histidine residue (pK 5–5.5) (4). In order to circumvent these difficulties and further clarify the situation, we have carried out a titration study of the lysozyme carboxylic residues using infrared absorption to monitor the degree of ionization. Carboxyl groups absorb in the spectral region of 1550–1710 cm^{-1}. The ionized residues have an absorption band around 1570 cm^{-1} while protonated carboxyls are characterized by a band near 1710 cm^{-1} (5). From the intensities of these two infrared bands it is possible to determine directly the fractions of carboxyls which are either ionized or un-ionized at any value of pH. This paper reports the results of such a study on lysozyme.

While the use of infrared absorption eliminates the problems inherent in potentiometric titration, it requires the use of special techniques because of two complications: (1) The bands characteristic of carboxyl groups appear as shoulders of the much stronger amide I band of the peptide links; (2) in aqueous solution the frequency region of interest is totally masked by strong H_2O absorption. These two problems are circumvented by using a differential infrared absorption technique, and substituting D_2O for H_2O as solvent (6). D_2O can be used as a solvent for proteins without altering their conformation (7). In the presence of D_2O, however, the pK values of ionizable groups are about 0.5 units higher on the pD scale than on the pH scale in H_2O (8, 9).

[1] Publication No. 824.

[2] This work was supported in part by the National Institutes of Health Grant GM 09410 and the National Science Foundation Grant GY 22632.

MATERIALS AND METHODS

Materials. Twice-crystallized lysozyme was purchased from Worthington (Lot LY644A)[3]; deuterium oxide (99.8%) was from Stohler. "DCl" (1.1 N) and "NaOD" (2.24 N) were prepared by dissolving reagent grade concentrated hydrochloric acid or sodium hydroxide pellets in D_2O; normalities were determined by titrations against standard acid or base. Protein concentration was determined spectrophotometrically, using the molar extinction coefficient, $\epsilon = 36{,}500$ (10).

pD measurements were made with a Radiometer TTTl meter equipped with a GK-2021B probe electrode maintained at 30°C. Standardization was with aqueous buffers at pH 4.01 (phthalate) and pH 6.50 (Radiometer phosphate). Immediately after standardization, a sample of the stock protein solution in D_2O (pD ca. 5.6) was read; equilibrium between electrode and D_2O solvent was reached after 15–30 min. This protein solution was used as a standard for the pD adjustments and measurements required in a set of titration experiments.

In order to obtain extensive exchange of hydrogen for deuterium, lysozyme was dissolved in D_2O at pD ca. 5, reacted overnight, and lyophilized. A stock solution was then prepared by dissolving the deuterated protein in D_2O and the pD was adjusted to the desired value for the reference solution.

Infrared measurements. For each experiment, 2 ml of stock solution lysozyme (43.9 g/liter) was adjusted to the proper pD by addition of concentrated NaOD or DCl and the volume was brought to 2.1 ml by further addition of D_2O or a solution of NaCl in D_2O. All additions were made with micropipets. The experimental and reference solutions were then introduced into two "matched" sealed cells of conventional design, with a nominal 0.1-mm pathlength, equipped with CaF_2 windows. Since the actual pathlengths of the cells were 0.093 and 0.089 mm, the 5% mismatch in protein concentration between the experimental sample and the reference stock solution was compensated by introducing each into the cell of proper thickness. The spectra were recorded on a Beckman Infrared Spectrophotometer, Model IR-4.

Difference spectra were obtained over the pD range of 1.0–10.0 using reference protein solutions with pD 7.75 and 4.40. Direct spectra were also measured at selected pD values. Typical direct

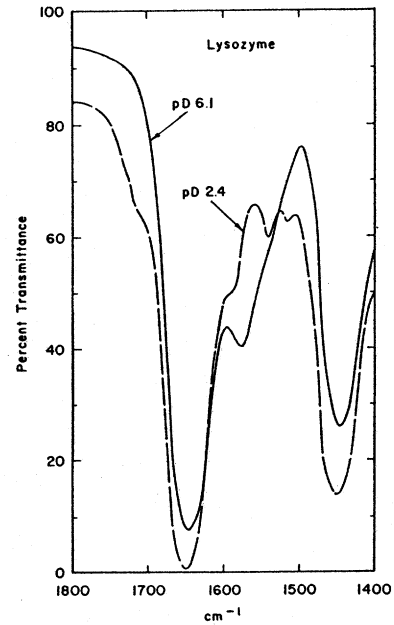

FIG. 1. Direct infrared spectra of lysozyme in D_2O at pD 2.4 and 6.1. The dashed line spectrum has been arbitrarily displaced by 10% transmittance.

and difference spectra are shown in Figs. 1 and 2. The direct spectra are characterized by a strong amide I band centered around 1650 cm^{-1}, with shoulders evident at 1560–1575 cm^{-1} and ca. 1710 cm^{-1}. The essential absence of the amide II band near 1550 cm^{-1} and the presence of a strong band near 1450 cm^{-1} indicate extensive deuteration of the peptide NH groups. The difference spectra are characterized by pD dependent bands at 1565 cm^{-1} and 1707 cm^{-1}; these correspond to COO^- and COOD absorption, respectively. The detail seen between 1620 and 1670 cm^{-1} must be due to pH perturbation of the backbone amide I band. At the protein concentration used, the amide I band in the direct spectrum was of such intensity that, at its peak, there remained 8–10% of the total energy, i.e., it had an optical density of 1.0–1.1. The amplitudes of the difference spectral bands, on the other hand, varied between 95 and 60% transmittance, i.e., their optical densities, ΔOD, varied between 0.020 and 0.200. Baseline controls were carried out periodically, using protein solutions of identical pD in the two cells, one of them having been diluted in a manner identical to the experimental samples. Typical baselines are shown in Fig. 2. There is essentially no detectable deviation from a smooth line in the regions of interest at 1565 and

[3] Mention of specific products or firms does not imply endorsement by the U. S. Department of Agriculture to the possible exclusion of others not mentioned.

1707 cm^{-1}. At 1650 cm^{-1}, i.e., in the region of the amide I band, mismatching by 2% transmittance can be seen. Since this corresponds to an optical density of approximately 0.01, the mismatching is seen to be no greater than 1% while reproducibility was considerably better.

Data analysis. The intensities of the difference bands were read relative to 1800 cm^{-1}, at which frequency there was no detectable difference spectrum. The values were normalized to 100% transmittance for the reference solution, converted to optical density units and corrected for the deviation of the optical densities at 1565 and 1707 cm^{-1} from that of 1800 cm^{-1} in the proper baseline. This was found to be necessary, since the baselines, while smooth, usually were not horizontal straight lines but displayed a slight frequency dependence, as seen in Fig. 2. The experimental error due to the reading of intensities is 3–5%. The infrared absorption intensities at the two frequencies were plotted as a function of pD and compared to theoretical titration curves, shown in Fig. 3.

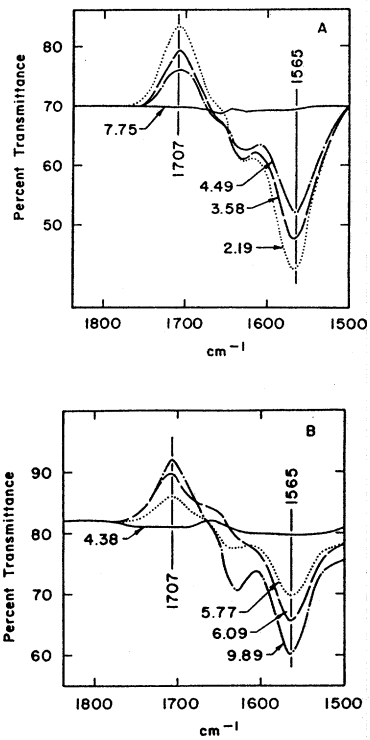

FIG. 2. Difference infrared spectra of lysozyme as a function of pD. (A) pD 7.75 reference; (B) pD 4.38 reference. The instrument was set so that the matched spectra at 1800 cm^{-1} were recorded at 65–85% transmittance; this permitted to record both positive and negative differences in a single scan. The numbers next to the curves indicate the pD at which the spectrum was recorded.

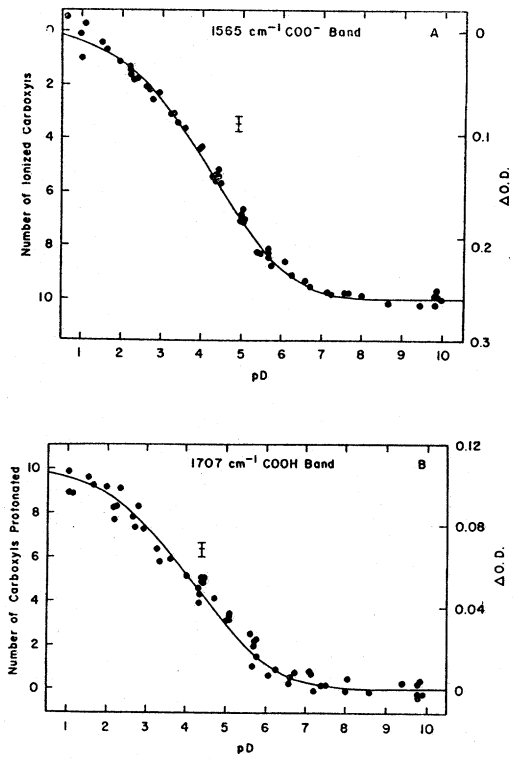

FIG. 3. Infrared titration curves of lysozyme carboxyls in D_2O. (A) 1565 cm^{-1} band; (B) 1707 cm^{-1} band. The dots are experimental points representing optical density differences from either the fully protonated (A) state or the fully ionized (B) state. The solid lines are calculated curves using the pK values reported in the text. The error bars correspond to the experimental errors in reading differential band intensities.

Calculation of carboxyl ionization. The fraction of ionized carboxyls in lysozyme, α, was calculated for a distribution of the groups over a number of classes of type i, with characteristic pK_i. The overall degree of carboxyl ionization, α, is given by

$$\alpha = \frac{\Sigma n_i \alpha_i}{\Sigma n_i}, \qquad (1)$$

where n_i is the number of groups of type i. The degree of ionization of each type of group, α_i, is given by the Linderstrøm-Lang equation, within the approximation of spherical symmetry and a

smeared charge distribution (11, 12)

$$\log \frac{\alpha_i}{1 - \alpha_i} = \mathrm{pH} - \mathrm{p}K_i + 0.868\ \bar{w}\ \bar{Z}$$
$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right), \quad (2)$$

where $\bar{Z}$ is the average net charge on the protein molecule, e is the electronic charge in the electrostatic units, D is the dielectric constant of the medium, k is Boltzmann's constant, T is the thermodynamic temperature, b is the radius of the protein molecule, a as the distance of closest approach of the center of a small ion to the center of the protein molecule, usually taken as $b + 2.5$ Å, and κ is the reciprocal thickness of the ionic atmosphere. For the calculation of the electrostatic work term, the value of the protein average net charge, $\bar{Z}$, as a function of pH was taken from the titration curve of Sakakibara and Hamaguchi (3). The ionic strength was taken as 0.135 throughout. The effective radius of the protein, b, was calculated from the radius of gyration determined in small-angle X-ray scattering experiments, using the relation

$$b = (5/3)^{1/2} R_g\ , \quad (3)$$

where R_g is the radius of gyration of the protein. The value of R_g used was 14.3 Å (13, 14). In carrying out these calculations, the known dimerization of lysozyme above pH 5 (15, 16) was taken into account. For this purpose the electrostatic interaction parameter of the dimer, w_d, was estimated by assuming that the dimer was a sphere with twice the volume of the monomer and had twice its charge, using the relation (12)

$$w_A Z_A = n w_M Z_M \frac{1 + \kappa a_M}{1 + \kappa n^{1/3}[(n^{1/3} - 1) b_M + a_M]}, \quad (4)$$

where the subscripts M and A stand for monomer and aggregate, respectively, and n is the degree of association. The term, $\bar{w}\bar{Z}$, was taken then as the average of the monomer and dimer contributions in proportions estimated from the reported association data. While this calculation is highly approximate, any uncertainties resulting from it are not significant, since dimerization becomes important only above pH 6, where the titration is 95% complete.

The experimental results were fitted with a set of 10 ionizable groups[4] of various pK values with the use of Eq. (1) and (2) and the above-described procedure. This calculation was repeated with various values of n_i and α_i until a good fit was obtained for the experimental curve. Comparison between theory and experiment required a value for the absorbance change developed through ionization of one group; this was taken to be the same for all groups and one-tenth of the total absorbance change between extreme acid and alkaline pH.

RESULTS

The results of the spectroscopic titration of lysozyme carboxyls are given in Fig. 3. The dots are experimental values of the absorbances calculated in each case as differences from the fully protonated (Fig. 3a) or fully ionized (Fig. 3b) states. The pH dependence of both the 1707 and 1565 cm^{-1} bands have shapes typical for titration curves of a number of nonidentical groups. The intensities of both bands vary in a monotone fashion over a broad pD range, from pD 1.0 to pD 8.0. Above pD 8.0, there is no more variation of the spectrum.

The data of Figs. 3a and 3b could be well-fitted with the following set of ionizable groups: one group with pK_D 2.5, three groups with pK_D 4.0, three groups with pK_D 5.0, two groups with pK_D 6.0, and one group with pK_D 7.0, where pK_D represents the pK in D_2O medium. Since in D_2O the pK values of the carboxyls are displaced by 0.4–0.5 units to the alkaline side (8, 9), these values correspond to intrinsic pK values in H_2O of 2.0, 3.5, 4.5, 5.5, and 6.5. The theoretical curves calculated using this set of pK values are shown by the solid lines of Fig. 3. The agreement with the experimental points is quite good and certainly within the error of of ±3 %. A point which should be mentioned is the fact that the manner of addition of DCl or NaOD to the stock solution caused the ionic strength to vary between individual experiments. This variation, however, was between the limits of 0.085 and 0.135, and it had only a secondary effect on w and the titration curve; calculations at several points

[4] Lysozyme has 10 carboxyl groups: the α-carboxyl, seven aspartic, and two glutamic. Residue 103 which had been thought to be aspartic (17, 18) is asparagine.[5] Recent potentiometric titrations in guanidine hydrochloride have shown that there are 10 ionizable carboxyls (19).

[5] Brown, J., personal communication.

have shown that the overall effect is no greater than ±0.1 ionizable group. Such an uncertainty is again fully within the limits of experimental error. Also, experimental results obtained at slightly different ionic strengths over the same pH range showed no systematic dependence on ionic strength.

DISCUSSION

Lysozyme is known to have 10 ionizable carboxyls. Various solution studies, as well as examination of the environment of these groups in the three-dimensional structure suggest a broad distribution of pK values. Such a distribution is observed in the present study. It is of interest to attempt an assignment of the particular pK values to the specific carboxyls.

The group with a pK of 2.0 is best ascribed as Asp 66. This residue is the only ionizable group completely buried within the protein structure (20, 21); it is hydrogen-bonded to two or three other polar groups, one of these being Tyr 53, which is also highly abnormal with pK ca. 12.5 (22); the free energy of unfolding shows at least one group of p$K < 2$ (23); the pH dependence of substrate binding shows that a group of pK ca. 2 is part of the active site (24), and Asp 66 is in the wing that moves in complex formation. The recent establishment of the number of carboxyl groups in lysozyme as 10 suggests that conclusions on the number of low pK groups from early titration experiments (see introductory paragraph), which were based on more than 10 ionizable groups, are not correct and that there is only one group of p$K \leq 2$, i.e., Asp 66. The other highly abnormal group, with pK 6.5, has been identified as Glu 35, which in accord with its high pK is in a nonpolar region of the lysozyme cleft (20, 21) and is not hydrogen bonded. Values of pK 4.5 have been assigned to Asp 101 through the pH dependence of saccharide binding (25) and to Asp 52 by titration of the ester derivative (26). Recent crystallographic experiments on the changes in protein structure with pH[6] suggest pK values ca. 3.5 for the α-carboxyl, Glu 7, and Asp 87. The other three groups with pK 4.5 or 5.5 represent normal or slightly abnormal carboxyls and are impossible to assign to specific residues at present.

[6] Beddell, C. R., and Phillips, D. C., personal communication.

REFERENCES

1. Tanford, C., and Wagner, M. L. (1954). *J. Amer. Chem. Soc.* **76**, 3331.
2. Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1960). *J. Amer. Chem. Soc.* **82**, 2154
3. Sakakibara, R., and Hamaguchi, K. (1968). *J. Biochem. (Tokyo)*, **64**, 613.
4. Piskiewicz, P., and Bruice, T. C. (1968). *Biochemistry* **7**, 3037.
5. Susi, H. (1969). *in* Structure and Stability of Biological Macromolecules (Timasheff, S. N., and Fasman, G. D., eds.), p. 575. Dekker, New York.
6. Susi, H., Zell, T., and Timasheff, S. N. (1959). *Arch. Biochem. Biophys.* **85**, 437.
7. Susi, H., Stevens, L., and Timasheff, S. N. (1967). *J. Biol. Chem.* **242**, 5460.
8. Bell, R. P. (1959). The Proton in Chemistry, Cornell University Press, Ithaca, New York.
9. Glasoe, P. K., and Long, F. A. (1960). *J. Phys. Chem.* **64**, 189.
10. Hartdegen, F. J. (1967). Ph.D. Thesis, University of Arizona.
11. Linderstrøm-Lang, K. O. (1924). *C. R. Trav. Lab. Carlsberg* **15**, No. 7.
12. Timasheff, S. N. (1970). *in* Biological Polyelectrolytes, (Veis, A., ed.), p. 1. Dekker, New York.
13. Krigbaum, W. R., and Kugler, F. R. (1970). *Biochemistry* **9**, 1216.
14. Pessen, H., Kumosinski, T. F., and Timasheff, S. N. (1971). *J. Agr. Food Chem.* **19**, 698.
15. Sophianopoulos, A. J., and Van Holde, K. E. (1964). *J. Biol. Chem.* **239**, 2516.
16. Bruzzesi, M. R., Chiancone, E., and Antonini, E. (1965). *Biochemistry* **4**, 1796.
17. Canfield, R. E. (1963). *J. Biol. Chem.* **238**, 2698.
18. Jolles, J., Jauregui-Adell, J., and Jolles, P. (1963). *Biochim. Biophys. Acta* **78**, 68.
19. Roxby, R., and Tanford, C. (1971). *Biochemistry* **10**, 3348.
20. Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967). *Proc. Roy. Soc. Ser. B* **167**, 365.
21. Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and

Sarma, V. R. (1967). *Proc. Roy. Soc. Ser.* B **167**, 378.

22. Tojo, T., Hamaguchi, K., Inanishi, M., and Amano T. (1966). *J. Biochem.* (*Tokyo*) **60**, 538.
23. Sophianopoulos, A. J., and Weiss, B. J. (1964). *Biochemistry* **3**, 1920.
24. Banerjee, S. K., and Rupley, J. A., submitted.
25. Rupley, J. A., Butler, L., Gerring, M., Hartdegen, F. J., and Pecoraro, R. (1967). *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1088.
26. Parsons, S. M., and Raftery, M. A. (1969). *Biochemistry* **8**, 4199.