IONIZATION BEHAVIOR OF THE CATALYTIC CARBOXYLS OF LYSOZYME *

S. M. Parsons† and M. A. Raftery

Church Laboratory of Chemical Biology
California Institute of Technology, Pasadena, California 91109

Received August 19, 1970

SUMMARY

The pH difference titration of a β-ethyl ester derivative of the Asp 52 residue of lysozyme relative to native lysozyme has been obtained. The difference curve reflects the ionization behavior of both Asp 52 and Glu 35. Four micro-constants describe the ionizations of the two interacting catalytic groups. The macroscopic ionization constant, including all forms of the enzyme, for Asp 52 is 4.5 while that for Glu 35 is 5.9 in 0.15 M KCl at 25°. The two groups have an electrostatic interaction equivalent to about 0.8 log units.

INTRODUCTION

Recently we prepared a derivative of hen egg-white lysozyme which consists of the β-ethyl ester of aspartic acid residue 52 in the amino acid sequence. It was one of several esters resulting from the aqueous reaction of triethyloxonium fluoroborate [$(C_2H_5)_3O^+BF_4^-$] with lysozyme. Its isolation and identification, as well as its inhibitor binding capability, enzymatic inactivity, and chemical properties have been described (1, 2). This communication reports the pH difference titration of the derivative when compared to native lysozyme. Four microconstants describing the

* Contribution No. 4119. Supported by U.S. Public Health Service Grant GM-14452.

† National Institutes of Health Predoctoral Fellow, 1969-1970.

ionizations of the interacting Asp 52 and Glu 35 carboxyls in the native enzyme are obtained.

EXPERIMENTAL

Solutions of carefully purified native and modified lysozyme (15 mg/ml) in 0.15 M KCl were matched in concentration to within 0.2% as determined by their ultraviolet difference spectrum utilizing matched 0.5 mm path length cells and a 0.1 OD slide wire. A two ml aliquot of each solution at 25° under nitrogen was titrated in a continuous manner from pH 9 to pH 3 with 0.150 N HCl. The titration curve for the ester derivative then was subtracted from that for native lysozyme.

RESULTS AND DISCUSSION

A control difference titration of lysozyme vs. lysozyme plus 0.6 mole ratio KO_2CCH_3 yielded data which was fit essentially perfectly with a pK of 4.60, the expected value. Also, the Asp 52 ester derivative remained enzymatically inactive after the titration, thus indicating no hydrolysis.

Figure 1 shows the results obtained from the ester difference titration. The breaking curve is readily explained as arising from the perturbation of a group of about pK 6 in native lysozyme to a lower value in the ester derivative. The perturbed group is certainly Glu 35 since it is located quite close to Asp 52 in the tertiary structure (3) and by other methods it has been shown to have a pK of about 6 (4). Absence of the negative electric field from Asp 52 in the ester derivative would lower the pK of Glu 35.

Utilizing the theoretical difference in titrant volumes expected from the Asp 52 side chain, the points were fitted with the sum of two positive and one negative ideal titration curves. Figure 1 was best fitted by pG_1 equal to 4.40 and pG_2 equal to 6.10 in native lysozyme, and pk_2 equal to 5.20 in the derivative. The meaning of G_1, G_2, and k_2 will be discussed below. A repeat titration on a separate preparation of the protein solutions yielded a curve

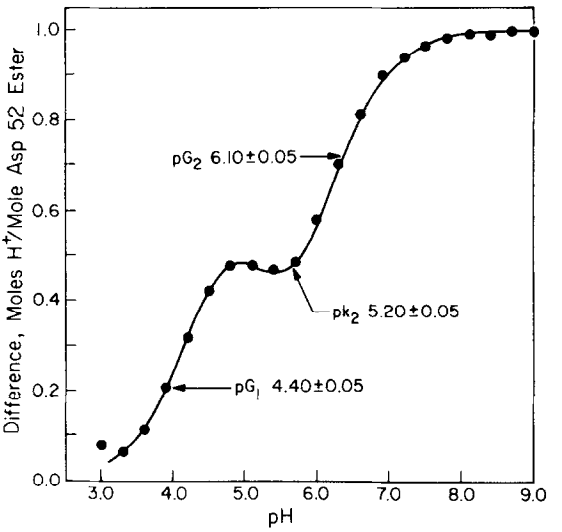

Figure 1. Difference titration of 2.00 ml of lysozyme vs. the Asp 52 ester derivative, both 15 mg/ml (about 1×10^{-3} M), in 0.150 M KCl at 25°C. The total untake of 0.150 N HCl from pH 9.000 to pH 3.000 was 547.0 small Agla micrometer units (0.1094 ml) for lysozyme and 489.5 small units (0.0979 ml) for the derivative. The expected difference due to one amino acid side-chain ester is 63 small Agla units which has been normalized to a span of one.

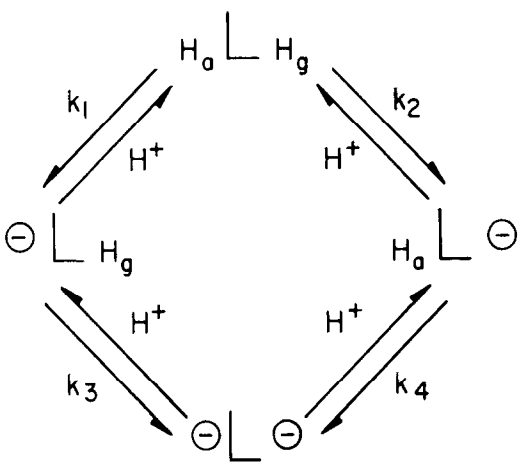

Figure 2. The two catalytic carboxyls of lysozyme considered as a dibasic acid. Subscripts a and g refer to Asp 52 and Glu 35. The predominate mode of ionization in the enzyme follows micro-constants k_1 and k_3 with $^{\ominus}LH_g$ the catalytically active species.

which was fitted as well as Figure 2 with values of 4.35, 6.05, and 5.25.

Usually, ideal titration curves are not expected in proteins because of a variable electrostatic potential. However, because of chloride ion binding (5) the total charge on lysozyme changes very little below pH 9. One could therefore hope that the relatively weak interactions with distal charged groups would average to a nearly constant electrostatic potential over the pH range 9 to 3 in the region of the active site. The excellent fit of the data in Figure 1 indicates that this is the case.

The usual interacting divalent acid ionization scheme as applied to Asp 52 and Glu 35 is shown in Figure 2. The "titration" constants for the two groups in the native enzyme, G_1 and G_2, were determined as if the two groups were an equivalent mixture of two simple monovalent acids. These values are related to two molecular dissociation constants by Equation 1a, b. The two molecular constants are dependent on the four micro-constants in Figure 2 according to Equation 2a, b (6).

$$K_1 = G_1 + G_2 \tag{1a}$$

$$K_1 K_2 = G_1 G_2 \tag{1b}$$

$$k_1 + k_2 = K_1 \tag{2a}$$

$$\frac{1}{k_3} + \frac{1}{k_4} = \frac{1}{K_2} \tag{2b}$$

All four micro-constants can be calculated by assuming that the titration constant obtained for Glu 35′ in the derivative is equal to k_2 in native lysozyme. A moderate error in this assumption would have little consequence on k_1 and k_3. The results are listed in Table 1 as pk values.

The electrostatic interaction between Asp 52 and Glu 35 is seen from Table 1 to be equivalent to 0.8 log units. Calculation of the macroscopic ionization curve of Asp 52 on the basis of Equation 3 shows it to be slightly asymmetric with the point of half ionization occurring at pH 4.5. This determination is the first assignment of the Asp 52 pK. A similar calculation

Table 1. Micro-constants for the Asp 52 and Glu 35 ionizations*

pk_1 (Asp 52)	4.40 4.60
pk_2 (Glu 35′)	5.25 5.20
pk_3 (Glu 35)	6.00 6.03
pk_4 (Asp 52)	5.15 5.29

*At 25°C in 0.15 M KCl as calculated by Equations 1 and 2 for the scheme in Figure 2 as applied to Figure 1, as well as a similar figure not shown, by assuming k_2. Possible error in pk_1 and pk_3 is estimated at ±0.10, pk_2 and pk_4 are more uncertain.

for Glu 35 gives pK 5.9, in agreement with previous values. These independent physical measurements of the properties of the catalytic carboxyls of lysozyme should prove valuable towards an understanding of the enzymatic mechanism.

$$\text{Fraction Asp 52 Ionized} = \frac{k_1[(H^+)+k_3]}{k_1[(H^+)+k_3] + (H^+)[(H^+)+k_2]} \qquad (3)$$

REFERENCES

1. Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Jr., Groff, T., Racs, J., and Raftery, M. A., Biochemistry 8, 700 (1969).
2. Parsons, S. M., and Raftery, M. A., Biochemistry 8, 4199 (1969).
3. Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R., Proc. Roy. Soc. Ser. B, Biol. Sci. 167, 378 (1967).
4. Dahlquist, F. W., and Raftery, M. A., Biochemistry 7, 3277 (1968).
5. Beychok, S., and Warner, R. C., J. Amer. Chem. Soc. 81, 1892 (1959).
6. Edsall, J. T., and Wyman, J., "Biophysical Chemistry", Academic Press, New York, Ch. 9, 1958.