Ionization Behavior of the Catalytic Carboxyls of Lysozyme. Effects of Temperature†

S. M. Parsons‡ and M. A. Raftery*

ABSTRACT: The pH difference titration of the β-ethyl ester derivative of the Asp-52 residue of lysozyme relative to native lysozyme has been obtained at 1.6, 25, and 40° in 0.15 M KCl. From the temperature dependence of the four microconstants describing the interacting Asp-52 and Glu-35 carboxyls, the heats and entropies of ionization at 25° are estimated to 3.5 kcal/mole and −9 e.u. for Asp-52 ionizing with Glu-35 protonated and 1.9 kcal/mole and −18 e.u. for Glu-35 ionizing with Asp-52 protonated. When these observed values are approximately corrected for the charge on the rest of the lysozyme molecule the values become more normal. These parameters are interpreted, with the aid of the X-ray crystallographic structure of lysozyme, to be indicative of the interactions of each group with its surrounding enzyme structure.

It was shown in the preceding paper (Parsons and Raftery, 1972a) that both Asp-52 and Glu-35 are in environments which may be classified as slightly hydrophobic. It would be of great value in describing the structure of the lysozyme active site if we could determine the precise thermodynamic nature of the two hydrophobicities. With the aid of the X-ray crystallographic map of the active site we could hope to interpret thermodynamic parameters successfully.

It has previously been shown that the ΔH of ionization for a group of pK = 6.2 in lysozyme, which can now be assigned to Glu-35, is about 3 ± 1 kcal/mole (Donovan *et al.*, 1961). A group observed by Kowalski and Shimmel (1969), which we believe to be Asp-52 (Parsons and Raftery, 1972a), also has a significant positive heat of ionization.

The difference titration between the aspartic acid residue 52 derivative and native lysozyme has been performed at several temperatures in order to obtain heats and entropies of ionization for the microconstants of the Asp-52 and Glu-35 ionizations. An attempt also has been made to correct the observed thermodynamics for the influence of the charge on the protein. The following paper in this series explores the effects of inhibitors and a substrate on the ionizations of the carboxyls in the lysozyme cleft (Parsons and Raftery, 1972b).

Experimental Section

Aliquots of the same solutions of the derivative and of native lysozyme used for the difference titration at 25.0° presented in Figure 2 of the preceding paper were used here. Titrations were performed at 1.6 and 40.0° in the manner previously described. Difference data were fitted as before with the sum of two positive and one negative titration curves. From these titration constants the four microconstants (Parsons and Raftery, 1972a, Figure 4) were calculated by assuming k_2.

Results

Difference Curves. The two difference curves obtained from identical solutions at 1.6 and 40° are shown in Figure 1. The curve obtained at 25° is shown in Figure 2 of the preceding paper (Parsons and Raftery, 1972a). There is a large shift toward acid pH in the difference titration at high temperature. The significant deviation below pH 4 at 1.6° from the three-parameter curve is attributed to experimental error because of the longer time required for electrode equilibration at low temperature.

Observed Heats and Entropies of Ionization for the Microconstants. The microconstants for Asp-52 and Glu-35 at 1.6, 25, and 40° are listed in Table I. Van't Hoff plots of the microconstants can be made in order to calculate apparent heats of ionization. The microconstants determined here are not strictly thermodynamic constants but rather apparent constants containing activity coefficients, thus yielding apparent heats and entropies of ionization.

The plots of pk_1, pk_2, and pk_3 are shown in Figure 2. The average observed heats of ionization and their two σ errors are 3.5 ± 1.4 kcal/mole for k_1, 1.9 ± 1.2 kcal/mole for k_2, 2.9 ± 0.6 kcal/mole for k_3, and 4.5 ± 3.2 kcal/mole for k_4 (Table II). The apparent entropies of ionization calculated from $\Delta S_{obsd} = (\Delta H_{obsd} - 2.3\ RT\ pk)/T$ at 25° are −9 ± 5 e.u. for k_1, −18 ± 4 e.u. for k_2, −18 ± 2 e.u. for k_3, and −9 ± 11 e.u. for k_4 (Table II).

Discussion

Observed Heats and Entropies of Ionization. Because of the large changes in heat capacity associated with carboxyl ionizations the van't Hoff plot is not strictly appropriate. However, by plotting data above and below 25°, a ΔH close to the correct value at 25° will be obtained from the van't Hoff slope. Caution should be exercised before accepting the plot. A significant change in the charge on the protein at the same pH at different temperatures would lead to a shift in pk's even though the actual heats of ionization were zero. Because the overall titration of lysozyme below pH 7 is only slightly dependent on temperature (Tanford and Wagner, 1954) and much of the dependence is accounted for by Glu-35 itself, the problem is not encountered here. It should be noted that the thermal denaturation temperature for lysozyme is well

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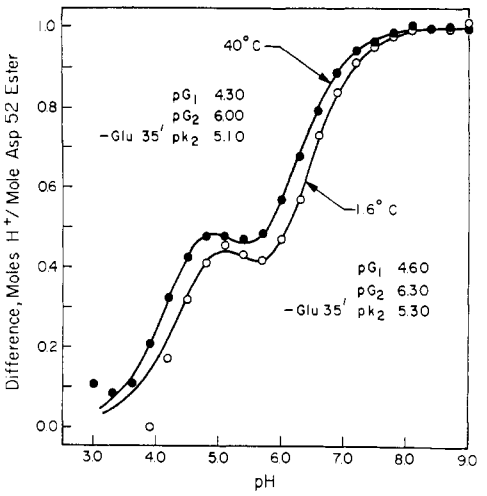

FIGURE 1: Difference titrations of lysozyme *vs.* the Asp-52 ester derivative at 1.6 and 40°. These titrations were performed on aliquots of the same solutions used for Figure 2 in the preceding paper.

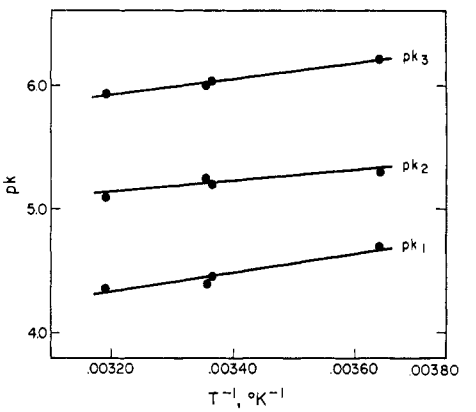

FIGURE 2: Van't Hoff plots of the data from Table I. The determined heats of ionization are listed in Table II along with their errors.

above 40° at all titrated pH values (Sophianopoulos and Weiss, 1964).

Heats and Entropies of Ionization in Uncharged Lysozyme. The observed apparent thermodynamic constants can be divided into two contributions, an "intrinsic" part, *e.g.*, ΔS^0, and an electrostatic part, *e.g.*, ΔS(el), as in eq 1a, b, and c.

$$\Delta F_{obsd} = \Delta F^0 + \Delta F(el) \quad (1a)$$

$$\Delta S_{obsd} = \Delta S^0 + \Delta S(el) \quad (1b)$$

$$\Delta H_{obsd} = \Delta H^0 + \Delta H(el) \quad (1c)$$

A Debye–Hückel expression for ΔF(el) was developed in the previous paper (Parsons and Raftery, 1972a) and is given here in modified form by eq 2. Each symbol was defined

$$\Delta F(el) = \frac{(Z-1)^2\epsilon^2 N}{2D_w}\left(\frac{\kappa}{1+\kappa a}\right) - \frac{(Z^2-2Z)\epsilon^2 N}{2D_w}\frac{1}{b} \quad (2)$$

previously and has conventional meaning. If one differentiates eq 2 with respect to temperature and takes ΔH as constant with respect to temperature, eq 3 is obtained for the electrostatic entropy. The term involving the differential of κ is

$$-\frac{d\Delta F(el)}{dT} = \Delta S(el) = \Delta F(el)\frac{d \ln D_w}{dT} \quad (3)$$

quite negligible in the derivative and has been dropped. The ΔF_H term in eq 5 of the preceding paper has not been included here in eq 1 since it is a small term at 0.15 M KCl whose temperature derivative is very small.

Westheimer and Kirkwood (1946) have discussed the accuracy of eq 3. It must be regarded as quite crude, exhibiting errors of up to a factor of two compared to experimental values measured for small molecules. However, the equation at least gives the correct sign for the electrostatic contribution in eq 1b, as explained below. Since ΔF(el) and d ln D_w/dT (Akerlöf, 1932) are both negative, the contribution to the observed entropy change because of the charge on lysozyme is positive. This is readily conceptualized. When an uncharged carboxylic acid ionizes, the charge on the carboxylate molecule increases by one unit with a consequent increase in hydration and decrease in entropy. When Asp-52 or Glu-35 ionizes, the net charge on the lysozyme molecule *decreases*. Thus, relative to a simple carboxyl group, there is less loss of entropy when Asp-52 or Glu-35 ionizes and the observed ΔS of ionization is more positive than an equivalent ionization in uncharged lysozyme.

The electrostatic free energy of ionization due to the rest of the protein, ΔF(el), readily is obtained from the difference between the pk in 0.15 M KCl and pk^0 (eq 1a, Table III). Using −0.0047°K (Akerlöf, 1932) in eq 3 we calculate

TABLE I: Temperature Dependence of the Microconstants for the Asp-52 and Glu-35 Ionizations.[a]

Temp (°C)	pk_1 (Asp-52)	pk_2 (Glu-35′)	pk_3 (Glu-35)	pk_4 (Asp-52)
1.6	4.69	5.30	6.21	5.60
25	4.40, 4.46	5.25, 5.20	6.00, 6.03	5.15, 5.29
40	4.36	5.10	5.93	5.19

[a] In 0.15 M KCl, calculated by assuming k_2 in eq 2 and 3 of Parsons and Raftery, 1972a, as applied to Figure 1 of this paper and Figure 2 of the previous paper as well as one other figure for 25° which is not shown.

TABLE II: Observed Thermodynamics of Ionization for Asp-52 and Glu-35.[a]

Ionization Step[b]	pk[c]	ΔH_{obsd}[d] (kcal/mole)	ΔS_{obsd}[e] (cal/deg-mole)
k_1 (Asp-52)	4.43	3.5 ± 1.4	−9 ± 5
k_2 (Glu-35′)	5.22	1.9 ± 1.2	−18 ± 4
k_3 (Glu-35)	6.01	2.9 ± 0.6	−18 ± 2
k_4 (Asp-52)	5.22	4.5 ± 3.2	−9 ± 11

[a] Determined from Figure 2. [b] Microconstants identified in Figure 4 of the preceding paper. [c] At 25° in 0.15 M KCl. [d] Observed apparent heat of ionization with 2 σ error. [e] Observed apparent entropy of ionization with 2 σ error.

TABLE III: Thermodynamics of Ionization for Asp-52 and Glu-35 in Uncharged Lysozyme.

Ionization Step	pk[a]	pk^0[b]	ΔF(el)[c] (kcal/mole)	ΔS(el)[d] (e.u.)	ΔH(el)[e] (kcal/mole)	ΔS^0[f] (e.u.)	ΔH^0[g] (kcal/mole)
k_1 (Asp-52)	4.42	5.30	−1.2	5.5	0.44	−14	3.1
k_2 (Glu-35′)	5.21	5.80	−0.81	3.8	0.32	−22	1.6

[a] Measured at 25° in 0.15 M KCl. [b] Obtained from Figure 7 of the preceding paper, the value which each pk would exhibit at zero ionic strength in uncharged lysozyme. [c] The electrostatic free energy difference corresponding to pk − pk^0. [d] The electrostatic entropy of ionization calculated from ΔF(el) d ln D_w/dT. [e] The electrostatic heat of ionization calculated from ΔF(el) + $T\Delta S$(el). [f] The corrected entropy of ionization at 25° that k would exhibit if lysozyme were not charged. [g] The corrected heat of ionization that k would exhibit if lysozyme were not charged.

ΔS(el), which with ΔF(el) gives us ΔH(el). Using these electrostatic values in eq 1b and 1c with the observed values we calculate ΔS^0 and ΔH^0. The corrected ionization parameters for Asp-52 and Glu-35 are listed in Table III. They must be regarded as approximate.

Aspartic Acid Residue 52. The observed heat and entropy of ionization for k_1 in Table II are both different from the values usually found in proteins. Typical values at 25° are −1.5 to +1.5 kcal per mole for ΔH and −18 to −21 e.u. for ΔS (Tanford, 1962). Since k_1 dominates the overall ionization of Asp-52, the thermodynamic values for k_1 are nearly those which would be deduced from any macroscopic measurement on Asp-52 (Kowalski and Shimmel, 1969).

These values do not arise from two enzyme states of measurably different structure, with the enthalpy and entropy changes spread over a large part of the cleft. Physical evidence, including optical rotation and viscosity (Jirgensons, 1958; Yang and Foster, 1955), inhibitor binding studies utilizing nuclear magnetic resonance (Dahlquist and Raftery, 1968), and the integrity of the Asp-52 ester derivative as evidenced by retained inhibitor binding (Parsons *et al.*, 1969), the small ultraviolet (uv) difference spectrum with native enzyme, and the titration data, all indicate that there is no measurable enzyme conformational transition coupled to the Asp-52 ionization. More refined studies could modify this conclusion.

The values for ΔH_{obsd} and ΔS_{obsd} become more normal when corrected for the lysozyme charge. However, the corrected ΔH^0 and ΔS^0 for k_1 in neutral lysozyme, 3.1 kcal/mole and −14 e.u., respectively, both still appear to be abnormally elevated, although the uncertainties in these numbers do not allow us to conclude this definitively. It is interesting, though, that the estimated values of these "solvent" determined parameters can be readily rationalized in terms of the possible interactions between Asp-52 and the surrounding protein structure as outlined below.

a. Blake *et al.* (1967) suggested that the formation of hydrogen bonds by ionized Asp-52 to Asn-46 and Asn-59 would be consistent with a lowering of the pK of Asp-52 and therefore with its catalytic role. Formation of these hydrogen bonds between side chains oriented by hydrophobic interactions would displace hydrogen-bonded water and lead to an increase in the entropy of the system (Nemethy *et al.*, 1963; Laskowski and Scheraga, 1954).

b. Lee and Richards (1971) have calculated the solvent accessibility of every atom in the lysozyme molecule on the basis of the X-ray coordinates. The Asp-52 β-carboxyl was relatively inaccessible, having an average accessibility of 9.8%. This is to be compared to a value of 35 to 40% for Asp incorporated within the model tripeptide Ala-Asp-Ala.

c. Some chemical properties of the Asp-52 β-ethyl ester derivative indicate that this carboxyl does not interact strongly with water. The ester group of the native derivative is inert to attack by aqueous hydroxylamine, hydrazine, and hydroxide ion, thus demonstrating rather tenacious steric hindrance around Asp-52 (Parsons and Raftery, 1969).

The high "intrinsic" ionization constant for Asp-52, the thermodynamics of ionization, the X-ray-determined tertiary structure, and the lack of chemical accessibility, all indicate that this part of the active site does not interact strongly with water. Also, the last point indicates that the bottom of the cleft does not exist as a motile structure which opens up even occasionally to water. Partial solvation of Asp-52 by the surrounding protein is not as effective as full hydration would be, as evidenced by the large positive heat of ionization of 3.1 kcal/mole, but the built-in entropy advantage partially compensates for this.

Minimal hydration is a quite desirable situation for a carboxyl group which must be maintained in the anionic state in an enzyme–substrate complex at acidic pH. If Asp-52 were strongly hydrated, many tightly bound water molecules would have to be displaced when substrate bound. It is unlikely that the bound saccharide could supply equal solvation. The result would be both an increased pK for Asp-52 when complexed and a decreased association constant for substrate when Asp-52 was ionized. On the other hand, if the substrate did supply the full solvation of Asp-52 so as to maintain a low pK, binding association and dissociation rates would be slowed. Thus built-in solvation of a buried catalytic group would seem to have enzymatic advantage. In the following paper in this series we show that bound inhibitors and substrates in fact do not change the pK of Asp-52. It seems very unlikely that a large dehydration step would be balanced out nearly exactly by quite different solvation modes in the enzyme–substrate complex.

Glutamic Acid Residue 35. The observed heats of ionization for Glu-35 listed in Table II are abnormally positive, but the entropies of ionization are within the normal range. Since they essentially dominate the Glu-35 ionization behavior, the observed parameters for k_3 are those which would be deduced from a direct observation of Glu-35 (Donovan *et al.*, 1961). Table III gives an estimate for these values in "neutralized" lysozyme. The crystallographic structure of lysozyme indicates that Glu-35 is in a hydrophobic region of the cleft which was characterized in the preceding paper (Parsons and Raftery, 1972a) by an "intrinsic" ionization constant of 5.8 for k_3. It is not surprising that decreased hydration is reflected by a positive ΔH^0 of ionization of 1.6 kcal/mole (Table III). An increased electrostatic work of charging Glu-35 makes

ΔH even more positive in the presence of ionized Asp-52 (2.9 kcal/mole, Table II). The somewhat abnormally negative value for ΔS^0 in uncharged lysozyme of -22 e.u., however, is surprising. Even the uncorrected normal value for ΔS_{obsd} of -18 e.u. indicates a larger decrease in entropy than one might have expected for formation of an anion in a poorly solvating medium. This indicates that either water in the lysozyme cleft, or part of the protein structure, or both, is specifically oriented by the ionization of Glu-35.

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Ionization Behavior of the Cleft Carboxyls in Lysozyme–Substrate Complexes†

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ABSTRACT: The pH difference titration of the β-ethyl ester derivative of the Asp-52 residue of lysozyme relative to native lysozyme has been obtained at 25° and 0.15 M KCl in the presence of several inhibitors and the substrate glycol chitin. The data reflect the ionizations of Asp-52, Glu-35, and Asp-101. The Asp-52 ionization changes very little in all the complexes while the Glu-35 ionization exhibits a pK of about 6.5 in the inhibitor complexes and a pK of 8.0 to 8.5 in the glycol chitin complex. Asp-101 in the native enzyme exhibits the expected pK changes due to hydrogen bond formation to bound inhibitors. Asp-101′ in the ester derivative complexes fails to form these hydrogen bonds.

The successful measurement of some properties of Asp-52 and Glu-35 in the free enzyme as reported in the two preceding papers (Parsons and Raftery, 1972a,b) prompted us to attempt similar determinations for complexes of lysozyme with inhibitors and a substrate. Such information clearly would be of utility in describing the roles of Asp-52 and Glu-35 in initiating catalysis.

The apparent pK of Glu-35 in NAG-βCH$_3$[1] and NAG$_3$ complexes with lysozyme has been determined to be about 6.5 by several methods (Dahlquist and Raftery, 1968; Dahlquist *et al.*, 1966). These complexes do not interact directly with the catalytic site. No physical information is available in the literature about the pK of Glu-35 when the enzyme is bound to substrates which interact intimately with the active site. As for Asp-52 there has been no pK previously assigned to it. The only data come from kinetic studies, which suggest a pK around 4. Since the binding of small inhibitors appears not to perturb the pK of Asp-52 (Dahlquist and Raftery, 1968; Dahlquist *et al.*, 1966) we expect it to exhibit a titration constant of about 4.4 in these complexes.

This paper reports on difference titrations carried out in the presence of methyl β-NAG, methyl β-chitobioside, chitotriose, methyl β(1–4)-chitotriosyl-β-D-glucopyranoside, and glycol chitin. The curves reflect the behavior of both

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[1] Abbreviations used are: NAG-βCH$_3$, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; NAG$_2$-βCH$_3$, methyl β-chitobioside; NAG$_3$, chitotriose; NAG$_3$-Glu-βCH$_3$, methyl β(1–4)-chitotriosyl-β-D-glucopyranoside; NAG-Glu-βC$_6$H$_4$NO$_2$, nitrophenyl 2-acetamido-2-deoxy-D-glucopyranosyl-β(1–4)-D-glucopyranoside.