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Protein-Salt Binding Data from Potentiometric Titrations of Lysozyme in Aqueous Solutions Containing KCl

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Abstract

An existing method for potentiometric titrations of proteins was improved, tested and applied to titrations of the enzyme hen-egg-white lysozyme in aqueous solutions containing KCl at ionic strengths from 0.1 M to 2.0 M at 25°C. Information about the protein's net charge dependence on pH and ionic strength were obtained and salt binding numbers for the system were calculated using a linkage concept.

For the pH range 2.5-11.5, the net charge slightly but distinctly increases with increasing ionic strength between 0.1 M and 2.0 M. The differences are most distinct in the pH region below 5. Above pH 11.35, the net charge decreases with increasing ionic strength.

Preliminary calculation of binding numbers from titration curves at 0.1 M and 1.0 M showed selective association of chloride anions and expulsion of potassium ions at low pH. Ion-binding numbers from this work will be used to evaluate thermodynamic properties and to correlate crystallization or precipitation phase-equilibrium data in terms of a model based on the integral-equation theory of fluids which is currently under development.

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1 Introduction

Salting-out, i.e. the precipitation of proteins from aqueous solutions by addition of inorganic salts, is a common technique in the recovery and purification of proteins. Nevertheless, there is so far no predictive model to describe the dependence of the phase behaviour on parameters such as salt type, ionic strength, temperature and pH.

Attempts to derive such a model within the framework of statistical mechanics require experimental determination of parameters describing both protein-protein and protein-salt interactions on the molecular level. Electrostatic interactions between protein molecules are affected by the presence of salt via dielectric screening. Protein-protein interactions are also known to be affected by specific ion binding.

A promising method for describing protein interactions in aqueous solutions is to use a two-body potential of mean force (PMF) incorporating the various modes of interaction present in aqueous protein-salt solutions.

For the electrostatic contribution to the PMF, information about the net (surface) charge dependence of the protein on temperature, pH and ionic strength are required. Data for these net charge dependences can be obtained from potentiometric titrations of proteins.

This information can also be used in sophisticated molecular-thermodynamic models for protein solution behavior. For a family of models based on the integral-equation theory of fluids (currently under development), the ion-binding numbers from this work will be required to evaluate thermodynamic properties and to correlate crystallization or precipitation phase equilibrium data.

The purpose of this work was to measure the net charge dependence of the enzyme hen-egg-white lysozyme on pH and ionic strength in aqueous solution containing KCl over the pH range 2.5-11.5 and the ionic-strength range 0.1-2.0 M at 25°C. Binding numbers for both cation and anion have been calculated from a linkage concept presented by Fraaije and Lyklema [7].

2 Binding Numbers from Titration Data

Binding of ions to a surface means that the concentration of the ions is higher in the interfacial region between the surface and the solution than in the bulk of the solution. Information about hydrogen-ion binding is directly obtained from acid-base titration of a protein; binding numbers for salt ions are then calculated from titration data at several different salt concentrations using a phenomenological approach (ESIN-MARKOV analysis).

2.1 Hydrogen Ion Binding

Binding of H^+ to the protein is assumed to be due to chemical association with one of the functional groups capable of an acid-base reaction (e.g. carboxyl and amino groups). Binding in the electrical double layer is neglected since the concentration of H^+ is much lower than the salt concentration (for salt concentrations in excess of 0.1 M). An equation often used to describe proteins as polyacids or polybases is

$$pH - \log \left(\frac{r_i}{n_i - r_i} \right) = pK_i^{int} + 0.868w\bar{Z} \quad (2.1)$$

where r_i is the number of protons dissociated from n_i units of a functional group i , pK_i^{int} is the intrinsic hydrogen-ion dissociation constant of this group, w is an electrostatic interaction factor and $\bar{Z}$ is the charge of the protein (with bound salt ions). [1] [4] [9] [20] [27] [28] [29].

In this work, the acid-base behavior of the protein is not described using equation (2.1). Rather, the *net charge* of the protein is examined as a function of pH and salt concentration.

2.2 Binding of Salt Ions

Binding of salt ions occurs in the electrical double layer of the protein, partly on the surface and partly in the diffuse layer. If the surface charge and geometry are known, the concentration of ions in the diffuse layer can be calculated by solving the POISSON-BOLTZMANN equation [7] [14]. This is not done here; instead, the overall binding number of salt ions is calculated as a function of pH and salt concentration using a thermodynamic approach described in section 2.3 below.

2.3 Phenomenological Description of Ion Binding

Fraaije and Lyklema [7] have proposed an approach to describe ion binding which allows calculation of the amounts of different ionic species that are bound to protein. Because this approach is completely phenomenological, it does not distinguish between different binding mechanisms; this approach is described in the following section.

2.3.1 Linkage Relations and Linkage Potentials

The concept of linkage was developed by Wyman and outlined by Schellman [22]. The basic idea is that the amount of bound ions of one species depends directly on the amount of bound ions of all the other species.

Figure 1: Model system (membrane equilibrium)

For the thermodynamic description of linkage, a reference system is considered which consists of two cells containing aqueous solutions in membrane equilibrium, where the membrane is ideally permeable to all components i except the protein (Figure 1). c_p is the concentration of protein in the left chamber (the right chamber contains no protein), c_i^L and c_i^R are the concentrations of the other species in the left and right chambers, respectively. P is the pressure in the right chamber, set equal to the external pressure and therefore an independent variable; and π is the osmotic pressure.

The GIBBS-DUHEM equations for both chambers are

$$N_p d\mu_p = -S^L dT - \sum_{i \neq p} N_i^L d\mu_i^L + V^L dP^L \quad (2.2)$$

$$N_w d\mu_w = -S^R dT - \sum_{i \neq w} N_i^R d\mu_i^R + V^R dP^R \quad (2.3)$$

where N_i are the mole numbers of the components in the system, the subscripts p and w denote protein and water, respectively. μ_i is the chemical potential of component i , S is the entropy and V the volume of a chamber. T is the system temperature which is constant for the whole system which is in thermal equilibrium. Because all components (but the protein) can cross

the membrane, in equilibrium their chemical potentials are identical for L and R

$$\mu_i^L = \mu_i^R \quad (i \neq p) \quad (2.4)$$

and therefore

$$d\mu_i^L = d\mu_i^R. \quad (2.5)$$

Combination of equations (2.2), (2.3) and (2.5) gives

$$N_p d\mu_p = -\Delta S^{(w)} dT + V^L dP^L - \frac{N_w^L}{N_w^R} V^R dP^R - \sum_{i \neq w, p} \Delta N_i^{(w)} d\mu_i \quad (2.6)$$

where for any extensive variable X an *excess extensive variable* is defined as

$$\Delta X^{(w)} \equiv X^L - X^R \frac{N_w^L}{N_w^R}. \quad (2.7)$$

A *reference-linkage potential* is defined by

$$\Lambda^r \equiv -\mu_p + \frac{\pi}{c_p}. \quad (2.8)$$

Application of VAN'T HOFF's law for low protein concentrations gives the total differential of Λ^r :

$$d\Lambda^r = s^{(w)} dT - v^{(w)} dP + \sum_{i \neq w, p} r_i^{(w)} d\mu_i - \frac{\pi}{c_p} d \ln c_p \quad (2.9)$$

where small letters denote excess extensive properties normalized with respect to N_p . The *binding number* $r_i^{(w)}$ is defined as the excess number of moles i per mole of protein. Equation (2.9) assumes that

$$V^L - \Delta V^{(w)} \approx V^L. \quad (2.10)$$

The linkage relation derived from this differential is

$$\left(\frac{\partial r_i^{(w)}}{\partial r_j^{(w)}} \right)_{\mu_{i \neq j}, P, T, c_p} = - \left(\frac{\partial \mu_j^{(w)}}{\partial \mu_i^{(w)}} \right)_{r_j^{(w)}, \mu_{k \neq i}, P, T, c_p}. \quad (2.11)$$

In the following, superscript (w) is omitted for convenience.

2.3.2 ESIN-MARKOV Coefficients

The linkage relation is a relation between binding numbers and chemical potentials. By rewriting the reference-linkage potential in terms of activities

and *ionic binding numbers* [7] for the species present in our system (H^+ , OH^- , K^+ , Cl^-), the linkage relations for these binding numbers can be written as

$$\left(\frac{\partial r_{K^+}}{\partial r_{a/b}}\right)_{a_s, c_p} = \frac{1}{2}(\beta - 1) \quad (2.12)$$

$$\left(\frac{\partial r_{Cl^-}}{\partial r_{a/b}}\right)_{a_s, c_p} = \frac{1}{2}(\beta + 1) \quad (2.13)$$

where $r_{a/b}$ is the *acid-base binding number* defined by

$$r_{a/b} \equiv r_{H^+} - r_{OH^-} \quad (2.14)$$

and can be identified with the net charge z of the protein. The ionic binding numbers are defined by

$$r_{K^+} \equiv r_{KOH} + r_{KCl} \quad (2.15)$$

$$r_{Cl^-} \equiv r_{HCl} + r_{KCl} \quad (2.16)$$

The ESIN-MARKOV coefficient β for our system is defined by

$$\beta \equiv - \left(\frac{\partial \ln a_{H^+}}{\partial \ln a_s} \right)_{r_{a/b}, c_p} \quad (2.17)$$

It is a measure of the extent of ion exchange and describes how the binding of hydrogen ion is balanced by expulsion of cations and co-binding of anions. Another possible definition of an ESIN-MARKOV coefficient that can be derived from the reference-linkage potential is [8]

$$\delta_{a/b} \equiv \left(\frac{\partial r_{a/b}}{\partial \log_{10} a_s} \right)_{pH, c_p} \quad (2.18)$$

2.3.3 Binding Isotherms

The parameter $\delta_{a/b}$ is accessible from hydrogen ion titration data in solutions of different ionic strengths. Cross-differentiation of the linkage relation, expressed in activity coefficients and ionic binding numbers yields

$$\left(\frac{\partial r_{Cl^-}}{\partial pH}\right)_{a_s} = \frac{1}{2} \left[- \left(\frac{\partial r_{a/b}}{\partial \log_{10} a_s} \right)_{pH} + \left(\frac{\partial r_{a/b}}{\partial pH} \right)_{a_s} \right] \quad (2.19)$$

Integration with respect to pH gives

$$r_{Cl^-}(pH) = r_{Cl^-}(pH^*) + \frac{1}{2} \left[r_{a/b} - \int_{pH^*}^{pH} \delta_{a/b} dpH \right] \quad (2.20)$$

which allows the calculation of the binding isotherm for Cl^- . Here pH^* is a reference point that requires separate information. Salt activity is obtained from

$$a_s = m_s \cdot \gamma_s^\bullet \quad (2.21)$$

where m_s is the molality of the solution and $\gamma_s^\pm$ can be taken from an activity coefficient model based on available data (see appendix B.2).

3 Potentiometric Titrations of Proteins in Aqueous Salt Solutions

3.1 Experimental Information from Potentiometric Titrations

Potentiometric titrations yield information concerning the change of pH, i.e. the change of hydrogen ion activity, a_{H^+}

$$\text{pH} \equiv -\log_{10} a_{H^+} \quad (3.1)$$

occurring when certain amounts of an acidic or alkaline titrant are added. Experimental data consist of the voltage response of an H^+ -sensitive electrode upon addition of titrant aliquots of specified volume. From the (modified) NERNST equation a relation between electrode potential E and pH is derived

$$E = E_0 + \frac{2.303RT}{F} \log_{10} a_{H^+} = E_0 - \frac{2.303RT}{F} \text{pH}. \quad (3.2)$$

E_0 is the electrode standard potential, R is the universal gas constant, T is the absolute temperature and F is FARADAY's constant.

E_0 varies for every electrode and therefore has to be determined by a calibration procedure.

The hydrogen ion activity coefficient defined with reference to an infinite dilute solution, is assumed to remain constant if the titration is conducted at a constant ionic strength. Therefore, equation (3.1) can be rewritten as

$$E = \underbrace{E_0 + \log_{10} \gamma_{H^+}}_{E_0^*} + \underbrace{\frac{2.303RT}{F}}_S \log_{10} c_{H^+} \quad (3.3)$$

or

$$E = E_0^* - S \cdot \text{p}^0\text{H} \quad (3.4)$$

where p^0H is defined as

$$\text{p}^0\text{H} \equiv -\log_{10} c_{H^+}. \quad (3.5)$$

c_{H^+} is the molar concentration of hydrogen ion. In some older articles, pH is defined by concentration, as p^0H is defined here. While the distinction between pH and p^0H may not be very important at conditions close to the standard state of ideal dilute solution where $\gamma_{H^+} = 1$, the difference becomes important at clearly non-ideal conditions, e.g. at high ionic strengths.

3.2 Calibration Titration (Blank Titration)

E_0^* and S can be determined by a calibration titration. To fit $(E - V_{\text{titr}})$ data, a H^+ or OH^- species balance can be used, depending on whether an acidic or alkaline calibration titration has been conducted.

$$N_{\text{H}^+} = N_{\text{H}^+}^{\text{init}} + N_{\text{H}^+}^{\text{add}} - N_{\text{H}^+}^{\text{cons}} \quad (3.6)$$

$$N_{\text{OH}^-} = N_{\text{OH}^-}^{\text{init}} + N_{\text{OH}^-}^{\text{add}} - N_{\text{OH}^-}^{\text{cons}} \quad (3.7)$$

where N are mole numbers, superscript $^{\text{init}}$ refers to the titration starting point, superscript $^{\text{add}}$ refers to the ions added in an aliquot of the titrant solution and superscript $^{\text{cons}}$ refers to the ions consumed by the water association reaction:

Stoichiometry demands that the number of OH^- ions consumed and the number of H^+ ions consumed must be equal at all times

$$N_{\text{OH}^-}^{\text{cons}} = N_{\text{H}^+}^{\text{cons}} \quad (3.9)$$

Because the acid (or base) contained in the titrant solution is totally dissociated, i.e.

$$N_{\text{H}^+}^{\text{add}} = c_{\text{acid}} V_{\text{titrant}}^{\text{add}} \quad (3.10)$$

$$N_{\text{OH}^-}^{\text{add}} = c_{\text{base}} V_{\text{titrant}}^{\text{add}} \quad (3.11)$$

it is possible to calculate the concentration of H^+ by solving the resulting equation for c_{H^+} :

$$c_{\text{base}} V_{\text{added}} = (V_{\text{init}} + V_{\text{added}}) \left(\frac{K_W^0}{c_{\text{H}^+}} - c_{\text{H}^+} \right) - V_{\text{init}} \left(\frac{K_W^0}{c_{\text{H}^+}^{\text{init}}} - c_{\text{H}^+}^{\text{init}} \right) \quad (3.12)$$

where $c_{\text{H}^+}^{\text{init}}$ is the initial proton concentration and K_W^0 is an "effective" water equilibrium constant, which is related to the activity-based water equilibrium constant, K_W ($= 10^{-13.998}$ at 298.15 K), by

$$K_W^0 \equiv K_W \cdot \frac{a_{\text{H}_2\text{O}}}{\gamma_{\text{H}^+} \gamma_{\text{OH}^-}} \quad (3.13)$$

If activity coefficients remain constant over the course of the titration,

$$K_W^0 = c_{\text{H}^+} c_{\text{OH}^-} \quad (3.14)$$

K_W^0 can be obtained from tabulated values for K_W and from the ionic activity function $\frac{\gamma_{\text{H}^+} \gamma_{\text{OH}^-}}{a_{\text{H}_2\text{O}}}$ as given by an activity model for electrolyte solutions,

e.g. the PITZER model (see appendix B.2).

With a given set of E_0^* and S , the voltage response for each data point (i.e. a certain amount of titrant solution added) can be predicted by equations (3.1) and (3.2). By minimizing the sum of the squared residuals between measured (during the course of a blank titration) and calculated response, optimal values for E_0^* and S can be determined. The quality of the calibration is indicated by a plot of experimental E versus experimental p^0H and the function $E = E_0^* - S \cdot p^0H$, as shown in Figure 2.

Figure 2: Sample calibration titration results

3.3 Protein Titrations

All activity coefficients are assumed to be the same (at a given temperature and ionic strength) in the solutions with and without protein if the protein concentration is sufficiently low [27]. Therefore, if E_0^* and S have been determined by a calibration titration, p^0H can be calculated from the voltage response by the equation

$$p^0H = -\frac{E - E_0^*}{S}. \quad (3.15)$$

From p^0H , H^+ and OH^- concentrations are known for the protein titration; they can be used in the species balance to give information concerning the number of ions changing the protein net charge. The species balance is

$$N_{OH^-} = N_{OH^-}^{init} + N_{OH^-}^{add} - N_{OH^-}^{cons} \quad (3.16)$$

but in the presence of protein, $N_{\text{OH}^-}^{\text{cons}}$ also contains the number of hydroxide ions that are consumed by reaction with protons dissociating from the protein, thereby changing the net charge of the protein:

$$N_{\text{OH}^-}^{\text{cons}} = -N_{\Delta z} + N_{\text{H}^+}^{\text{cons}}. \quad (3.17)$$

Since the concentration of protein in a given solution can be determined (e.g. by measurement of specific UV adsorbance) and its molecular weight is known, the change of net charge per molecule can be calculated from

$$\Delta z = \frac{N_{\Delta z}}{N_{\text{protein}}^{\text{init}}} = \frac{N_{\Delta z} M_{\text{protein}}}{c V_{\text{solution}}^{\text{init}}} \quad (3.18)$$

where M_{protein} is the molecular weight, c is the mass concentration of the protein and V_{init} is the volume of the solution at the beginning of the titration. The results obtained are the *relative* net charge of the protein versus $p^0\text{H}$. The calculation of binding numbers, as described in section 2 requires information on *absolute* net charge z versus pH . It is therefore necessary to calculate γ_{H^+} from an activity coefficient model, since

$$\text{pH} = p^0\text{H} - \log_{10} \gamma_{\text{H}^+} \quad (3.19)$$

and to know z for a particular pH or $p^0\text{H}$.

4 Materials and Methods

4.1 Protein

Lysozyme used for the titration experiments was purchased from Sigma Chemical Company, St. Louis, USA [catalog number L-2879, Grade VI: Chloride, from Chicken Egg White, 3 x crystallized, not dialyzed or lyophilized; lot # 15H7090]. Polyacrylamide gel electrophoresis (PAGE) showed that this product contained small amounts of ovalbumin and two other higher-molecular weight proteins. A purification procedure was therefore applied.

4.2 Sample Preparation

Preparation of an aqueous solution of lysozyme of a certain ionic strength and protein concentration followed three separate steps: size exclusion chromatography, ultrafiltration and dialysis. These procedures are described in the following.

4.2.1 Size Exclusion Chromatography

A gel permeation chromatography (GPC) method was used both to purify L-2879 lysozyme and to estimate the extent of its impurities. The apparatus consisted of a glass column (Kontes, Diameter 4.8 cm $\times$ 60 cm) with a top flow adapter, packed with Toyopearl HW50F size-exclusion resin (TosoHaas); a ConstaMetric III HPLC pump (LDC/Milton Roy); a flow-through absorbance detector unit (LinearTM UV-106) operating at 280 nm and connected to an Omniscribe chart recorder (Houston Instrument) and a Minipulse 2 peristaltic pump (Gilson) which supplied running buffer to the reference chamber of the UV absorbance flow cell.

NANOpure water, degassed and adjusted to pH 3 with HCl was used as a running buffer. For each separation, 10 ml of a solution of lysozyme dissolved to 80 g/l in this buffer and brought back to pH 3 were loaded onto the column through a 0.8 μ m Millex-PF filter tip. The pH of the lysozyme solution was readjusted to 3.0 prior to loading the column since it had been observed that the pH after dissolving L-2879 to 80 g/l in the acidified water was approximately 3.9. The typical column pressure was $\approx$ 15 psig, at a flowrate of 10 ml/min. A detailed description of this GPC method is in [12].

Eight fractions were collected from a separation and analyzed by gel electrophoresis. The results showed that fractions collected after point (C) in the chromatogram (Figure 3) did not contain ovalbumin in considerable amounts. (A) is probably a lysozyme-ovalbumin aggregate. Harvesting was finished at (D). At (B) the signal was reduced using a potentiometer (the omniscribe recorder only allows reduction by order of magnitudes, but a reduction by $\approx$

Figure 3: Result from SEC

0.3 was found to be adequate). The flat top of the lysozyme peak is due to saturation of the UV detection unit at such high protein concentrations.

4.2.2 Ultrafiltration

The protein concentration of the pure lysozyme fraction from the SEC column was typically ≈ 3 g/l. Such a low concentration is not desired because the relative errors of both $N_{\Delta z}$ and c_{protein} increase with decreasing protein concentration. Therefore, samples were concentrated by approximately a factor of 3 (≈ 10 g/l) using pressure-driven ultrafiltration through DIAFLO YM10 (Diameter 76 mm, MWCO 10,000) ultrafiltration membranes (Amicon, Beverly, USA). The final volume of ≈ 40 -50 ml was sufficient for subsequent titrations.

4.2.3 Dialysis

Finally, the ultrafiltrated solution was dialyzed against 2 liters of a solution containing the necessary amount of KCl to achieve the desired ionic strength. SpectraPor membrane tubing (Flat Width 100 mm; Diameter 64 mm; MWCO 6-8,000) was used to prepare the dialysis bags, which were clamped and rotated in the KCl solution for at least 12 hours to establish equilibrium. It was assumed that, due to the small concentration of protein, activity coefficients for the transferring components were equal in both compartments of the system and therefore also their respective concentrations.

Another effect of dialysis is the removal of undesired molecules of MW < 6-8,000 which may be present in the protein solution.

4.3 Titration Apparatus

4.3.1 Hardware

Figure 4 shows the apparatus used for the protein titrations, the calibration titrations and the titrant concentration measurements. Thick lines signify flow of fluids while thin lines signify flow of information (voltage signal). The respective solutions were loaded into a 100-ml jacketed reaction vessel and agitated by a magnetic stirrer. The titrant aliquots, typically 0.1 ml, were injected through a fritted line by a Dosimat 665 (Metrohm, Westbury, NY) precision pump. A Ross combination semimicro electrode, model BN 8001 (Orion, Boston, MA) was used to measure pH. The electrode was attached to a Chemtrix 60A pH meter acting as a voltmeter for visual checking of the electrode signal. An IBM-compatible PC equipped with an A/D conversion board was used both to control the Dosimat injections and to record the voltage signal from the pH meter. To keep the temperature of the vessel steady at 25° C, a constant temperature bath with a thermostatted heater-mixer unit (Fisher Thermomix, Model 730) was used, the total water volume was $\approx$ 4 L.

The headspace of the titration vessel was blanketed with argon to prevent a change of pH due to CO₂ partitioning into the solution. Argon from a high-pressure cylinder was passed through a column containing Drierite and acid-gas-specific molecular sieves to remove residual CO₂, then through a gas washing bottle containing pure deionized water to re-saturate the argon and prevent evaporation of the sample from the titration vessel.

A specially designed plexiglas lid, equipped with ports for the electrode, injection line and argon inlet/outlet, rested on an O-ring on the top of the titration vessel. An airtight seal was provided by compression of the lid, O-ring and vessel by an adjustable locking ring. Compared to the equipment used before [5], the new equipment provided better control of temperature, tightness of the reaction cell and, most important, required a 5 times smaller amount of all chemicals used.

Figure 4: Titration apparatus

4.3.2 Software

To take full advantage of the possibilities provided by using a computer controlled titration apparatus, the existing software for both calibration (CAL-TITR) and protein (TITRATE) titrations was modified in a number of ways, allowing greater flexibility in conducting the titrations, giving more certainty for the results and making the whole procedure of gathering data more straightforward.

CAL-TITR

This program controls the titration of the blank solution and collects (V_{titr} , E) data. A number of parameters can be pre-set and it is also possible to interrupt the course of the program to change them. The parameters include:

- The volume of titrant added per step.
- The total volume of titrant to be added.
- The number of voltage readings gathered to calculate one value. 6000 readings per value was found to give a "stable" signal.¹

Figure 5: Voltage signals

- Parameters n and ΔE_{max} for determination of equilibrium (Figure 4.3.2).
- The time between injecting titrant and beginning voltage reading.

¹A double averaging is done: a certain number of readings are used to calculate one value. n of these values are then used to calculate the actual data point.

Figure 6: Determination of equilibrium

The average for the last n values is calculated and taken as the equilibrium data point if none of the n values differs more than $\Delta E_{\max}$ from this average.

The CAL-TITR program generates two ASCII files: a *.DAT which contains the (V_{titr} , E) data points and a *.SIG which contains the "raw data", i.e. all (averaged) values of the electrode for each titration point. This can be used to check if the readings scatter and if the system is equilibrated. The calibration parameters E_0^* and S are not calculated by CAL-TITR. They were calculated as described in section 3 with standard software for spreadsheet calculations.

TITRATE

This program controls the titration of the protein solution, yielding (V_{titr} , E) data. Again, a number of parameters can be pre-set and also be changed during the titration. They are basically the same as for the CAL-TITR program, with the exception that instead of a certain total volume of titrant, the end p⁰H of the titration can be chosen (E_0 and S have to be determined before and are entered manually).

Again, two ASCII files are generated, *.DAT and *.SIG, containing the (V_{titr} , E) data and the (averaged) electrode signal. Calculation of net charge versus pH, commonly called the *titration curve* of a protein, was done with a spreadsheet calculation.

4.3.3 Titrant Solutions

If activity coefficients are to remain unchanged during the course of the titration, the ionic strength must be held constant. Therefore, the titrant solutions must have the same ionic strength as the titrated solution itself.

Titrant solutions were HCl and KOH solutions with a concentration of 0.1 M, brought to the desired ionic strength by adding a sufficient amount of KCl. The contribution of H^+ and OH^- to the ionic strength is neglected since $2 < pH < 12$ and $c_{salt} \geq 0.1$ M; the amount of salt in moles to be added is

$$N_{salt} = V \left(I - \frac{c_{titrant}}{2} \right) \quad (4.1)$$

if the ionic strength be held constant. V refers to the volume of titrant solution prepared, $c_{titrant}$ is the concentration of acid or base in the titrant solution.

The concentration of H^+ and OH^- of the titrant solutions was determined by first titrating the alkaline titrant against a standard solution containing a known amount of potassium hydrogen phthalate (done at least 3 times) and then titrating the acidic titrant against a solution containing a known amount of alkaline titrant (done at least 2 times). A solution of 0.15 % phenolphthalein in ethanol was used as indicator.

4.4 Titration Procedure

4.4.1 Calibration Titration

A solution of KCl with the desired ionic strength was prepared in a 250-ml volumetric flask. The reaction vessel was first rinsed with distilled water and then with NANOpure water. It was connected to the heater-mixer unit and a steady temperature was provided before beginning with the actual experiment. The vessel was sealed with the rubber ring and the lid, and then tightened. A stirring bar (length 1 cm) was cleaned and put into the vessel. Using a transfer pipet, 50 ml of the KCl solution were then loaded into the vessel; stirring was applied and the solution was degassed with argon until the voltage reading changed no more than 1 mV per minute before beginning the titration.

Each calibration titration proceeded from low to high p^0H , titrating with KOH of known concentration. This method was observed to yield greatest accuracy in the electrode response. 2 ml of the acid titrant were injected first, acidifying the solution to $p^0H \approx 2.5$. Then the titration was conducted with alkaline titrant, using an aliquot volume of 0.1 ml and a total amount of 4 ml alkaline titrant added. This corresponded to an upper p^0H of ≈ 11.4 . With a rate of 6000 readings per p^0H point, equilibrium for each aliquot was usually established after 5 to 7 p^0H points.

4.4.2 Protein Titration

25 ml of the protein solution obtained from dialysis were loaded into the titration vessel, using a transfer pipet. Again, a steady temperature was established using the heater/mixer unit; the solution was degassed and the cell covered with argon before the titration was started.

An acidic titration down to $p^0H 2.5$ was conducted, followed by an alkaline titration of the resulting solution up to $p^0H 11.5$. Titrations beyond these pH limits were not conducted since the titration curves of lysozyme exhibit asymptotic behavior at extremes of p^0H , requiring long experiment times and large solution volumes to obtain only a little more information. Titrations were conducted using titrant aliquots of 0.1 ml. For most points of the titration, pH equilibrium, defined as $\Delta E_{\max} < 0.1$ mV, was achieved within 60 seconds after adding each aliquot.

5 Experimental Results and Discussion

5.1 Stability of Voltage Signals

In the beginning of the titration experiments it was a severe problem to obtain a stable voltage signal without noise. A moderate stirring speed (just enough to produce a small vortex), temperature control within $\pm 0.1^\circ\text{C}$ and degassing of the solutions all contributed to a stable signal. At higher ionic strengths the signal was more stable.

5.2 Calibration Titrations

Results for S were always a little lower than the ideal value for 298.15 K, 59.16 mV but never lower than 58.66 mV giving a deviation of $< 1\%$. It seems that extended usage of the electrode constantly diminishes the value of S . A cleaning procedure for the electrode should be tried in future experiments to find out whether this is caused by protein buildup on the electrode or simply by extended usage.

Values for E_0 were in the range of 377–395 mV. A decrease with repeated electrode usage was also observed here and the correspondence between ionic strength and the difference in E_0^* is recognizable. The sum of the squared residuals in the calibration calculation $\sum_i (E_{\text{calc}} - E_{\text{meas}})_i^2$ was always typically around 25 (mV)². Since 40 data points were obtained in each calibration, this is a mean absolute deviation of 0.8 mV for each data point, so that the relative deviation is clearly less than 1%, since mV values for E are in the magnitude of $O(10^2)$. The "worst" calibration yielded a SSR of 80 (mV)², i.e. 1.4 mV mean absolute deviation per data point.

5.3 Titrations of Hen-Egg-White Lysozyme

Solutions of hen-egg-white lysozyme of ionic strengths 0.1 M, 0.15 M, 0.2 M, 0.5 M, 1.0 M and 2.0 M were titrated with HCl and KOH titrant solutions over a p^0H range of 2.5 to 11.5, corresponding to a pH range of approximately 2.6 to 11.6. Solutions were titrated from a $p^0H \approx 7$ to the acidic end first and then back over the full p^0H range. In some experiments, a separate titration from $p^0H \approx 7$ to the alkaline end was also conducted. At each ionic strength, at least 3 titrations were conducted to make sure that reproducible results were obtained.

5.3.1 Net Charge Curves (Titration Curves)

Figure 7: Example for a titration curve

Figure 5.3.1 shows a titration curve from a lysozyme titration conducted at 0.1 M. pH values are obtained from electrode potential measurements and net charge is calculated as described in section 3.

As expected from the pK_a 's of the functional groups (side chain carboxyl and amino groups), the biggest changes in net charge occur in the regions below pH 4 and above pH 10. The number of titrated groups over the full range is 17. This equals the results given in [29]. Unlike the absolute net charge, the total number of titrated groups does not depend on the charge reference point, but equation (3.18) indicates that it depends on the correct determination of protein concentration and the value for the molecular weight of the protein. For ordinate positioning of the titration curve, a ref-

erence point must be known or assumed.

A common method in colloid titrations is to position the titration curves from different ionic strengths relative to each other and then to obtain the point of zero charge (p.z.c.)² as the intersection point of the curves [11], [14]. However, this is not possible for our data. Since protein solutions in this work were obtained from ultrafiltration and dialysis, the starting pH's of the solutions could not be positioned relative to each other. Therefore, the assumption [8] that the p.z.c. equals the isoionic point, $pI_i = 11.35$ was used.

Investigation of Hysteresis Effects

To determine whether the titration curves represent thermodynamic equilibrium, a few titrations were checked for reversibility with respect to pH in both the acidic and alkaline region. No hysteresis was observed in titrations to p^0H 2.5 and back. However, for titrations in which the lower p^0H limit was extended to 2.0, hysteresis was observed upon titration with alkaline titrant, indicating irreversible changes to the protein (e.g. denaturation, partial hydrolysis). For this reason, the titration region was limited to 2.5-11.5.

Reproducibility of Titration Curves

At each ionic strength up to 0.5 M, between 3 and 5 repetitions of the titration were performed (i.e. different solutions used). For each ionic strength, there were at least 3 titration curves which matched each other very well, i.e. calculated net charge differed by less than $\pm 2.5\%$ from that calculated from the other two curves at the same p^0H . At each ionic strength, the titration curve that was closest to the arithmetic mean of the three curves was later used for the ion-binding calculation. Figure 5.3.1 shows as an example the titration curves from three titrations for 0.15 M. For 1.0 M and 2.0 M, only two titrations were conducted that showed the same reproducibility ($< 2\%$ relative deviation).

²For the definitions of p.z.c., isoionic and isoelectric point see Appendix A

Figure 8: Titration curves for 0.15 M

Effect of Protein Concentration

The target value of the initial protein concentration in all titrations was 10 g/l ($\approx 1\%$ protein by weight). It was possible to achieve this concentration with fair accuracy with ultrafiltration. However, subsequent volume changes during equilibrium dialysis could not be controlled. The result was that the initial protein concentration varied between 7 and 12 g/l. However, the net charge *per molecule* should not be affected by the concentration of protein at such dilute concentrations. In fact, no effect of protein concentration on z could be observed.

Effect of Ionic Strength

Figure 9 shows the group of titration curves all referenced to a p.z.c. of 11.35, i.e. the isoionic point for lysozyme [8]. Net charge increases with decreasing pH as well as with increasing ionic strength. Above the p.z.c., the net charge decreases with increasing ionic strength; sometimes this is referred to as "titration curves rotating around the p.z.c.".

Figure 9: Titration curves for several ionic strengths

Figure 10: Titration curves at low and high pH

Separate plots for two "extreme" pH regions (Figure 10) show that the net charge decreases almost linearly with increasing pH between 2.5 and 3.5 and that the slope is the same for all ionic strengths. Between 3.5 and 4.5, the titration curves begin to approach each other and at 4.5 they start to intersect. Above the p.z.c. 11.35, increasing ionic strength means *decreasing* net charge. Both effects might be explained by the increased screening of electrostatic repulsion between charged groups of same sign on the protein surface with increasing ionic strength. With increasing ionic strength the presence of several charged groups of the same sign becomes less unfavorable and therefore the absolute net charge increases.

Figure 11: Titration curves for 0.1 M, 1.0 M and 2.0 M

Figure 11 shows a titration curve for $I = 2.0$ M in comparison to 0.1 M and 1.0 M. The net charge is larger than for 1.0 M for pH below 5 following the trend for 0.1-1.0 M but since there were only 2 titrations conducted at 2.0 M and they differed considerably, the results were not included in the site-binding calculations.

5.3.2 Calculation of Binding Numbers

For the calculation of binding numbers, the method given in [8] is used with the difference that consistent titration data for 5 ionic strengths in the range of 0.1 M to 1.0 M are available whilst only data for 2 ionic strengths were used in [7].

ESIN-MARKOV coefficients for pairs of titration curves were calculated at ionic strengths I_2 and I_1 , using the approximative equation

$$\delta_{a/b} \approx \left(\frac{r_{a/b}(I_2) - r_{a/b}(I_1)}{\log_{10}(a_s(I_2)) - \log_{10}(a_s(I_1))} \right)_{\text{pH}} \quad (5.1)$$

In this approximation, $\delta_{a/b}$ must be assumed to be constant in the observed range of ionic strength. Using equation (2.20), binding isotherms for Cl^- were calculated. If data for the ionic strength between the two titration curves were not available, a mean titration curve was calculated by arithmetic averaging.

Figure 12: Binding number for Cl^-

Figure 12 shows the calculated binding numbers for Cl^- as a function of pH with ionic strength as a parameter. The co-binding of Cl^- increases with decreasing pH, but there is no trend for an increase or decrease of Cl^- binding with ionic strength.

From the condition, that the overall charge of {protein + bound anions and cations} has to equal zero, the binding number for K^+ is calculated simply by

$$r_{K^+} = r_{Cl^-} - z. \quad (5.2)$$

Figure 13: Binding number for K^+

Figure 13 shows the result for the K^+ binding number, which is negative and could therefore also be called an *expulsion number*. The expulsion of K^+ increases with decreasing pH but the effect of ionic strength does not follow any discernible trend.

6 Conclusion

In this work, potentiometric titrations of hen-egg-white lysozyme were conducted at 25°C over the p⁰H range 2.5-11.5 for ionic strengths from 0.1 M to 2.0 M to obtain information about this protein's net charge dependence on pH and ionic strength and to calculate salt-binding information from a linkage concept.

Equipment and procedure for these titrations were modified in a number of ways to obtain results of substantially greater accuracy and reproducibility than previously possible. The titration method now developed should be apt for the conduction of titration experiments with solutions of different proteins and salts.

For the p⁰H range of 2.5-11.5, it was shown that the net charge slightly but distinctly increases with increasing ionic strength for ionic strengths between 0.1 M and 1.0 M.

Preliminary binding-number calculations showed that chloride anions are selectively associated with the protein at low pH and potassium ions are consequently expelled from the region surrounding the protein. As pH increases towards the point of zero charge, selective ion binding decreases. No clear trend in ion binding with ionic strength was observed using the method of Fraaije and Lyklema [7]. Further, theoretical work is in progress to address the experimental observations reported here.

If binding numbers are calculated from titration curves at 0.1 M and 1.0 M, maximum binding numbers of ≈ 10 for Cl⁻ and ≈ 11 for K⁺ are obtained for pH ≈ 2.5 . The success of these calculations depends strongly on the change of net charge with changing ionic strengths and on the method of referencing the titration curves. If possible, curves should be referenced to each other to find the point of zero charge.

7 Nomenclature

Latin Symbols

a_i	activity of species i
c_i	molar concentration of species i
m_i	molality of species i
r_i	binding number of species i
s	normalized excess extensive entropy
v	normalized excess extensive volume
z	net charge
x_i	mole fraction of species i
E	electrode potential
E_0	electrode standard potential
E_0^*	effective electrode reference potential
F	FARADAY's constant
I	ionic strength
K_W	water equilibrium constant ($10^{-13.998}$ at 298.15 K)
K_W^0	"effective" water equilibrium constant
M	molecular weight
$\mathcal{M}$	mass
M	mol/l
N_i	mole number of species i
P	system pressure
R	universal gas constant
S	system entropy
S	effective electrode slope
T	absolute temperature
V	volume
V_i	partial molar volume of species i
X	any extensive property
$\Delta X^{(w)}$	excess extensive property

Greek Symbols

β	ESIN-MARKOV coefficient, defined by eq. (2.17)
γ_i	activity coefficient of species i
$\delta_{a/b}$	ESIN-MARKOV coefficient, defined by eq. (2.18)
μ_i	chemical potential of species i
π	osmotic pressure
ρ	molar density
Λ^r	reference-linkage potential
Φ_V	apparent molar volume

Superscripts

0	referring to concentrations
add	added
cons	consumed
init	at titration startpoint
L	left chamber of reference system
R	right chamber of reference system
$^{(w)}$	excess extensive property with respect to water

Subscripts

$_{a/b}$	acid/base
$_i$	any component
$_p$	protein
$_s$	salt
$_w$	water

A Isoionic Point, Isoelectric Point, P.Z.C.

- The *isoionic point* pI_i of a protein is defined as the pH of a solution of protein in pure water without any salt where the charge of the protein molecule is only balanced by H^+ and OH^- as counterions. It can be determined experimentally by deionization of a protein-salt solution [28].
- The *isoelectric point* pI_e is defined as the pH where a protein in an aqueous saline solution has zero total net charge (i.e. with the ions bound in the STERN layer and without those in the diffuse layer); it can be determined at low ionic strength by isoelectric focusing [1] [10] [14].
- The *point of zero charge* (p.z.c.) is the pH, where the net charge of the protein molecule is zero (without any surface-bound salt ions) [14].

The three points can be very close to each other or far apart, depending on the individual properties of the protein.

B Properties of Electrolyte Solutions

B.1 Concentration Variables

Several concentration variables are in use to describe the composition of an electrolyte solution and it depends on the purpose which one is most comfortable to use. The *mole fraction* x is defined as the mole number of a species in the system divided by the total number of moles

$$x_i \equiv \frac{N_i}{\sum_i N_i} \quad (\text{B.1})$$

The *molarity* c of an electrolyte solution is defined as the number of moles of electrolyte divided by the volume of the solution

$$c_i \equiv \frac{N_i}{V} \quad (\text{B.2})$$

The *molality* m is defined as the number of moles of electrolyte divided by the mass of the solvent

$$m_i \equiv \frac{N_i}{\mathcal{M}_1} \quad (\text{B.3})$$

If molarity is the parameter, it can be necessary to calculate the corresponding molality, e.g. for the calculation of activity coefficients. To do so, information about the volume dependence of the solution on its composition is required. Strictly, the relation between c_2 and m_2 in a solution containing one salt (component 2) is

$$m_2 = \frac{c_2 \frac{V_1}{M_1}}{1 - c_2 V_2} \quad (\text{B.4})$$

where V_i denote partial molar volumes. Using the concept of an *apparent molar volume* Φ_V which is given for an aqueous KCl solution by the empirical relation [13]

$$\Phi_V = 26.81 + 1.86\sqrt{c_2} + 0.2c_2 \quad (\text{B.5})$$

where c_2 has the units mol/l and Φ_V has the units l/mol, the molality can be calculated by

$$m_2 = \frac{c_2}{\rho_1(1 - \Phi_V c_2)} \quad (\text{B.6})$$

B.2 Activity Coefficients

Activity coefficients in electrolyte solutions are usually defined on a *molal* basis because it is not dependent on the volume of the solution. We calculate activity coefficients with the PITZER model as described in [30]. The full equations given for single-ion activity coefficients given are

$$\begin{aligned} \ln \gamma_C = & z_+^2 f^\gamma + \sum_a m_a \{ 2B_{Ca} + (2 \sum_c m_c z_c) C_{Ca} \} + \sum_c m_c (2\theta_{Cc} + \sum_a m_a \psi_{Cca}) \\ & + \sum_c \sum_a m_c m_a (z_+^2 B'_{ca} + |z_+| C_{ca}) + \frac{1}{2} \sum_a \sum_{a'} m_a m_{a'} \psi_{Caa'} \end{aligned} \quad (\text{B.6})$$

$$\begin{aligned} \ln \gamma_A = & z_-^2 f^\gamma + \sum_c m_c \{ 2B_{cA} + (2 \sum_a m_a z_a) C_{cA} \} + \sum_a m_a (2\theta_{Aa} + \sum_c m_c \psi_{Aac}) \\ & + \sum_c \sum_a m_c m_a (z_-^2 B'_{ca} + |z_-| C_{ca}) + \frac{1}{2} \sum_c \sum_{c'} m_c m_{c'} \psi_{cc'A} \end{aligned} \quad (\text{B.7})$$

Mixing parameters for a solution of four mixed ions (K^+ , Cl^- , H^+ and OH^-) are not available. Since concentrations of H^+ and OH^- are very small compared to the concentration of KCl , the molalities m_{H^+} and m_{OH^-} are neglected, so several terms in the original equations vanish including the ones containing the mixing parameters. Further, m_{K^+} and m_{Cl^-} are set equal to the ionic strength. This is not exactly true, but the differences are negligible. The working equations are

$$\ln \gamma_{\text{K}^+} = f^\gamma + 2I(B_{\text{KCl}} + C_{\text{KCl}}I) + I^2(B'_{\text{KCl}} + C_{\text{KCl}}) \quad (\text{B.8})$$

$$\ln \gamma_{\text{Cl}^-} = f^\gamma + 2I(B_{\text{KCl}} + C_{\text{KCl}}I) + I^2(B'_{\text{KCl}} + C_{\text{KCl}}) \quad (\text{B.9})$$

$$\ln \gamma_{\text{H}^+} = f^\gamma + 2I(B_{\text{HCl}} + C_{\text{HCl}}I) + I^2(B'_{\text{KCl}} + C_{\text{KCl}}) \quad (\text{B.10})$$

$$\ln \gamma_{\text{OH}^-} = f^\gamma + 2I(B_{\text{KOH}} + C_{\text{KOH}}I) + I^2(B'_{\text{KCl}} + C_{\text{KCl}}) \quad (\text{B.11})$$

The parameters B and B' and f^γ are calculated by

$$B = \beta_0 + \frac{2\beta_1}{\alpha_1^2 I} \left[1 - (1 + \alpha_1 \sqrt{I}) e^{-\alpha_1 \sqrt{I}} \right] \quad (\text{B.12})$$

$$B' = \frac{2\beta_1}{(\alpha_1 I)^2} \left[-1 + \left(1 + \alpha_1 \sqrt{I} + \frac{\alpha_1^2}{2I} \right) e^{-\alpha_1 \sqrt{I}} \right] \quad (\text{B.13})$$

$$f^\gamma = -A_\phi \left[\frac{\sqrt{I}}{1 + 1.2\sqrt{I}} + \frac{2}{1.2} \ln(1 + 1.2\sqrt{I}) \right]. \quad (\text{B.14})$$

The parameters β_0 , β_1 , C , α_1 and A_ϕ are taken from [30]:

$A_\phi = 0.39228$			
	KCl	KOH	HCl
β_0	0.04835	0.1298	0.1775
β_1	0.2122	0.320	0.2945
C	-0.00084	0.0041	0.0008
α_1	2	2	2

If the activity coefficient on a molal basis is known, the activity coefficient on a *molar* basis can be calculated by

$$\gamma_2^{\text{molar}} = \frac{m_2}{\rho \cdot c_2} \gamma_2^{\text{molal}} \quad (\text{B.15})$$

where ρ is the molar density of the solution and can be calculated with the apparent molar volume method described above.

C Experimental Procedures

The following pages provide the experimental procedures for sample preparation and titrations in detail and can be used as a guideline for future experiments.

Preparation of Titrant Solutions

- Clean and rinse titrant reservoir bottles and syringes.
- Weigh the amount of KCl necessary to maintain a constant ionic strength, according to equation (4.1).
- Prepare 0.1 N titrant solution by dissolving the KCl in 1 N HCl (KOH, respectively) stock solution (1/10 of the final titrant volume to obtain a 0.1 N solution) in a volumetric flask. Top off the flask with NANOpure water and mix thoroughly.
- Rinse the reservoir bottle and the syringe with titrant solution.
- Degas the titrant solution, seal reservoir bottle.
- Dispense the volume of a syringe (≈ 22 ml) several times to flush air and remaining old titrant solution from the lines.

Titration Procedures

For all titration procedures one should make sure that a constant temperature is established. Therefore at the beginning of every series of experiments (standardization, calibration, titration), the vessel should be connected to the temperature bath and the heater/mixer/pump unit should be started and kept going. It is recommended to interrupt the water circulation only for cleaning of the vessel.

Further, before starting any titration, the injection lines should be flushed with titrant to flush out air bubbles and rinsed with NANOpure water to clean off titrant from the surface.

Alkaline Titrant Standardization

- Prepare 250 ml of a solution of potassium hydrogen phthalate (KHP) and KCl at the ionic strength where the titrations will be conducted. Record the mass of KHP (M_{KHP}). The mass of KHP to bring up the solution to the desired ionic strength is

$$M_{\text{KCl}} = \left(0.251 \cdot I - \frac{M_{\text{KHP}}}{M_{\text{KHP}}} \right) \quad (\text{C.1})$$

- Transfer 50 ml of this solution to the reaction vessel and add 6 drops of a 0.15% phenolphthalein solution in ethanol. Apply stirring at high speed.
- Titrate with the KOH titrant using dosimat modes 1 and 2 until the equivalence point (defined as the point where the pink color does not disappear after 1 minute) is reached and record the volume of titrant added, V_{base} .
- The concentration of the alkaline titrant is then calculated by

$$c_{\text{base}} = \frac{M_{\text{KHP}}}{M_{\text{KHP}} V_{\text{base}}} \quad (\text{C.2})$$

where $M_{\text{KHP}} = 204.22 \text{ g/mol}$.

- Repeat the procedure at least two times.

Acidic Titrant Standardization

- Prepare 250 ml of a solution of KCl at the ionic strength where the titrations will be conducted.
- Transfer 50 ml of this solution to the reaction vessel and add a few drops of a 0.15% phenolphthalein solution in ethanol. Apply stirring at high speed.
- Add $V_{\text{acid}} = 3 \text{ ml}$ of the acidic titrant using dosimat mode 3.
- Titrate with the alkaline titrant using dosimat modes 1 and 2 until the equivalence point (defined as the point where the pink color does not disappear after 1 minute) is reached and record the volume of titrant added, V_{base} .
- The concentration of the acidic titrant is then calculated by

$$c_{\text{acid}} = \frac{c_{\text{base}} \cdot V_{\text{base}}}{V_{\text{acid}}} \quad (\text{C.3})$$

- Repeat the procedure at least two times.

Calibration Titration

- Transfer 50 ml of blank solution to the reaction vessel. Switch on magnetic stirrer at medium speed.
- Seal the vessel and degas the solution for several minutes until the potential does not change more than 1 mV per minute.

- Activate blanketing with the water-saturated argon.
- Add 2 ml of acidic titrant to the solution using dosimat mode 3.
- Change titrant reservoirs.
- Program a volume of 0.1 ml using dosimat mode 7.
- Start the CAL-TITR program for the titration with alkaline titrant.
- Place pH meter on 'standby', remove electrode from solution, rinse it and place it in the storage buffer.

Protein Titration

- Transfer 25 ml of protein solution to the reaction vessel. Switch on magnetic stirrer.
- Seal the vessel and degas the solution for 5 minutes. Be careful not to cause too much foaming.
- Activate blanketing with the water-saturated argon.
- Program a volume of 0.1 ml using dosimat mode 7.
- Start the TITRATE program for the titration with acidic titrant.
- After reaching the low pH endpoint, change titrant reservoirs and start TITRATE for a titration with alkaline titrant.

D Titration Data

In the following, the results of all conducted titrations are given, including the results of the calibration. A hypothetical net charge is calculated for the blank titration and included in the net charge plots to show the quality of the calibration.

Titration at 0.1 M (11/12/96)

c_{KOH} [mol/L]	: 0.1004	c_{HCl} [mol/L]	: 0.0998
E_0^* [mV]	: 379.25	S [mV]	: 58.88
I [kg ² mol ⁻²]	: 0.6245	$\sum_i (\Delta E)^2$ [mV ²]	: 25.5

V_{init} [ml]	: 20	c_p [g/l]	: 9.98
$V_{\text{acid}}^{\text{total}}$ [ml]	: 1.8	p^0H	: 7.18 ... 2.47
$V_{\text{base}}^{\text{total}}$ [ml]	: 4.4	p^0H	: 2.47 ... 11.52

Titration at 0.1 M (11/09/96)

c_{KOH} [mol/L]	: 0.1003	c_{HCl} [mol/L]	: 0.0998
E_0^* [mV]	: 379.36	S [mV]	: 58.92
I [kg ² mol ⁻²]	: 0.6245	$\sum_i (\Delta E)^2$ [mV ²]	: 30.9

V_{init} [ml]	: 25	c_p [g/l]	: 6.96
$V_{\text{acid}}^{\text{total}}$ [ml]	: 1.8	p^0H	: 6.68 ... 2.47
$V_{\text{base}}^{\text{total}}$ [ml]	: 4.6	p^0H	: 2.47 ... 11.50

Titration at 0.1 M (11/05/96)

c_{KOH}	[mol/L]	: 0.1003	c_{HCl}	[mol/L]	: 0.0998
E_0^*	[mV]	: 380.81	S	[mV]	: 58.89
I	[kg ² mol ⁻²]	: 0.6245	$\sum_i (\Delta E)^2$	[mV ²]	: 17.6

V_{init}	[ml]	: 25	c_p	[g/l]	: 9.76
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.2	p^0H		: 6.96 ... 2.47
$V_{\text{base}}^{\text{total}}$	[ml]	: 5.4	p^0H		: 2.47 ... 11.50

Titration at 0.15 M (11/21/96)

c_{KOH}	[mol/L]	: 0.1022	c_{HCl}	[mol/L]	: 0.1006
E_0^*	[mV]	: 377.11	S	[mV]	: 58.66
I	[kg ² mol ⁻²]	: 0.5924	$\sum_i (\Delta E)^2$	[mV ²]	: 19.6

V_{init}	[ml]	: 25	c_p	[g/l]	: 8.70
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.0	p^0H		: 6.64 ... 2.49
$V_{\text{base}}^{\text{total}}$	[ml]	: 5.1	p^0H		: 2.49 ... 11.51

Titration at 0.15 M (11/20/96)

c_{KOH}	[mol/L]	: 0.1022	c_{HCl}	[mol/L]	: 0.1006
E_0^*	[mV]	: 377.55	S	[mV]	: 58.71
I	[kg ² mol ⁻²]	: 0.5924	$\sum_i (\Delta E)^2$	[mV ²]	: 18.4

V_{init}	[ml]	: 25	c_p	[g/l]	: 8.59
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.0	p^0H	:	: 7.37 ... 2.49
$V_{\text{base}}^{\text{total}}$	[ml]	: 5.0	p^0H	:	: 2.49 ... 11.50

Titration at 0.15 M (11/19/96)

c_{KOH}	[mol/L]	: 0.1022	c_{HCl}	[mol/L]	: 0.1006
E_0^*	[mV]	: 377.74	S	[mV]	: 58.78
T	[kg ² mol ⁻²]	: 0.5924	$\sum_i(\Delta E)^2$	[mV ²]	: 18.9

V_{init}	[ml]	: 20	c_p	[g/l]	: 9.62
$V_{\text{acid}}^{\text{total}}$	[ml]	: 1.7	p^0H	:	6.82 ... 2.50
$V_{\text{base}}^{\text{total}}$	[ml]	: 4.3	p^0H	:	2.50 ... 11.51

Titration at 0.2 M (11/15/96)

c_{KOH} [mol/L]	: 0.1006	c_{HCl} [mol/L]	: 0.1007
E_0^* [mV]	: 378.82	S [mV]	: 58.91
I [kg ² mol ⁻²]	: 0.5733	$\sum_i (\Delta E)^2$ [mV ²]	: 21.9

V_{init} [ml]	: 25	c_p [g/l]	: 7.48
$V_{\text{acid}}^{\text{total}}$ [ml]	: 1.9	p^0H	: 6.70 ... 2.46
$V_{\text{base}}^{\text{total}}$ [ml]	: 5.0	p^0H	: 2.46 ... 11.52

Titration at 0.2 M (11/14/96)

c_{KOH}	[mol/L]	: 0.1006	c_{HCl}	[mol/L]	: 0.1007
E_0^*	[mV]	: 379.02	S	[mV]	: 58.89
I	[kg ² mol ⁻²]	: 0.5733	$\sum_i (\Delta E)^2$	[mV ²]	: 21.2

V_{init}	[ml]	: 25	c_p	[g/l]	: 9.05
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.1	p^0H		: 6.94 ... 2.50
$V_{\text{base}}^{\text{total}}$	[ml]	: 5.4	p^0H		: 2.50 ... 11.50

Titration at 0.2 M (11/13/96)

c_{KOH} [mol/L]	: 0.1006	c_{HCl} [mol/L]	: 0.1007
E_0^* [mV]	: 378.51	S [mV]	: 58.84
I [kg ² mol ⁻²]	: 0.5733	$\sum_i (\Delta E)^2$ [mV ²]	: 20.9

V_{init} [ml]	: 25	c_p [g/l]	: 8.59
$V_{\text{total acid}}$ [ml]	: 2.1	p^0H	: 7.31 ... 2.49
$V_{\text{total base}}$ [ml]	: 5.3	p^0H	: 2.49 ... 11.50

Titration at 0.2 M (09/13/96)

c_{KOH}	[mol/L]	:	0.0995	c_{HCl}	[mol/L]	:	0.0996
E_0^*	[mV]	:	390.34	S	[mV]	:	59.01
I	[kg ² mol ⁻²]	:	0.5733	$\sum_i (\Delta E)^2$	[mV ²]	:	11.0

V_{init}	[ml]	:	20	c_p	[g/l]	:	10.38
$V_{\text{acid}}^{\text{total}}$	[ml]	:	1.9	$p^0\text{H}$		:	7.13 ... 2.47
$V_{\text{base}}^{\text{total}}$	[ml]	:	4.5	$p^0\text{H}$		:	2.47 . . 11.47

Titration at 0.2 M (09/11/96)

c_{KOH}	[mol/L]	: 0.0995	c_{HCl}	[mol/L]	: 0.0996
E_0^*	[mV]	: 397.83	S	[mV]	: 59.19
I	[kg ² mol ⁻²]	: 0.5733	$\sum_i (\Delta E)^2$	[mV ²]	: 21.9

V_{init}	[ml]	: 20	c_p	[g/l]	: 7.59
$V_{\text{acid}}^{\text{total}}$	[ml]	: 1.6	$p^0\text{H}$		: 7.26 ... 2.49
$V_{\text{base}}^{\text{total}}$	[ml]	: 3.7	$p^0\text{H}$		: 2.49 ... 11.48

Titration at 0.5 M (11/16/96)

c_{KOH}	[mol/L]	: 0.1000	c_{HCl}	[mol/L]	: 0.1007
E_0^*	[mV]	: 378.68	S	[mV]	: 58.82
I	[kg ² mol ⁻²]	: 0.5491	$\sum_i (\Delta E)^2$	[mV ²]	: 28.1

V_{init}	[ml]	: 25	c_p	[g/l]	: 9.01
$V_{\text{total acid}}$	[ml]	: 2.3	p^0H		: 7.36 ... 2.48
$V_{\text{total base}}$	[ml]	: 5.7	p^0H		: 2.48 ... 11.50

Titration at 0.5 M (09/24/96)

c_{KOH}	[mol/L]	: 0.1000	c_{HCl}	[mol/L]	: 0.1005
E_0^*	[mV]	: 389.41	S	[mV]	: 59.00
T	[kg ² mol ⁻²]	: 0.5491	$\sum_i (\Delta E)^2$	[mV ²]	: 25.8

V_{init}	[ml]	: 25	c_p	[g/l]	: 9.50
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.4	p^0H		: 7.70 ... 2.48
$V_{\text{base}}^{\text{total}}$	[ml]	: 13.2	p^0H		: 2.48 ... 12.01

Titration at 0.5 M (09/18/96)

c_{KOH} [mol/L]	: 0.1006	c_{HCl} [mol/L]	: 0.1012
E_0^* [mV]	: 389.37	S [mV]	: 59.02
I [kg ² mol ⁻²]	: 0.5491	$\sum_i (\Delta E)^2$ [mV ²]	: 12.7

V_{init} [ml]	: 20	c_p [g/l]	: 11.00
$V_{\text{acid}}^{\text{total}}$ [ml]	: 2.1	p^0H	: 7.67 ... 2.49
$V_{\text{base}}^{\text{total}}$ [ml]	: 5.1	p^0H	: 2.49 ... 11.51

Titration at 1.0 M (10/01/96)

c_{KOH} [mol/L]	: 0.0993	c_{HCl} [mol/L]	: 0.0994
E_0^* [mV]	: 390.21	S [mV]	: 58.94
T [kg ² mol ⁻²]	: 0.5826	$\sum_i (\Delta E)^2$ [mV ²]	: 37.5

V_{init} [ml]	: 25	c_p [g/l]	: 9.21
$V_{\text{acid}}^{\text{total}}$ [ml]	: 1.9	$p^0\text{H}$	: 7.20 ... 2.49
$V_{\text{base}}^{\text{total}}$ [ml]	: 4.5	$p^0\text{H}$	: 2.49 ... 11.50

Titration at 1.0 M (09/27/96)

c_{KOH}	[mol/L]	: 0.0993	c_{HCl}	[mol/L]	: 0.0994
E_0^*	[mV]	: 391.65	S	[mV]	: 59.11
I	[kg ² mol ⁻²]	: 0.5826	$\sum_i (\Delta E)^2$	[mV ²]	: 31.7

V_{init}	[ml]	: 20	c_p	[g/l]	: 9.91
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.0	p^0H	:	: 7.42 ... 2.47
$V_{\text{base}}^{\text{total}}$	[ml]	: 4.8	p^0H	:	: 2.47 ... 11.52

Titration at 2.0 M (10/11/96)

c_{KOH} [mol/L]	: 0.1001	c_{HCl} [mol/L]	: 0.0995
E_0^* [mV]	: 395.08	S [mV]	: 59.05
I [kg ² mol ⁻²]	: 0.5826	$\sum_i (\Delta E)^2$ [mV ²]	: 80.8

V_{init} [ml]	: 20	c_p [g/l]	: 6.26
$V_{\text{acid}}^{\text{total}}$ [ml]	: 1.6	$p^0 H$	: 6.85 ... 2.46
$V_{\text{base}}^{\text{total}}$ [ml]	: 3.4	$p^0 H$	: 2.46 ... 11.54

Titration at 2.0 M (10/07/96)

c_{KOH}	[mol/L]	: 0.1001	c_{HCl}	[mol/L]	: 0.0996
E_0^*	[mV]	: 394.22	S	[mV]	: 59.13
I	[kg ² mol ⁻²]	: 0.5826	$\sum_i (\Delta E)^2$	[mV ²]	: 31.7

V_{init}	[ml]	: 25	c_p	[g/l]	: 12.13
$V_{\text{acid}}^{\text{total}}$	[ml]	: 2.6	p^0H		: 6.63 ... 2.55
$V_{\text{base}}^{\text{total}}$	[ml]	: 5.9	p^0H		: 2.55 ... 11.51

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