pH titration of native and unfolded ß-trypsin: evaluation of the ΔΔG⁰ titration and the carboxyl pK values

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Abstract

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The stabilizing free energy of ß-trypsin was determined by hydrogen ion titration. In the pH range from 3.0 to 7.0, the change in free energy difference for the stabilization of the native protein relative to the unfolded one ($\Delta\Delta G^0$ titration) was 9.51 ± 0.06 kcal/mol. An isoelectric point of 10.0 was determined, allowing us to calculate the Tanford and Kirkwood electrostatic factor w. This factor presented a nonlinear behavior and indicated more than one type of titratable carboxyl groups in ß-trypsin. In fact, one class of carboxyl group with a $pK = 3.91 \pm 0.01$ and another one with a $pK = 4.63 \pm 0.03$ were also found by hydrogen ion titration of the protein in the folded state.

Introduction

It is important to obtain thermodynamic data and parameters with high accuracy and precision when determining the free energy of the protein folding-unfolding process. The contribution of whole noncovalent free energy to protein stabilization can be accurately measured by using pH titration (1) and solvent denaturation data (2) for the folded and unfolded proteins.

Bovine ß-trypsin has been used in our laboratory as a model protein for kinetic and thermodynamic studies because of its well-known close-to-spherical tertiary structure determined by X-ray diffraction (3,4), and relatively easy purification procedures (5-7). Although a good amount of kinetic data is available in the literature for this typical serine protease, little is known about its thermodynamics in solution. Thus, we are developing a major effort in order to collect data and to probe the thermodynamic stability of this enzyme. The determination of the factor w and the net charge as a function of pH requires the accurate determination of the isoelectric point (pI) of this enzyme by different techniques. On the other hand, some complementary quantities can only be determined by invoking certain procedures which lie outside the scope of thermodynamics. One example is the classic theory of Debye-Hückel (8), which has been extended to hydrogen ion titrations of folded proteins treated as impenetrable spherical ions (9-11).

The aim of the present study was to col-

lect potentiometric data in the pH range of 3.0 to 7.0 for the native and unfolded forms of ß-trypsin in order to determine the change in free energy of the unfolding process (ΔG^0_U) as a function of pH and the electrostatic factor w associated with the titration of carboxylates probing the adequacy of the impenetrable sphere model for ß-trypsin.

Material and Methods

Proteins and chemicals

Bovine ß-trypsin was purified on SP-Sephadex C-50 (5-7) from a commercial preparation purchased from Sigma (lot 129F0367; St. Louis, MO). Before each experiment, a sufficient amount of about 7.5 mg ß-trypsin was additionally purified by affinity chromatography on Sepharose-1,4-butanediol-diglycidylether-4-amino-benzamidine. The column (4.2 cm high x 1.3 cm in diameter) was equilibrated with a solution of 2.0 mM $CaCl_2$ in NH_4OH, pH 8.5. Lyophilized ß-trypsin was dissolved in 5.0 mM Tris-HCl, pH 8.0, containing 2.0 mM $CaCl_2$ and added to the column. After washing the column with 15.0 ml of equilibrium solution, the protein was eluted with HCl, pH 3.0, containing 2.0 mM $CaCl_2$. The enzymatic activity was followed with benzoyl-L-arginine-4-nitroanilide (L-BAPA, Sigma) as substrate (12). The concentration of ß-trypsin was determined at pH 3.0 by measuring the absorbance at 280 nm and using a molar extinction coefficient of 40,000 mol^{-1} cm^{-1}. The active site concentration was determined by titrating the enzyme with 4-nitrophenyl-4-guanidino benzoate (NPGB) (13). High purity guanidino-hydrochloride (GU-HCl) was obtained by double recrystallization in ethanol-water (14).

Standards and solutions

A freshly prepared CO_2-free Tris-hydroxymethyl-aminomethane primary stan-dard (Tris; Merck, Darmstadt, Germany) was used to standardize the HCl solutions. The equivalence points were determined using Gran plots (15). These standardized HCl solutions contained 140 mM KNO_3 and 20 mM $CaCl_2$ (solution A) or additionally 6.0 M GU-HCl (solution B), and were used as titrants of native and denatured enzyme, respectively.

Potentiometric titrations

The potentiometric measurements (16) were made using a Micronal B-375 precision pH meter with 1/1000 pH unit sensitivity, equipped with a Metrohm-combined Ag/AgCl glass electrode. The titrant was dispensed with a Radiometer ABU 13 autoburette. The titration vessel was flushed with nitrogen and the temperature in the titration vessel was controlled with an ultrathermostated Hetofrig circulation bath at 25.00 ± 0.01^oC. During the experiments this gas was continuously passed through a saturated $Ba(OH)_2$ solution, followed by 2 M phosphoric acid and then solution A or B as indicated. Immediately before starting the titrations, the pH meter was calibrated with standard certified buffers at pH 4.000 ± 0.002 and pH 7.000 ± 0.002.

The pH titrations of native ß-trypsin were carried out as follows: the amounts of protein at a concentration of approximately 5 mg/ml dialyzed at 4^oC with solution A in HCl, pH 3.0, were diluted with the solution used in the dialysis to 5 ml and filtered with Millipore 0.02-μm membranes as preceding steps. It is important to emphasize that no significant loss of activity of folded ß-trypsin at pH 3.0 was observed for five to seven days. Afterwards, the protein titrations and backtitrations of 4-ml samples were interposed between three blank titrations of the solution used to dialyze the protein. The initial pH at the beginning of the titration was adjusted to slightly over 7 by adding a few μl of freshly prepared CO_2-free 0.2 M

KOH. Volumes of 5-15 µl titrant were then consecutively added under continuous stirring and nitrogen gas atmosphere, and the pH was recorded at 15- to 45-s intervals between additions.
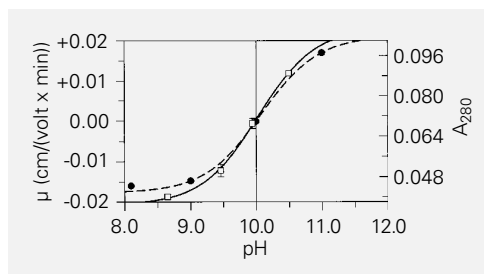
For the titration of the denatured form of the protein, aliquots of 0.5 ml of ß-trypsin in solution A containing HCl, pH 3.0, were diluted to 5 ml with a solution of 6.67 M GU-HCl in HCl, pH 3.0, containing 140 mM KNO_3 and 20 mM $CaCl_2$. The subsequent steps included dialysis with solution B prepared in HCl, pH 3.0, followed by filtration. The titration procedures were identical to those described above.

Isoelectric point (pI) determination

The pI of ß-trypsin was determined by gel binding (17) in an SP-Sephadex suspension equilibrated with 20 mM carbonate buffer in five pH sets from 8.5 to 11.0. A protein stock solution, 0.069 mM in 1.0 mM HCl containing 20 mM $CaCl_2$, was diluted five times. Aliquots of 2 ml of this protein solution were added to an equal volume of gel suspension and gently mixed for 3, 4 or 5 min. After these times each mixture was filtered and the pH recorded. Immediately after the pH measurements, the protein solutions were added 1:1 to 100 mM ß-alanine buffer, pH 3.0, containing 140 mM KNO_3 and 20 mM $CaCl_2$. Absorbance at 280 nm was then measured and the specific activity on L-BAPA (12) was compared to that of the initial dilution of the enzyme.

Calculations

The number of H^+ protons bound per mole of protein (ν) as a function of pH was calculated by subtracting the number of H^+ equivalents consumed in the blank titrations from the number of H^+ equivalents consumed in the protein titrations. The correct subtractions were performed after an 8th degree polynomial fitting and interpolation

Figure 1 - Determination of ß-trypsin isoelectric point. The continuous line represents the best fit to the data (open squares) for the isoelectric point determination by protein binding to SP-Sephadex C-50 gel (A_{280}) *vs* pH. The pH value at which 50% of the protein is bound to the gel corresponds to a pI value of 10.03 ± 0.08. The discontinuous line (filled circles) represents the best fit for the migration (µ) in cm/(volt x min). The migration equal to zero was obtained at pH 10.08 ± 0.09.

at the same pH values for both blank and protein titration curves. All the fittings and calculations were performed using the GraFit version 3.0 program.

Results and Discussion

The additional purification of ß-trypsin by affinity chromatography revealed about 10% inert material in the lyophilized protein obtained from the preceding SP-Sephadex C-50 step. Our ß-trypsin samples were 99% pure by active site titration and by the constant A_{410}/A_{280} ratio (specific activity) observed in the affinity chromatography effluent.

Isoelectric point determination is a simple task for many proteins. Nevertheless, ß-trypsin presents a characteristic autolytic behavior (5) at alkaline pH. The determined pI = 10.0 (Figure 1) is a reliable result since exposure to the alkaline pH ranges did not exceed 6 min and no decrease in specific activity relative to the starting samples was observed after the experiments. The amount of protein bound to the gel was not affected by incubation times from 3 to 5 min. The pI = 10.0 was also found by Cellogel electrophoresis of the major migrating band, in spite of a small extent of autolysis observed.

Figure 2 shows the results of potentiometric titrations. The number of bound protons at pH 7.0 was taken arbitrarily as zero for native and unfolded proteins. The data for native and unfolded ß-trypsin include the best 8th degree polynomial fit for reversible titration and backtitration curves of the protein. The observed differences between titra-

Figure 2 - ß-Trypsin titration curves of ν moles of hydrogen ion bound per mole of protein as a function of pH. The area under the curves delimited by filled squares and open circles corresponds to the titration free energy changes of folded ß-trypsin (ΔG^0_N), and unfolded ß-trypsin (ΔG^0_U), respectively. The shaded area corresponds to the change of noncovalent free energy associated with the native to denatured conformational transition of ß-trypsin expressed as $\Delta\Delta G^0_{U-N} = 9.51 \pm 0.06$ kcal/mol. The superposed curve (filled circles) corresponds to the theoretical curve for the unfolded protein.
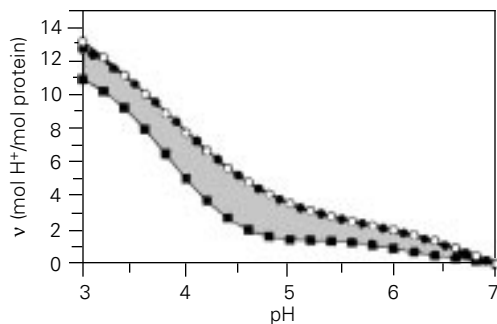
tions and backtitrations for both native and unfolded enzyme were smaller than 1%. Protein autolysis was avoided by shortening the time of exposure to pHs from 7.0 to 8.0 and no decrease in active center was observed after the experiments. The area under the curves in Figure 2 corresponds to the titration free energies calculated by the equation:

$$\Delta G^0 = 2.303\, RT \int \nu\, dpH(\nu) \qquad \text{(Eq. 1)}$$

Equation 1 describes the chemical work (ΔG^0_N or ΔG^0_U; Figure 2) of transferring a number of protons (ν) to a protein molecule as a function of pH.

Since during the unfolding of a protein with GU-HCl there is a breakdown of buried weak bonds and changes of pK values, it is necessary to measure the proton absorption by this major conformational change at a fixed pH value. This amount of absorbed hydrogen ions was determined by first unfolding native protein at pH 3.0 with GU-HCl solution at the same pH, followed by backtitration of the unfolded protein solution to the initial pH value (pH = 3.0), and the obtained $\Delta\nu = 1.11 \pm 0.08$ was used to correct the titration curve position of the unfolded protein relative to the native one.

After doing so, we obtained the corresponding difference between areas for the proper noncovalent free energy calculations expressed as $\Delta\Delta G^0_{U-N} = \Delta G^0_U - \Delta G^0_N$. The ΔG^0_N value found for native ß-trypsin titration was 17.81 ± 0.04 kcal/mol at the pH range from 3.0 to 7.0. Unfolded ß-trypsin

titration showed a $\Delta G^0_U = 27.33 \pm 0.05$ kcal/mol for the same pH range. The value of $\Delta\Delta G^0_{U-N} = 9.51 \pm 0.06$ kcal/mol reveals the total change in noncovalent free energy entrapped by the native protein stabilization. Theoretical titration curves (18) for unfolded proteins are described by equation 2:

$$\nu_{(pH)} = \Sigma_i n_i \{ [(10^{-(pK_i-pH)})/(1+10^{-(pK_i-pH)})] - [(10^{-(pK_i-7)})/(1+10^{-(pK_i-7)})] \} \qquad \text{(Eq. 2)}$$

where n_i and pK_i are the number and the pK of the i titratable group, respectively. The fitted calculated values for ß-trypsin were used to generate the superimposed curve in Figure 2. The difference between the fitted experimental and theoretical curves was less than 1%. Table 1 shows the calculated pK for some unfolded proteins (19) and those obtained here for ß-trypsin.

The native protein titration curve also showed two sets of titratable carboxylates: one presenting a pK = 3.91 ± 0.01 and the other with pK = 4.63 ± 0.03 values. These values were obtained by statistical comparison (F test) of equations for one and two pK values. This latter equation showed the best curve fit and its F test was significant at the 0.01 level. The 3 basic histidines should have higher pKs and/or reduced accessibility at the studied pH range, since only a fraction of the imidazoles was titrated and the α-NH$_2$ was assumed to be a hidden group, being completely buried.

The major structural titratable groups of ß-trypsin in the 3 to 7 pH range are the ß- and γ-carboxyl groups. Of these groups, the Asp102 ß-carboxyl group is also a component of the catalytic triad of the active site. The electrostatic factor w was calculated for the native ß-trypsin carboxyl titration data. These experimental w data were further compared to the predicted w values for the enzyme treated both as an impenetrable sphere (model I) (9,10), or as a sphere without the charge-free shell impenetrable by small ions (model II) (11). The equation used for the treatment of the experimental data was

$pH-\log[x_i/(1-x_i)] = (pK_{int})_i - 0.868wZ$ (Eq. 3)

The equations describing w for model I and model II are equations 4 and 5, respectively:

$w = (\varepsilon^2/2Dk_bT)[(1/R)-\kappa/(1+\kappa a)]$ (Eq. 4)

$w = (\varepsilon^2/8\pi D_0Dk_bT)[(1/R)-\kappa/(1+\kappa R)]$ (Eq. 5)

The symbols in the equations are as follows: x_i is the average degree of dissociation; pK_{int} is the pK of the i titratable group in the absence of interaction; Z is the net charge of the protein; ε is the electronic charge ($\varepsilon = 4.8032 \times 10^{-10}$ ues); D is the dielectric constant of the medium (D = 73.78); D_0 is the dielectric constant in the vacuum; k_b is the Boltzmann's constant (1.381×10^{-16} erg· deg^{-1}); R is the sphere radius in cm calculated from $4/3\pi R^3 = 0.73$ M/N_{av}; κ is the Debye-Hückel parameter in cm^{-1}; and a is the distance in cm of closest approach of ions to the center of the protein sphere. The Debye-Hückel formulation for the parameter κ is:

$\kappa = [(8\pi N_{av}\varepsilon^2)/(1000Dk_bT)]^{1/2} I^{1/2}$ (Eq. 6)

where N_{av} is the Avogadro number ($N_{av} = 6.023 \times 10^{23}$) and I is the ionic strength. The κ value we used for solvent A was calculated by proper interpolation (D = 73.78) of tabulated D values (8).

Table 2 shows the results of experimental w data compared to theoretical values obtained for model I and model II. The w values given in parentheses are the calculated values for the radius R equal to protein radius R_p. The experimental w value for ß-trypsin (Figure 3) was calculated by linear regression of the lowest slope of a curve of $pH - \log[x_i/(1-x_i)] = (pK_{int})_i - 0.868wZ$, since the observed w for this protein is not a constant independent of Z. Even doing so, the calculated pK_{int} is higher than the experimental values in spite of the close numerical value of experimental to predicted w of model I. This fact, as discussed in the literature (19,20), may be due to different factors such as chloride binding, ionic strength,

wrong number of charge, discrete charge distribution over the protein surface, and changes in volume and/or shape of the molecule during titration. The lower values found for the experimental pK could be explained by the presence of basic residues near some acidic residues. In summary, the nonlinear behavior of the w parameter for ß-trypsin as a function of Z indicates the presence of two classes of carboxylates. This assumption was used to calculate the pK values of 4.63 and 3.91 of the native trypsin titration fitted curve shown in Figure 2. These data are in agreement with the X-ray structure (4) (Protein Data Bank - pdb1tld entry) in which three

Table 1 - Intrinsic pK values of titratable groups at 25°C.

a = Values obtained according to Ref. 19.

Type of group	Model compounds[a]	Proteins in 6 M GU-HCl[a]	ß-Trypsin in 6 M GU-HCl and $\Gamma/2$ = 0.2 M
α-Carboxyl	3.8	3.1	3.1
Aspartyl carboxyl	4.0	3.9	3.93
Glutamyl carboxyl	4.4	4.35	4.2
Imidazole	6.3	6.4	6.5
α-Amino	7.5	7.4	7.4

Table 2 - Calculated and experimental w and intrinsic pK values for the titration data of ß-trypsin.

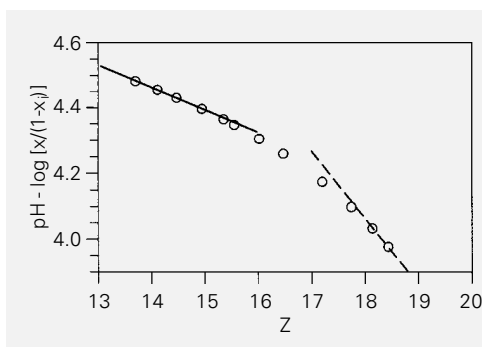| Experimental w	Calculated w (model I)	Calculated w (model II)	Intrinsic pK values
0.066 ± 0.001 (lower slope)	0.067	0.084	5.39 ± 0.02
0.175 ± 0.007 (higher slope)	(0.051)	(0.033)	7.20 ± 0.13

Figure 3 - Evaluation of w and intrinsic pK values. The protein net electric charge Z is related to the dissociation degree of the carboxyl group x_i by the linear equation: $pH - \log[x_i/(1-x_i)] = (pK_{int})_i - 0.868 wZ$. The intrinsic pK is given by the Y intercept. The nonlinear shape of experimental data (open circles) suggests more than one class of titratable carboxyl groups in the protein.

ion pairs were described, i.e., N-terminal to Asp194, C-terminal to Lys87, C-terminal to Lys107 and His57 to Asp102. Thus, since ionic pairs with basic residues tend to stabilize carboxylates, the active center residue Asp102 is more likely to be included in the class of lower pK. In fact, the neutron structure of trypsin (21) pointed to a protonated imidazole at the His57 residue in support of the Asp102···His57 ion pair idea. Quite independently of the model used, the pH titration data permit the calculation of the corresponding $\Delta\Delta G^0$ in the pH range studied. This value incorporates any molecular event that happens when the enzyme is titrated. So, once the stability of the protein at a fixed pH value (in the pH range studied) is determined, one can quickly evaluate the stability of the enzyme as a function of pH; this is the main goal of our current experimental efforts.

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