Characterization of ß-trypsin at acid pH by differential scanning calorimetry

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Abstract

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Trypsin is a serino-protease with a polypeptide chain of 223 amino acid residues and contains six disulfide bridges. It is a globular protein with a predominance of antiparallel ß-sheet and helix in its secondary structure and has two domains with similar structures. We assessed the stability of ß-trypsin in the acid pH range using microcalorimetric (differential scanning calorimetry) techniques. Protein concentrations varied in the range of 0.05 to 2.30 mg/ml. Buffer solutions of 50.0 mM ß-alanine and 20.0 mM $CaCl_2$ at different pH values (from 2.0 to 4.2) and concentrations of sorbitol (1.0 and 2.0 M), urea (0.5 M) or guanidinium hydrochloride (0.5 and 1.0 M) were used. The data suggest that we are studying the same conformational transition of the protein in all experimental situations using pH, sorbitol, urea and guanidinium hydrochloride as perturbing agents. The observed van't Hoff ratios ($\Delta H_{cal}/\Delta H_{vH}$) of 1.0 to 0.5 in the pH range of 3.2 to 4.2 suggest protein aggregation. In contrast, $\Delta H_{cal}/\Delta H_{vH}$ ratios equal to one in the pH range of 2.0 to 3.2 suggest that the protein unfolds as a monomer. At pH 3.00, ß-trypsin unfolded with Tm = 54°C and ΔH = 101.8 kcal/mol, and the change in heat capacity between the native and unfolded forms of the protein (ΔCp) was estimated to be 2.50 ± 0.07 kcal mol^{-1} K^{-1}. The stability of ß-trypsin calculated at 298 K was ΔG_D = 5.7 kcal/mol at pH 3.00 and ΔG_D = 15.2 kcal/mol at pH 7.00, values in the range expected for a small globular protein.

Introduction

The biological activity of proteins depends on the structure and three-dimensional conformation of their polypeptide chains. These conformations are the result of the thermodynamically driven folding of the linear amino acid sequence, allowing proteins to change from a nonfunctional and unordered structure to a well-defined, compact and active form.

Although there is a great deal of interest in protein folding, we still have much to learn about the mechanisms involved in the process. The reversible process of protein folding and unfolding can be studied by various sensitive methods capable of detecting changes in protein structure. According to Jackson and Brandts (1), the two-state denaturation process that considers only the conformational extremes, i.e., the native and denatured forms of the protein, can be used for the analysis of small and simple three-

dimensional structure proteins. Thermodynamic analysis is simplified if one can describe the denaturation process as a simple transition between two macroscopic states (native and denatured). In contrast, for large proteins, an increase in temperature induces unfolding of isolated domains, an event that cannot be regarded as a simple two-state process (2).

The success of thermodynamic analysis of protein folding and unfolding is directly dependent on the method of determination of the thermodynamic parameters and the characteristics of the protein analyzed. Differential scanning calorimetry (DSC) is an experimental tool capable of determining the calorimetric parameters ΔG_D, ΔH_D, ΔS_D and ΔCp_D associated with a given conformational change of the protein induced by temperature variation. A special type of microcalorimeter with adequate technical characteristics for data acquisition from aqueous protein solutions of relatively low concentration is necessary for DSC. The DSC apparatus integrates hardware and software which allow data acquisition, analysis, and deconvolution (3).

In this context, ß-trypsin is a very interesting model for the microcalorimetric study of a serine protease since it exhibits 223 amino acid residues that are presented in two similar domains (4), its three-dimensional structure is known, it possesses high reversibility for the type of denaturation utilized (thermal), and shows great stability in its native form. Trypsin stability has been investigated using solvent denaturation (5,6) and calorimetry (7), but these authors did not adequately explore different experimental conditions. Thus, the objective of the present investigation was to study the thermodynamic characteristics of the reversible thermal denaturation of ß-trypsin under several solvent conditions and in the presence of different protein concentrations in the acid range (pH 2.0 to 4.5) using DSC. This is part of an ongoing research project that ultimately aims to compare the stability of ß-trypsin with its zymogen under similar experimental conditions and to describe the activation process of a typical serine protease from a thermodynamic point of view.

Material and Methods

Purification of ß-trypsin. Trypsin was purified by the chromatographic procedure described by Schroeder and Shaw (8) and Higaki and Light (9). For purification, 250 mg of commercial bovine trypsin (Sigma, St. Louis, MO, USA) was dissolved on an ice bath in 5.0 ml Tris-HCl buffer

(100.0 mM Tris-HCl, 20.0 mM calcium chloride, 100.0 mM sodium chloride and 1.0 mM benzamidine), pH 7.10. The solution was applied to a SE-Sephadex C-50 column (2.0 x 110.0 cm; 345.0 cm^3 volume) and was eluted with the same buffer (isocratic) at a constant flow of 7.0 ml/h. The fractions were collected using an automatic collector (Pharmacia-LKB Frac-100, New York, NY, USA), read at 280.0 nm in a spectrophotometer (Shimadzu UV 160-A, Kyoto, Japan) and submitted to electrophoresis to determine their purity (peaks). The amidase activity of each fraction pool was determined as described below.

Electrophoresis. The purified fractions were submitted to electrophoresis by the method of Laemmli (10). The following standard markers (Sigma) were added to all electrophoretic runs: 116-kDa ß-galactosidase, 97.4-kDa rabbit muscle ß-phosphorylase, 66-kDa bovine albumin, 45-kDa swine heart fumarase, and 29-kDa carbonic anhydrase. The gels were stained with silver by the method of Tunon and Johansson (11) and with Coomassie blue by the method of Hames and Rickwood (12).

Amidase activity was determined by the method of Antonini and Ascenzi (13) using DL-BAPNA as substrate. After SE-Sephadex C-50 chromatography and determination of amidase activity, selected fractions were submitted to dialysis for 8 h at 4ºC using membranes of 6000-8000 Da exclusion limit, against 1.0 mM HCl, pH 3.00. The dialyzed samples were lyophilized and stored at -20ºC.

Active site titration. The active site of the enzyme samples was titrated with small modifications (14). In this method, ß-trypsin catalyzes the hydrolysis of 4-nitrophenyl-4-guanidinebenzoate. The first catalytic step is several-fold faster than the second step, permitting the spectrophotometric detection of the acyl-enzyme at 410 nm. A reference cuvette with Veronal buffer and a sample cuvette with the same buffer plus trypsin were used. The values of 40,000 and 16,595 M^{-1}cm^{-1} were adopted as the molar extinction coefficient (ϵ) for ß-trypsin (read at 280.0 nm) and 4-nitrophenol at pH 8.30 (read at 410.0 nm), respectively. The equation used for calculation was

$$[\text{Active site}]\% = \frac{\Delta A_{280}/40.000}{\Delta A_{410}/16.595 \text{ x f}} \text{ x } 100\% \tag{Eq. 1}$$

where f = enzyme dilution factor.

Thermal denaturation of ß-trypsin in acid media. The microcalorimetric study of ß-trypsin denaturation at pH 2.00 to 4.20 was performed by the methods of Privalov and Potekhin (2) and Freire et al. (15) using the MicroCal

Ultrasensitive VP-DSC apparatus and standard software for data acquisition and analysis. For analysis in acid media the ß-trypsin samples, resuspended in ß-alanine buffers at pH 2.00, 2.25, 2.50, 2.75, 3.00, 3.25 and 3.50 (50.0 mM ß-alanine and 20.0 mM $CaCl_2$), 4.00 and 4.20 (50.0 mM sodium acetate and 20.0 mM $CaCl_2$) and determined spectrophotometrically at 280.0 nm to be approximately 1.0 mg/ml, were degassed for 30 min and then loaded onto the DSC MicroCal apparatus. Runs were performed with a scan rate of 60ºC/h. The changes in enthalpy (ΔH) did not depend on the scan rate used in the range of 30º to 90ºC per hour. The data obtained were subtracted from a baseline of water against water or buffer against buffer (ß-alanine at the corresponding pH values) before normalization of the protein concentration.

Microcalorimetry of ß-trypsin at pH 3.00 in the presence of a protein denaturant or stabilizer. The microcalorimetric runs of ß-trypsin with a destabilizing or stabilizing agent were carried out by the method described above. Guanidinium hydrochloride was used as a denaturing agent at 0.25, 0.50 and 0.75 M in ß-alanine buffer, pH 3.00 (50.0 mM ß-alanine, 20.0 mM $CaCl_2$) (16), and sorbitol was used as a stabilizing agent at 0.5, 1.0, 1.5 and 2.0 M in ß-alanine buffer, pH 3.00 (50.0 mM ß-alanine, 20.0 mM $CaCl_2$) (16).

Results and Discussion

It is not possible to directly assess the stability of ß-trypsin at neutral pH due to the autolysis of this proteolytic enzyme. The alternative is to assess stability within the acid pH range (pH 2.00 to 4.50) and to calculate the stability of the enzyme in the neutral range according to the thermodynamic cycle described below (Figure 1).

The analysis of experimental DSC data (Cp x temperature) allows the evaluation of two types of enthalpy variation (2): i) calorimetric enthalpy ($ΔH_{cal}$), which represents the area under the denaturation curve, minus the contribution of the variation of heat capacity (ΔCp) between the native and denatured forms of the protein. This is a model-independent parameter and the units are cal/mol of protein (monomer), but can also be expressed as cal/mol dimer, if necessary. ii) van't Hoff enthalpy ($ΔH_{vH}$), which reflects the shape of the denaturation curve and describes the dependence of the equilibrium constant (K_D) as a function of temperature. It is a model-dependent parameter expressed as cal/mol of cooperative units, responsible for the observed conformational transitions.
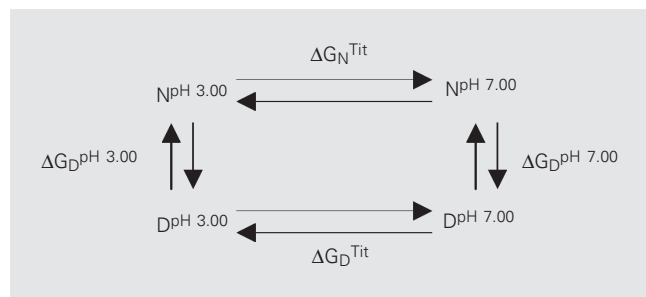

Figure 1. Thermodynamic cycle for ß-trypsin, where: $ΔG_D^{pH\ 3.00}$ = measured stability of the protein at pH 3.00; $ΔG_D^{pH\ 7.00}$ = calculated stability of the protein at pH 7.00; $ΔG_D^{Tit}$ = free energy change of titration of the denatured species from pH 3.00 to 7.00; $ΔG_N^{Tit}$ = free energy change of titration of the native species from pH 3.00 to 7.00.

An important experimental parameter is the $ΔH_{cal}/ΔH_{vH}$ ratio, which represents the ratio

$$\frac{\dfrac{Cal}{\text{mol of protein (monomer)}}}{\dfrac{Cal}{\text{mol of cooperative units}}} = \frac{\text{mol of cooperative units}}{\text{mol of protein (monomer)}}$$

(Eq. 2)

For a monomer, a two-state transition is described by a $ΔH_{cal}/ΔH_{vH}$ value = 1.0, i.e., the monomer is the cooperative unit responsible for the observed conformational transition. In the case of a dimer, the ratio is $ΔH_{cal}/ΔH_{vH}$ = 0.5, i.e., we have 0.5 mol of cooperative units per mol of monomer. For a monomer with two domains of similar size, when uncoupled, a $ΔH_{cal}/ΔH_{vH}$ ratio = 2.0 should be expected, i.e., 2 mol of cooperative units per monomer would be detected, assuming that the domains make similar contributions to the overall $ΔH_{cal}$ value (2).

At pH 3.00 the reversibility of the thermal denaturation of ß-trypsin was 90% or higher. For this protein the denaturation process was evaluated as a two-state transition, as can be seen in Figure 2 which shows a quite symmetrical transition curve. At this pH, the denaturation process was assumed to be a two-state transition because the $ΔH_{cal}/ΔH_{vH}$ ratio was very close to one.

ß-Trypsin at pH values ranging from 2.00 to 3.00 exhibited a $ΔH_{cal}/ΔH_{vH}$ ratio always close to one (Figure 3), indicating that the enzyme is a monomer and that the domains are coupled under these conditions. These results agree with those reported by Brumano et al. (5), who showed that the thermal denaturation of ß-trypsin at pH 2.80 was a two-state transition with a $ΔH_{cal}/ΔH_{vH}$ value close to one.

On the other hand, the DSC runs performed at pH values ranging from 3.20 to 4.20 revealed that the $\Delta H_{cal}/\Delta H_{vH}$ ratio ranged from 1.0 to 0.5 (Figure 3), suggesting protein dimerization. Indeed, literature data suggest ß-trypsin dimerization at pH values around 4.00 (17), although Tishchenko and Gorodkov (7), in experiments with ß-trypsin in the 2.5
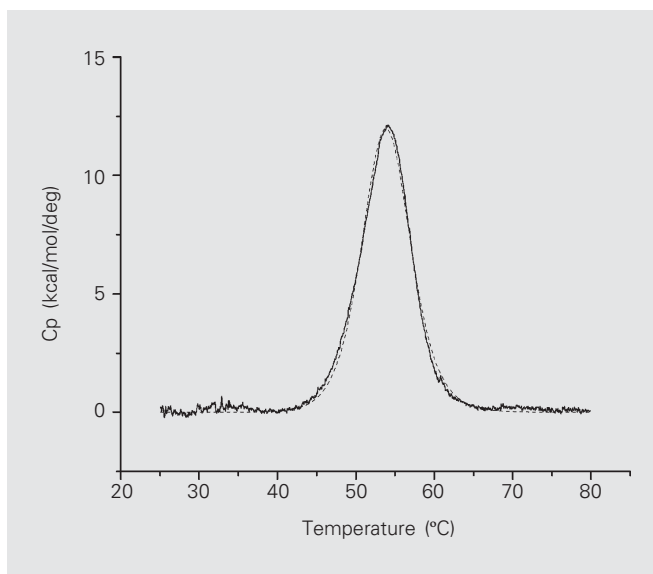

Figure 2. Acid denaturation curve of ß-trypsin. The proteins were 1.0 mg/ml in 50.0 mM ß-alanine and 20.0 mM CaCl$_2$ buffer, pH 3.00. Data were obtained by differential scanning calorimetry. The solid line shows data processed after subtraction of the baseline and normalization of protein concentration. The dashed line indicates the fitting of the data to the mathematical model of a two-state transition.
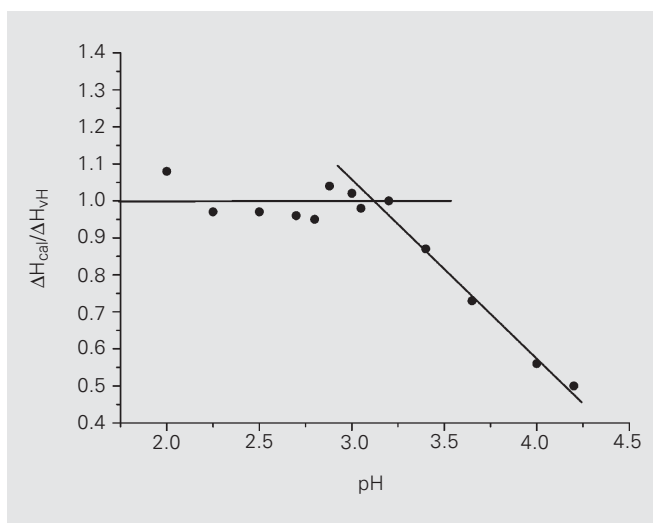

Figure 3. $\Delta H_{cal}/\Delta H_{vH}$ values for ß-trypsin in the acid pH range. Buffers: 50.0 mM ß-alanine with 20.0 mM CaCl$_2$, except at pH 4.00 and 4.20 where 50.0 mM acetate with 20.0 mM CaCl$_2$ buffers were used. ß-Trypsin concentration was 1.0 mg/ml.

to 4.0 pH range, observed that the $\Delta H_{cal}/\Delta H_{vH}$ ratio was always approximately one.

In an attempt to clarify this point, the $\Delta H_{cal}/\Delta H_{vH}$ ratio was determined at constant pH 3.70 (50.0 mM ß-alanine and 20.0 mM CaCl$_2$). At this pH the $\Delta H_{cal}/\Delta H_{vH}$ ratio was about 0.75, i.e., between the extreme values 1.0 (100% of proteins are monomers) and 0.5 (100% of proteins are dimers). Figure 4 shows the results obtained at the protein concentration range of 0.05-2.30 mg/ml. While the protein concentration varied, the protein aggregated under these conditions, since the ratio tended to reach lower values with high protein concentrations, while it tended to reach 1.0 when the protein concentration tended to be zero.

Assuming that under these conditions the protein shows an equilibrium of 2 monomers ⇔ dimer, the dimerization constant can be calculated using the following equation

$$K_D = \frac{[Dimer]}{[Monomer]^2} = \frac{(1-f)}{f^2 * [P]} \qquad \text{(Eq. 3)}$$

where [P] is the total protein concentration and f is the fraction of the total protein as a monomer. Solving this equation for f leads to a quadratic equation that can be solved by retaining only the positive f value, whereas the other possible solution involves a negative f value that has no physical meaning. For the observed $\Delta H_{cal}/\Delta H_{vH}$ ratio, we can assume that

$$(\Delta H_{cal}/\Delta H_{vH})_{obs} = f * (\Delta H_{cal}/\Delta H_{vH})_{monomer} + (1-f)* (\Delta H_{cal}/\Delta H_{vH})_{dimer}$$
$$\text{(Eq. 4)}$$

Recall that $(\Delta H_{cal}/\Delta H_{vH})_{monomer} = 1.0$ and $(\Delta H_{cal}/\Delta H_{vH})_{dimer} = 0.5$.

By combining this equation with the f value obtained previously, we obtain the following equation for $(\Delta H_{cal}/\Delta H_{vH})_{obs}$ as a function of total protein concentration.

$$(\Delta H_{cal}/\Delta H_{vH})_{obs} = 0.5 - (1/4*K_D*[P]) + (1 + 4*K_D*[P]^{1/2}/(4*K_D*[P])) \qquad \text{(Eq. 5)}$$

Simulations showed that the limiting value of this equation was 0.5 when the protein concentration tended to reach high values, and 1.0 when the protein concentration tended to be zero.

When this equation was fitted to the $(\Delta H_{cal}/\Delta H_{vH})_{obs}$ values (Figure 4) it did fit reasonably well protein concentrations lower than 1.0 mg/ml, but did not fit the experimental data observed at higher protein concentrations. We

believe that this departure suggests the presence of higher aggregate states (trimers, tetramers, etc.) that cannot be accounted for by this equation.

For a two-state transition, at the transition temperature (Tm), $\Delta G_{(Tm)} = 0$ cal/mol ($K_D = 1.0$). Thus,

$$\Delta G_{(Tm)} = \Delta H_{(Tm)} - Tm \times \Delta S_{(Tm)} = 0 \therefore \Delta S_{(Tm)} = \frac{\Delta H_{(Tm)}}{Tm}$$

(Eq. 6)

The dependence of $\Delta H_{(Tm)}$ on Tm allows the evaluation of ΔCp, which is the variation of the heat capacity between the native and denatured forms of the protein. Hence:

$$\Delta Cp = \frac{d\Delta H}{dT}$$

(Eq. 7)

Integrating between the limits (assuming constant ΔCp in the temperature range studied):

$$\int_{Tm}^{T} d\Delta H = \Delta Cp \int_{Tm}^{T} dT$$

(Eq. 8)

$$\therefore \Delta H_{(T)} = \Delta H_{(Tm)} + \Delta Cp (T - Tm)$$

(Eq. 9)

Analogously, it can be demonstrated that

$$\Delta S_{(T)} = \Delta S_{(Tm)} + \Delta Cp \, 1n \, (T/Tm)$$

(Eq. 10)

Once $\Delta H_{(Tm)}$, Tm and ΔCp are known, the protein stability $\Delta G_{(T)}$ in the temperature range of interest can be calculated according to the following equation (18):

$$\Delta G_{(T)} = \Delta H_{(Tm)}(1 - T/Tm) - \Delta Cp[(Tm - T) + T * \ln (T/Tm)]$$

(Eq. 11)

At pH 3.00, ß-trypsin denaturation presented a Tm of 54ºC and a ΔH of 101.8 kcal/mol. The variation in heat capacity between the native and denatured forms of the protein (ΔCp) was estimated to be 2.50 ± 0.07 kcal mol^{-1} K^{-1} for the ß-trypsin monomer. The values used for this evaluation presented $\Delta H_{cal}/\Delta H_{vH}$ ratios close to one. In some runs, sorbitol was used as the protein stabilizer and guanidinium hydrochloride or urea as a destabilizer (Figure 5). These compounds are known to affect the stability of proteins (16) by changing the relative stabilities of the native and unfolded forms of the protein. The observation

of a straight line fitting well all the data suggests that we are studying the same conformational transition of the protein in all experimental situations using pH, sorbitol, urea and guanidinium hydrochloride as perturbing agents. The stability curve calculated for ß-trypsin at pH 3.00 is shown in Figure 6.

The ΔG value of ß-trypsin denaturation calculated from the DSC at pH 3.00 and extrapolated to 298 K was 5.7 kcal/mol, as can be observed in Figure 6. The result for ß-trypsin was similar to that obtained by Günther (19) by solvent (urea) denaturation at the same pH and temperature (6.10 ± 1.03 kcal/mol).

It is known that protein folding is a thermodynamically directed process depending on the primary protein structure and can produce stable intermediates with structures sufficiently similar to the native form of the protein (molten globule) (20,21). In the case of small proteins the denaturation process is quite simple, with a transition between two states, i.e., native and denatured. For large proteins and with some domains the denaturation process can present some intermediate states between the native and the denatured forms (21). Based on the $\Delta H_{cal}/\Delta H_{vH}$ values observed
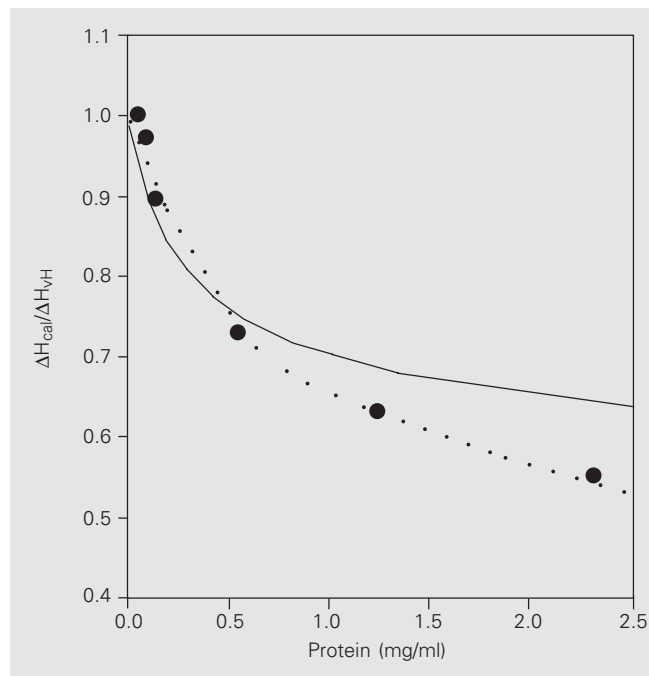

Figure 4. $\Delta H_{cal}/\Delta H_{vH}$ values for ß-trypsin at pH 3.70 as a function of protein concentration. The buffer was 50.0 mM ß-alanine and 20.0 mM CaCl$_2$, pH 3.70. Dotted line = fourth degree polynomial fitted to the data to guide the eyes. Solid line = equation 5 fitted to the data to show the predicted values due to a putative dimerization process. Protein concentration was 0.05 to 2.3 mg/ml.
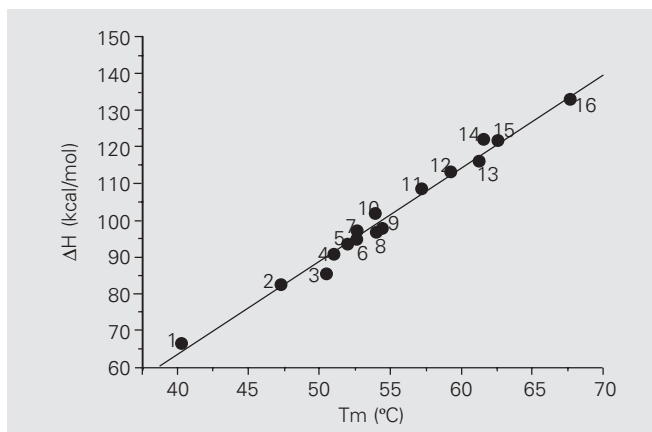
Figure 5. ΔH_{cal} versus Tm (ºC) at different pH values, concentrations of sorbitol at pH 3.00, of guanidinium hydrochloride at pH 3.00, of urea at pH 3.00, and different buffers. Protein concentration was 1.0 mg/ml except when the influence of protein concentration was tested. NaCl (0.2 M) was used in one experiment at pH 3.00. In all experiments the buffer was 0.1 M ß-alanine except when glycine or acetate was used. The slope was $\Delta Cp = 2.50 \pm 0.07$ kcal mol^{-1} K^{-1}. Run conditions: 1) 1.00 M guanidinium hydrochloride, pH 3.00; 2) 0.70 M guanidinium hydrochloride, pH 3.00; 3) 0.50 M guanidinium hydrochloride, pH 3.00; 4) 0.50 M urea, pH 3.00; 5) pH 2.70; 6) pH 2.80; 7) pH 2.85; 8) pH 2.90; 9) 0.20 M NaCl, pH 3.00; 10) ß-alanine, pH 3.00; 11) pH 3.20; 12) glycine, pH 3.00; 13) 0.1 mg/ml protein, pH 3.70; 14) acetate buffer, pH 3.00; 15) 1.0 M sorbitol, pH 3.20; 16) 2.0 M sorbitol, pH 3.14. In all experiments, ΔH_{cal} was equal to ΔH_{vH}.
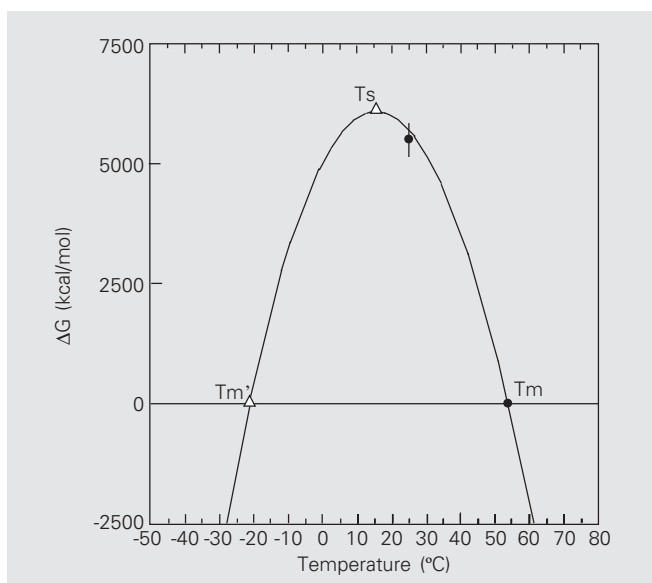

Figure 6. Stability curve of ß-trypsin: ΔG_D as a function of temperature (ºC), at pH 3.00, where Tm is the heat denaturation temperature, Tm' is the theoretical cold denaturation temperature and Ts is the temperature of maximum stability of the protein under these experimental conditions. The value at 25ºC, 6.10 ± 1.03 kcal/mol, was obtained by solvent denaturation with urea as denaturant (21); the data were analyzed as a two-state transition.

in the 2.00 to 3.00 pH range, the process of thermal denaturation of ß-trypsin occurs as a transition between two states. Although ß-trypsin has two similar domains, the enzyme denatures as a cooperative unit in the denaturation process. This behavior was already shown in studies using DSC for observation of the denaturation of ß-trypsin at pH 2.80. This was attributed to the close similarity and strong interaction between the two domains (5). As the pH increased from 3.20 to 4.20, ß-trypsin tended to aggregate, as suggested by a $\Delta H_{cal}/\Delta H_{vH}$ ratio tending to 0.5. This dimerization was the consequence of deprotonation of some carboxyl groups causing an interaction on the surface of molecules. Dimerization can be reversed by dilution of the protein solution (17).

Martins et al. (6) determined the thermodynamic values of the denaturation of ß-trypsin at pH 3.00, with better adjustment for a three-state transition, using solvent denaturation with urea. Ruan et al. (22) showed the presence of the intermediate molten globule in the denaturation of trypsin under pressure. Thus, the results of the cited reports are consistent with the existence of one or more intermediates in the denaturation of ß-trypsin. However, Ruan et al. used pressure denaturation and did not purify the sample of bovine trypsin, and other trypsin isoforms were present, while Martins et al. (6) used solvent denaturation with urea. Bulaj and Otlewski (23) worked with commercial trypsin, trypsinogen and ß-trypsin and concluded that the process of denaturation of these proteins fitted a two-state transition, although they described a kinetically complex denaturation process.

Finally, it is worthwhile to calculate the stability of ß-trypsin at neutral pH. From the thermodynamic cycle (Figure 1) we obtain

$$...... \Delta G_N^{Tit} + \Delta G_D^{pH\ 7.00} = \Delta G_D^{Tit} + \Delta G_D^{pH\ 3.00} \qquad \text{(Eq. 12)}$$

$$\therefore \ \Delta G_D^{pH\ 7.00} = (\Delta G_D^{Tit} - \Delta G_N^{Tit}) + \Delta G_D^{pH\ 3.00} = (\Delta\Delta G^{Tit}) + \Delta G_D^{pH\ 3.00}$$
$$\text{(Eq. 13)}$$

The titration of the native form of this protein from pH 3.00 to 7.00 is affected by any change in the state of the protein, including the aggregation that occurs around pH 4.5, so that the thermodynamical cycle shown in Figure 1 is still valid, and it is known that this enzyme is monomeric at neutral pH. In the present study, the $\Delta G_D^{pH\ 3.00} = 5.71 \pm 0.06$ kcal/mol was assessed at pH 3.00 and at 25ºC. The $\Delta\Delta G^{Tit}$ term was found to be 9.51 ± 0.06 kcal/mol (24) when the native

and unfolded forms of the protein were independently titrated from pH 3.00 to 7.00 at 25ºC. Based on these values, the calculated stability of ß-trypsin at pH 7.00 was 15.22 ± 0.07 kcal/mol, a value that is typical for small globular proteins at neutral pH. It is important to realize that we cannot obtain this value directly at pH 7.00 due to autolysis of the protein.

We have come a long way from the work of Anson and Mirsky (25), the first description of the effect of temperature on the equilibrium between the native and denatured forms of a preparation of trypsin. Their data were obtained

only at pH 2.0 using a quite different technique. Our work is an extensive thermodynamic study of ß-trypsin denaturation at acid pH that significantly extends our knowledge of the thermodynamical behavior of this important protein. It would be instructive to compare the stability of ß-trypsin with its zymogen under similar experimental conditions (at pH 7.00 and 25ºC) and describe the activation process of a typical serine protease from a thermodynamic point of view. This is the subject of an ongoing research project in our laboratory.

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