Stability Studies on Derivatives of the Bovine Pancreatic Trypsin Inhibitor

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ABSTRACT: Gibbs energy, enthalpy, and entropy data were determined for two selectively modified analogues of bovine pancreatic trypsin inhibitor (BPTI) to provide a model free set of thermodynamic parameters that characterize (a) the energetic and entropic contributions of the 14–38 disulfide bridge and (b) the variation of the overall stability resulting from the introduction of two negative charges into the positions 14 and 38. The two BPTI analogues studied were BPTI having Cys-14 and Cys-38 carboxymethylated (BPTI-RCOM) and BPTI having Cys-14 and Cys-38 carboxamidomethylated (BPTI-RCAM). They were obtained from native BPTI by reduction, followed by modification of the sulfhydryl groups with iodoacetic acid or iodoacetamide, respectively. The temperature dependence of all thermodynamic parameters of BPTI is drastically altered in the absence of the third disulfide bridge. Even the apparently minute difference of two dissociable carboxyl groups instead of uncharged amide groups in positions 14 and 38 has surprisingly large effects on the temperature dependence of the stabilization enthalpy. The Gibbs energy of BPTI at pH 2, 25 °C, decreases by approximately 70% when the 14–38 disulfide bond is cleaved. BPTI-RCOM is more stable than BPTI-RCAM in the whole pH range studied. The difference of −4 kJ/mol at pH 2, 25 °C, is reduced to −2.7 kJ/mol at pH 5, 25 °C. This finding demonstrates that the presence of two negative charges reduces the higher stability of BPTI-RCOM slightly; however, the overall effect of the two charges is still a stabilization. High-pressure liquid chromatography and gel electrophoresis studies on multiply unfolded samples suggested that incorrect refolding and/or association occurred in the cooling period after thermal unfolding. These side reactions could be shown not to interfere with the calorimetric measurements.

The recent advances in genetic engineering techniques provide us with potentially powerful tools for protein design. Tailor-made DNA sequences whose translation products are predictable with respect to stability, structure, and function can well be envisaged to revolutionize the industrial, agricultural, and medical impact of biotechnology. However, there is still a serious obstacle that prevents rational protein design: Essentially, we lack quantitative information on the covalent and noncovalent forces contributing to the maintenance of the structure and function of proteins. Although it has been realized since the pioneering work of Anfinsen (Anfinsen & Scheraga, 1975) that the amino acid sequence determines the properties of proteins, there is no quantitative scale available at present that would, e.g., permit the prediction of the energetic contribution of a change in the degree of intramolecular covalent linkage or of a charge to the overall stability of a protein. More generally speaking, the knowledge of exactly how amino acid sequence influences the stability, structure, and function of a protein is still pretty much in its infancy.

To fill this gap with some quantitative data, we continued our studies on BPTI[1] (Moses & Hinz, 1983) and modified the number of disulfide bridges and the charge of the molecule in a defined manner. We studied the influence of these modifications on the overall stability of the protein over a wide range of pH values. In this study we report some quantitative thermodynamic data on the role of the 14–38 disulfide bridge as well as on the contribution of two negative charges in the same position.

For that purpose we studied two derivatives of BPTI, BPTI-RCAM and BPTI-RCOM. In both these analogues, the 14–38 disulfide bridge has been reductively cleaved and re-formation of the bond has been prevented by introducing carbaminomethyl and carboxymethyl groups, respectively. Thus both BPTI-RCAM and BPTI-RCOM have only two internal disulfide bridges; they differ, however, by the presence of two negative charges at the 14–38 carboxyl groups of BPTI-RCOM at neutral pH. The overall solution structures of BPTI-RCAM, BPTI-RCOM, and native BPTI are similar according to laser Raman (Brunner et al., 1974) and NMR spectroscopic evidence (Snyder et al., 1976; Wüthrich & Wagner, 1978; Wagner et al., 1979a,b; Roder et al., 1985a).

BPTI and its analogues are particularly attractive objects for a correlation between energetics, structure, and function, since due to its small size and high stability, BPTI has become a widely accepted, well-characterized model system in biochemical and biophysical research. Crystal as well as solution structure is exceptionally well-known from X-ray, neutron diffraction (Wlodawer et al., 1984), and hydrogen-exchange studies (Deisenhofer & Steigemann, 1974; Huber et al., 1971; Masson & Wüthrich, 1973; Karplus & McCammon, 1979; Wüthrich & Wagner, 1978, Wagner & Wüthrich, 1979, 1982; Pershina & Hvidt, 1974; Hvidt & Pedersen, 1981; Richarz et al., 1979, 1980; Roder et al., 1985a,b; Havel & Wüthrich, 1985). Thermodynamics of unfolding have been described in a recent study (Moses & Hinz, 1983).

BPTI is a monomeric protein (Figure 1) consisting of 58 amino acid residues that are cross-linked by three disulfide bonds at positions 14–38, 30–51, and 5–55 (Kassel & Laskowski, 1965). The molecular weight is 6512. Amino acids 16–36 form a double-stranded antiparallel β-sheet, while at the N-terminus there is a short α-helix from amino acid 47

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; BPTI-RCOM, BPTI having Cys-14 and Cys-38 carboxymethylated; BPTI-RCAM, BPTI having Cys-14 and Cys-38 carboxamidomethylated.

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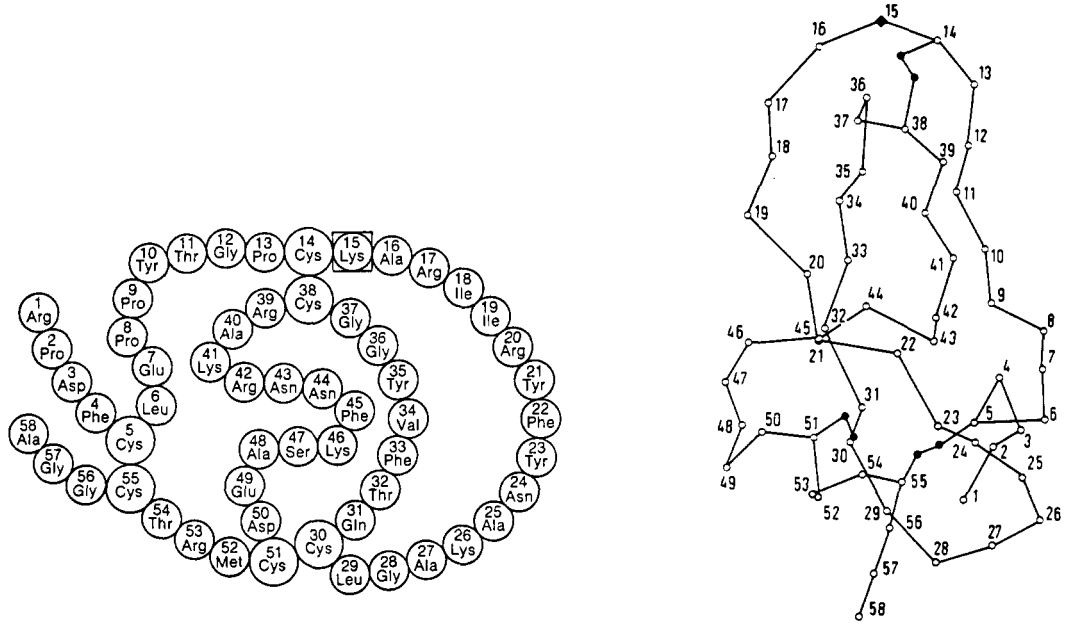

FIGURE 1: Primary and tertiary structure (Kassel & Laskowski, 1965; Huber et al., 1971) of BPTI. BPTI-RCAM and BPTI-RCOM are obtained by reductive cleavage of the disulfide bridge between Cys-14 and Cys-38 and reaction with ICH_2CONH_2 and ICH_2COOH, respectively.

to amino acid 56. (Deisenhofer & Steigemann, 1975.) The isoelectric point of BPTI is close to 10.5 (Kassel, 1970).

Materials and Methods

Materials

BPTI (Trasylol) was obtained as a generous gift from the Farbenfabriken Bayer AG. The BPTI derivatives BPTI-RCAM and BPTI-RCOM have been prepared essentially according to published procedures involving selective reduction of the disulfide bond 14–38 with sodium borohydride (Kress & Laskowski, 1967) and reaction of the resulting sulfhydryl groups with iodoacetamide or iodoacetic acid, respectively (Kress et al., 1968). The derivatives were purified by cation-exchange chromatography on CM-Sephadex C-25 (10 mM borate buffer, pH 8.6, linear gradient from 0 to 0.6 M NaCl). Desalting on Sephadex G-25 (eluent 0.1 M acetic acid) and lyophilization yielded proteins that were homogeneous as judged from amino acid analysis, polyacrylamide gel electrophoresis, and analytical high-performance cation-exchange chromatography (Wenzel et al., 1985). The concentration of the protein was determined photometrically, with a Shimadzu UV-110-02 spectrophotometer, using an absorption coefficient at 280 nm of $E^{1\%,1cm} = 8.25$. pH values of the solutions were adjusted by using a WTW pH digi 510 pH meter (Wissenschaftliche Technische Werkstätten, Weilheim, Federal Republic of Germany). The buffer used was 1 mM potassium phosphate and 0.1 M NaCl. Deviations from this choice of buffer are noted in the text.

Methods

(*a*) *Calorimetry.* Temperature dependence of the apparent specific heat capacity has been determined with an electronically modified differential scanning microcalorimeter, DASM-1M (Privalov et al., 1975). Each measurement with protein was preceded by a buffer run, to establish the base line and the calibration constant. The protein concentrations used varied between 0.5 and 2.7 mg/mL, but most frequently 1 mg/mL solutions were employed.

Typically the measurements were started at 10 °C and carried out, depending on pH, up to 90 or 110 °C. Heating rates of 0.2, 1, and 2 deg/min were used, in order to check for kinetic effects. Since no influence of the heating rate on the transition properties was observed, the majority of measurements was carried out with 1 K/min. Each protein solution was subjected to up to five heating and cooling cycles to study the influence of repeated heating on the macromolecule. All measurements were performed with an excess pressure of 1 atm on the solution, to avoid vaporization of the solvent. Heat capacity and temperature data were collected every 0.1 K and stored by a computer.

For the evaluation of ΔH values from the heat capacity vs. temperature curves, three programs were routinely used. The base line of the transition peak was approximated (a) by a straight line, (b) by a step function at half-transition temperature T_m, or (c) by a program, kindly provided by E. Freire, which is based on the thermodynamic analysis of the sequential transitions (Freire & Biltonen, 1978). All three methods yielded identical results within error limits of ±5–10%.

The molecular weights used for calculation of molar quantities are M_r 6628 (BPTI-RCAM) and M_r 6630 (BPTI-RCOM).

The van't Hoff enthalpy was calculated from the calorimetric data according to

$$\Delta H_{vH} = 4RT_m^2 \Delta c_{p,m} \Delta h_{cal}^{-1} \quad (1)$$

where T_m is the temperature at 50% unfolding of the protein in absolute units, $\Delta c_{p,m}$ is the excess specific heat capacity at T_m, and Δh_{cal} is the corresponding specific enthalpy change.

(*b*) *UV Studies.* A Gilford spectrophotometer 2400, equipped with a thermal programmer, was used to observe unfolding of the protein with increasing temperature. The decrease of tyrosine absorption at 287 nm was employed as measure of the degree of denaturation. Scans were made between 10 and 100 °C at a heating rate of 1 K/min. van't Hoff enthalpies have been derived from the absorption vs. temperature curves by assuming a two-state transition. A plot of the degree of transition α vs. temperature yields a transition curve, the slope of which at T_m is proportional to ΔH_{vH} according to

$$\Delta H_{vH} = 4RT_m^2 (d\alpha/dT)_{T_m} \quad (2)$$

(*c*) *SDS Gel Electrophoresis.* SDS-containing polyacrylamide gels (13%, 0.4% SDS) were used, before and after the measurements, to check whether the protein had been de-

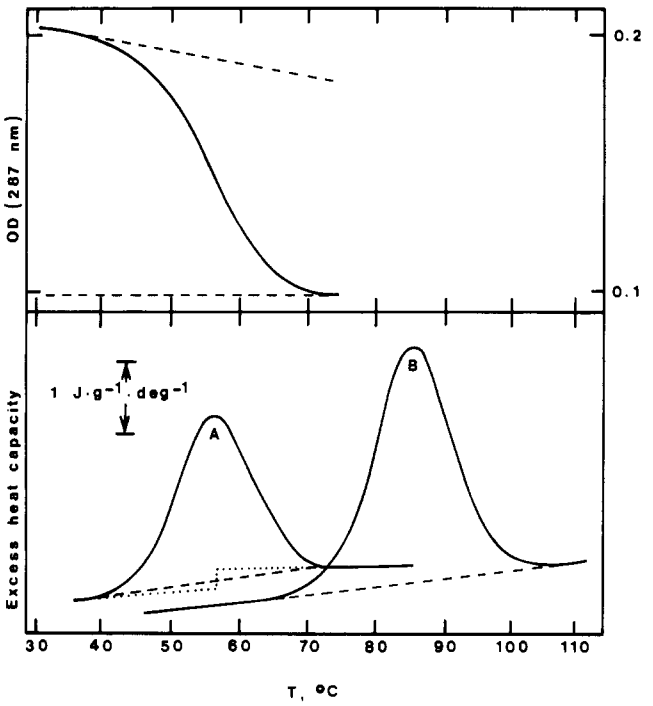

FIGURE 2: Heat capacity and optical transition curves of native BPTI (B) and BPTI-RCAM (A) at pH 2, in 1 mM potassium phosphate and 0.1 M NaCl. The upper part shows the UV transition profile of BPTI-RCAM, and the lower part shows heat capacity vs. temperature curves. The dashed and dotted lines denote the various base lines employed for evaluation of the transition curves. Due to the small heat capacity change involved in unfolding of native BPTI (0.015 J/(g·K); Moses & Hinz, 1983), the step-function base line would not visibly differ from the dashed base line in curve B.

graded by the temperature scans (Laemmli, 1970). Sample size was 20 μg.

(*d*) *Native Gel Electrophoresis.* Polyacrylamide gels (6%) were used to test for aggregation or incorrectly refolded species after the calorimetric or spectrophotometric studies (Laemmli, 1970).

(*e*) *HPLC Measurements.* A RP18 column, particle pore size 10 nm (Serva), was used in connection with a Spectra-physics HPLC apparatus to check for free amino acids or small peptides in the sample solutions after repeated heating and cooling cycles.

RESULTS

All measurements were performed in 1 mM potassium phosphate buffer containing 0.1 M NaCl. Typical transition curves, obtained at pH 2, of native BPTI (curve B) and BPTI-RCAM (curve A) are shown in Figure 2. The drastically lower stability caused by cleavage and carboxamidomethylation of the 14–38 disulfide bond is clearly demonstrated by the downshift of the transition temperature.

Variation of Transition Temperatures of BPTI-RCAM and BPTI-RCOM with pH. In order to determine the dependence of ΔH on T_m, different transition temperatures were generated by changing the pH of the solution. Figure 3a,b gives the experimental points and the theoretical curves for the variation of T_m of BPTI-RCAM and BPTI-RCOM. The curves have been calculated on the basis of a least-squares procedure by using

$$\Theta_m = (\Theta_l + \Theta_u 10^{pH-pK})/(1 + 10^{pH-pK}) \quad (3)$$

and varying Θ_u, Θ_l (the upper and lower temperatures, respectively), and pK to minimize the square of the deviations. The resulting values are for BPTI-RCAM, Θ_u = 77.7 °C, Θ_l = 57.5 °C, Θ_z = 67.6 °C, and pK_z = 3.42; for BPTI-RCOM, Θ_u = 79.6 °C, Θ_l = 59.6 °C, Θ_z = 69.6 °C, and pK_z = 3.57. Θ_z is the temperature at the inflection point pK_z of the T_m vs. pH curve. Comparison with native BPTI shows that the

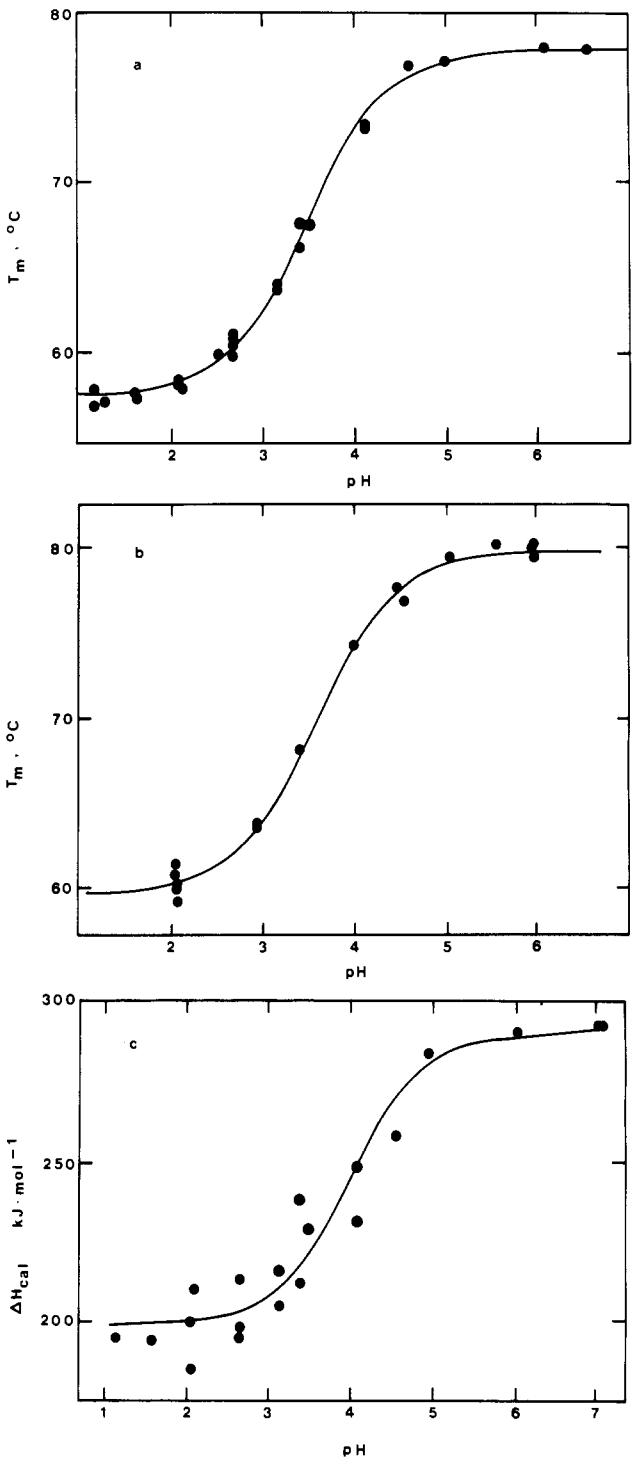

FIGURE 3: Variation of transition temperature and transition enthalpy with pH. (a) Dependence of transition temperature of BPTI-RCAM on pH. The T_m values have been fitted by a one-proton titration curve by using eq 3. The inflection point occurs at pH 3.42, and the lower and upper transition temperatures are Θ_l = 57.5 °C and Θ_u = 77.7 °C, respectively. (b) Dependence of transition temperature of BPTI-RCOM on pH. The curve has been fitted in the same manner as that of BPTI-RCAM; the inflection point occurs at pH 3.57, and the lower and upper transition temperatures are Θ_l = 59.6 °C and Θ_u = 79.6 °C, respectively. (c) Dependence of transition enthalpy of BPTI-RCAM on pH. The experimental ΔH values have been fitted by using an equation analogous to eq 3, employing a limiting ΔH value of 200 kJ/mol at lower pH and a ΔH of 290 kJ/mol at high pH; the inflection point occurs at pH 3.96.

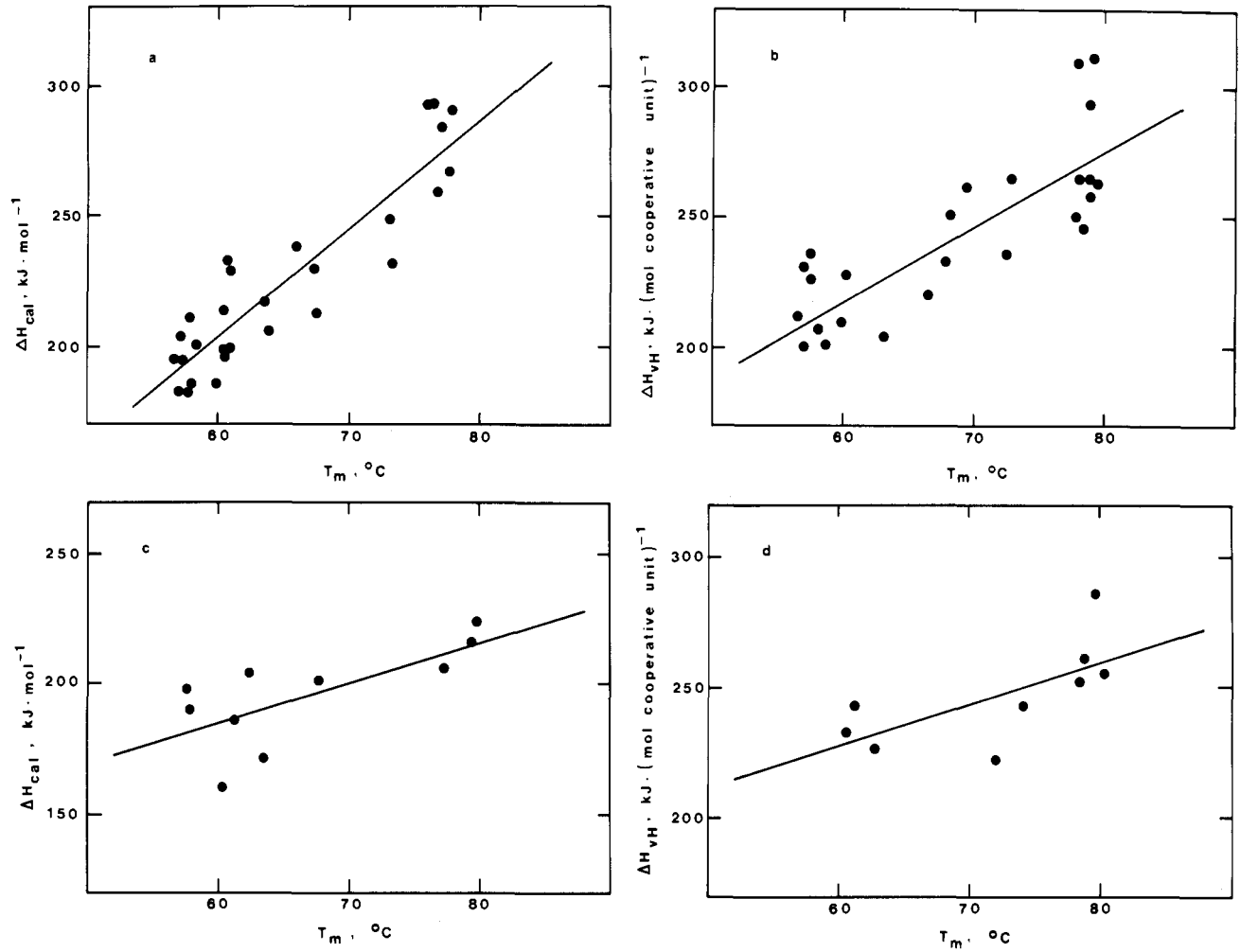

FIGURE 4: Variation with temperature of the calorimetric, ΔH_{cal}, and van't Hoff enthalpies, ΔH_{vH}, in 1 mM potassium phosphate and 0.1 M NaCl: (a) BPTI-RCAM; (b) BPTI-RCAM; (c) BPTI-RCOM; (d) BPTI-RCOM. The straight lines represent least-squares fits of the data. (b) Derived from optical measurements; (d) Derived from calorimetric measurements by using eq 1.

transition temperatures of the two analogues at pH = 2 are lower by approximately 30 °C.

The knowledge of the stabilization enthalpy ($\Delta H_{st} = -\Delta H_{cal}$) at the transition temperature (T_m) and its pH dependence [$dT_m/d(pH)$] allowed us to estimate the difference in the number of protons Δn bound to the native and denaturated forms of the two derivatives of BPTI, according to (Privalov et al., 1969)

$$[dT_m/d(pH)]_T = -\Delta n 2.303 R T_z^2/\Delta H_{cal} \quad (4)$$

which is valid for a two-state transition. T_m and T_z are in K. T_z is Θ_z + 273.15, using the Θ_z values given for eq 3. The change in the number of protons, Δn, on denaturation was 1.15 for BPTI-RCAM and 1.18 for BPTI-RCOM. The value of native BPTI was 1.1 (Moses & Hinz, 1983). This shows that the cleavage of the 14–38 disulfide bond does not significantly influence the proton release during denaturation.

Variation of Transition Enthalpies with Temperature: Experiments on BPTI-RCAM. Microcalorimetric measurements of the transition enthalpies, employing protein solutions of different pH values, resulted in the ΔH values given in Figure 4a. The experimental points can be approximated by the linear equation

$$\Delta H_{cal} = 4.19\Theta_m - 48.2 \text{ (kJ/mol)} \quad (5)$$

where Θ_m is in degrees Celsius.

The heat capacity change of 4.19 kJ/mol of BPTI-RCAM differs significantly from that of native BPTI, which was shown to exhibit an extraordinary small ΔC_p of 0.096 kJ/mol (Moses & Hinz, 1983), thus rendering ΔH_{cal} nearly temperature independent within the temperature range studied.

Because pH changes above pH 5 or below pH 2 do not affect T_m (see Figure 3), one should expect that ΔH_{cal}, as a function of pH, also remains constant. Figure 3c shows this assumption to be correct.

Variation of van't Hoff Enthalpies of BPTI-RCAM with Temperature. The van't Hoff enthalpies, obtained from the calorimetric curves, are a measure of the sharpness of the transition. The ratio of the van't Hoff and the calorimetric ΔH values provides a measure of cooperativity. For RCAM the ratio was found to be $\Delta H_{vH}/\Delta H_{cal} = 1.1 \pm 0.1$ over the whole transition range. This finding demonstrates that no thermodynamically relevant intermediates occur and that the transition is very nearly of two-state nature. This conclusion is supported by the results of the UV studies exhibited in Figure 4b. The experimental ΔH_{vH} can be approximated by

$$\Delta H_{vH} = 2.9\Theta_m + 44.6 \text{ (kJ/mol)} \quad (6)$$

Although there is no complete correspondence between the calorimetric and optical studies, the agreement is fair in view of the greater difficulties involved in assigning unique base lines to the UV transition profiles.

Experiments on BPTI-RCOM. In the experiments involving BPTI-RCOM, we tested also a 50 mM potassium phosphate buffer containing 50 mM NaCl; however, the results were identical with those obtained with 1 mM potassium phosphate–0.1 M NaCl buffer.

Table I: Selected Thermodynamic Stability Parameters of Native BPTI, BPTI-RCAM, and BPTI-RCOM[a]

	Θ (°C)	pH	BPTI	BPTI-RCAM	BPTI-RCOM
ΔH_{st} (kJ/mol)	25		−285.3	−56.6	−131.3
ΔH_{st} (kJ/mol)	110		−293.47	−412.7	−263
Δh_{st} (J/g)	25		−43.8	−8.7	−19.8
Δh_{st} (J/g)	110		−45.06	−63.4	−39.7
ΔS_{st} [J/(mol·K)]	25	2	−791.5	−148	−385
ΔS_{st} [J/(mol·K)]	110	2	−809.8	−1199	−770
Δs_{st} [J/(g·K)]	25	2	−0.122	−0.022	−0.06
Δs_{st} [J/(g·K)]	110	2	−0.125	−0.181	−0.116
ΔS_{st} [J/(mol·K)]	25	5	−757	−111	−350
ΔS_{st} [J/(mol·K)]	110	5	−780	−1160	−740
Δs_{st} [J/(g·K)]	25	5	−0.116	−0.017	−0.059
Δs_{st} [J/(g·K)]	110	5	−0.120	−0.175	−0.112
ΔG_{st} (kJ/mol)	25	2	−49.3	−12.6	−16.6
ΔG_{st} (kJ/mol)	110	2	+19	+46.3	+33.3
ΔG_{st} (kJ/mol)	25	5	−59.7	−23.5	−26.2
ΔG_{st} (kJ/mol)	110	5	+5.7	+32.4	+20.8
Δg_{st} (J/g)	25	2	−7.57	−1.9	−2.1
Δg_{st} (J/g)	110	2	+2.92	+7.0	+5.03
Δg_{st} (J/g)	25	5	−9.17	−3.55	−3.96
Δg_{st} (J/g)	110	5	+0.875	+4.9	+3.14
K	25	2	4.33×10^8	161	810
K	25	5	2.88×10^{10}	1.3×10^4	3.9×10^4

[a] ΔG_{st}, ΔH_{st}, and ΔS_{st} refer to molar quantities, while Δg_{st}, Δh_{st}, and Δs_{st} denote specific quantities. K is defined as (native state)/(denatured state).

Whereas the variation of T_m with pH is very similar to that found for BPTI-RCAM, the dependence on temperature of the transition enthalpy of BPTI-RCOM is significantly different, as shown in Figure 4c. At Θ_m = 59 °C (pH 2) both derivatives require an enthalpy of about 200 kJ/mol for unfolding, while at 80 °C ΔH of BPTI-RCAM is 287 kJ/mol; that of BPTI-RCOM, however, is only 217 kJ/mol. This difference is caused by the lower temperature dependence of the transition enthalpy of BPTI-RCOM which is described by

$$\Delta H_{cal} = 1.55\ \Theta_m + 92.5\ (\text{kJ/mol}) \quad (7)$$

The variation with temperature of the van't Hoff enthalpies (Figure 4d) obtained from the calorimetric transition curves can be summarized by

$$\Delta H_{vH} = 1.57\Theta_m + 134\ [\text{kJ/(mol of cooperative unit)}] \quad (8)$$

Calculation of Thermodynamic Stability Parameters. The knowledge of the thermodynamic parameters ΔH, T_m, and ΔC_p permits model-free calculations of the Gibbs energy function of the BPTI derivatives, according to

$$\Delta G_{st}(T) = -\Delta G_d(T) = -\Delta H_d(1 - T/T_m) - \Delta C_{p,d} T \ln (T_m/T) + \Delta C_{p,d}(T_m - T) \quad (9)$$

where T_m is the respective temperature of half-denaturation at a fixed pH value in absolute units, ΔH_d is the calorimetrically determined molar denaturation enthalpy at T_m, and $\Delta C_{p,d}$ is the corresponding temperature coefficient of ΔH_d. Entropy values have been obtained from the relationship $\Delta G_{st} = \Delta H_{st} - T\Delta S_{st}$. The subscripts refer to stabilization parameters.

Assuming that the calorimetric parameters are independent of concentration and can be equated with standard quantities, an equilibrium constant K for the folding reaction can be estimated, from the relationship $\Delta G_{st}° = -RT \ln K$.

A summary of selected thermodynamic stability parameters is presented in Table I. Figure 5 illustrates the temperature dependence of the Gibbs energy, enthalpy, and entropy of BPTI-RCAM and BPTI-RCOM at pH 3 to allow a comparison with the data on BPTI (Moses & Hinz, 1983).

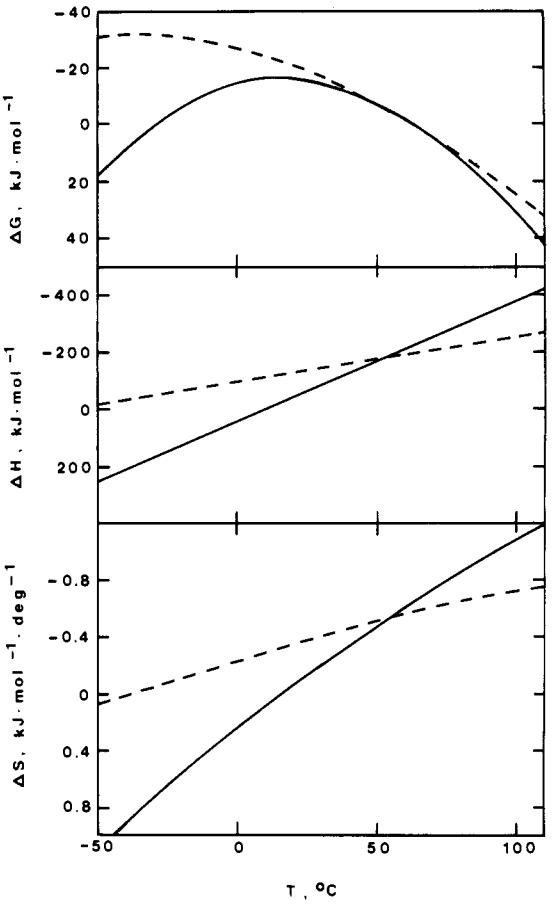

FIGURE 5: Dependence on temperature of molar Gibbs energies, enthalpies, and entropies of stabilization of BPTI-RCAM and BPTI-RCOM (dashed lines) in 1 mM potassium phosphate buffer, containing 0.1 M NaCl, pH 3.

Effects of Repeated Thermal Unfolding: (a) Second and Further Calorimetric Heatings of BPTI-RCAM. Integration and shape analysis of transition curves of samples subjected to two or more heating and cooling cycles provided the following picture. The van't Hoff enthalpy which is independent of concentration was found to be practically identical with that observed in the respective first heatings. The observed dependence on temperature can be described by

$$\Delta H_{vH} = 2.53\Theta_m + 91.4\ (\text{kJ/mol}) \quad (10)$$

This fact, and the identity of the dependence of T_m on the pH in the first and second measurements, illustrates that the substance, whose transition is observed in the second and following runs, is identical with the original sample. It has been perfectly refolded; only the amount has been reduced by each heating cycle.

As mentioned before, the calorimetric enthalpy, ΔH_{cal}, of BPTI-RCAM increased with increasing T_m according to eq 5. However, in the second measurements $\Delta H_{cal}'$ decreases with increasing T_m, according to (Figure 6a)

$$\Delta H_{cal}' = -4.26\Theta_m + 418\ (\text{kJ/mol}) \quad (11)$$

This result suggests that the actual heat required to denature the sample is continuously decreased with each additional heating. To understand this finding Figure 6b,c shows successive calorimetric scans of BPTI-RCAM at pH 2.5 and pH 5. At low pH (Figure 6c) the shape of the transition curves is practically maintained and only the area under the peak decreases, whereas at high pH (Figure 6b) the original peak around 76 °C is progressively decreased and a low-temperature peak appears at approximately 52 °C. Both results indicate

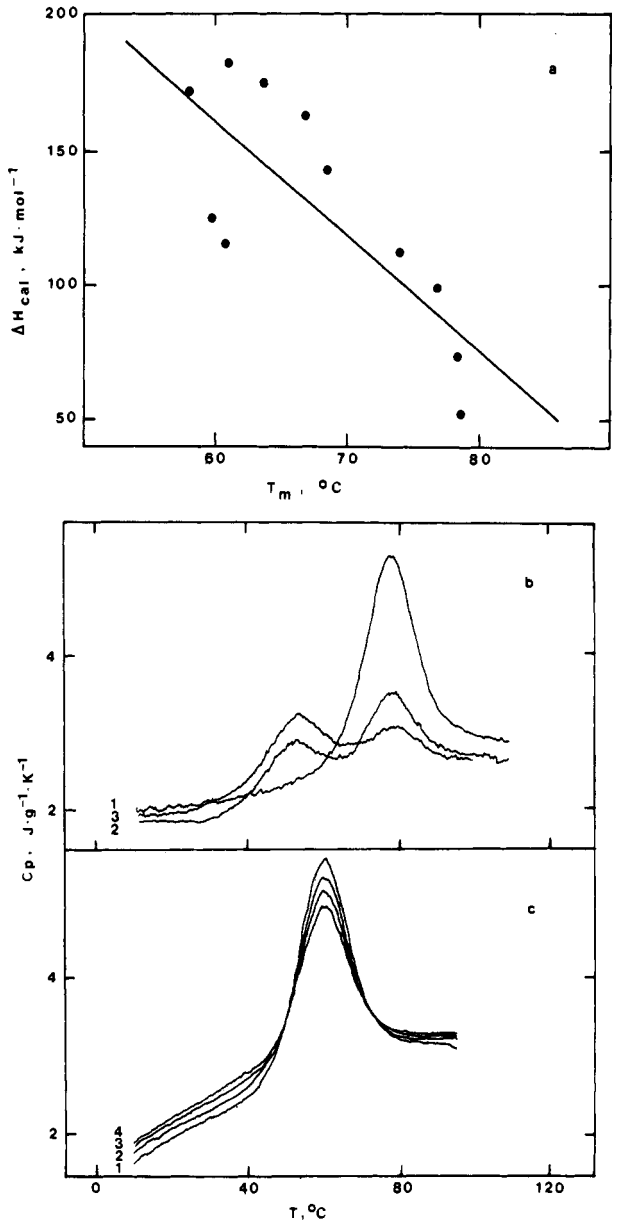

FIGURE 6: Effects of repeated unfolding on transition reversibility. (a) Variation with temperature of the calorimetrically observed enthalpy of second heatings of BPTI-RCAM. (b) Transition profiles of BPTI-RCAM in 1 mM potassium phosphate buffer, containing 0.1 M NaCl, pH 5; three successive runs of the same sample are shown. (c) Transition profiles of BPTI-RCAM in 1 mM potassium phosphate buffer, containing 0.1 M NaCl, pH 2.5; four successive runs of the sample are shown. The numbers in (b) and (c) refer to the sequence of calorimetric scans.

that the original amount of native protein is progressively reduced by the heating cycles.

(b) Purity Tests of BPTI-RCAM and BPTI-RCOM after Repeated Heating. To check whether the proteins were altered by repeated heating and cooling cycles, we studied samples before and after the measurements by HPLC, native gel electrophoresis, and SDS gel electrophoresis.

HPLC Experiments. To test whether repeated heatings resulted in partial hydrolysis of the protein associated with the appearance of free amino acids or small peptides, we performed the following experiment. Aliquots of solutions of BPTI-RCAM and BPTI* (a BPTI derivative that contains the three native disulfide bonds but a cleaved 15–16 peptide bond) were subjected for 60 min to 100 °C at pH 7. Both the native and heated samples were then dialyzed for 18 h by using a benzoylated dialysis tubing of cutoff M_r 2000. This tubing prevents the proteins from crossing the membrane but permits free dialysis of amino acids or small peptides. The dialysate was concentrated at 55 °C and then subjected to HPLC analysis employing a RP18 column (pore size 10 nm; Serva). The pattern of the peaks was identical for the heated and native samples. This result suggests that no major degradation into amino acids or small peptides has occurred as a result of heating.

Gel Electrophoresis. Probes of the native proteins taken before the measurements showed sharp bands, while heated samples no longer yield sharp bands, but blurred ones. The width of the bands increases with the number of heating cycles undergone by the sample.

SDS Gel Electrophoresis. In the presence of SDS a different result was obtained. Untreated and heated samples move the same distance. The heated samples showed no additional or broadened bands.

Discussion

These studies on BPTI derivatives continue an attempt to establish experimentally thermodynamic stability parameters by investigating a well-characterized protein. This approach might be generally useful for both the understanding and future predictive methods of protein structure and function on the basis of sequence information. The small size and the extreme specific stability of native BPTI render the protein a particularly good model system, where the relative effects of various specific alterations, such as changes in the number of disulfide bridges, single amino acid modifications, introduction of charges, exchange of amino acids, or covalent bond cleavage, will be particularly pronounced.

Reversibility of the Transition. As Figure 6 illustrates, the BPTI analogue shows no perfect reversibility of the transition. The degree of nonreversibility increases with increasing pH. This finding can be understood, if one recalls that BPTI is strongly positively charged at low pH values. With increasing ionization of carboxyl groups at higher pH values, repulsion of the unfolded chains will be reduced with a concomitant increase in the tendency to either refold incorrectly or aggregate. Both processes are probably responsible for the blurred bands in native gel electrophoresis. The irreversibility of the transitions does not interfere with the correctness of the thermodynamic parameters derived from the first heating curves, since incorrect refolding or aggregation appear to occur during the cooling cycle that lasts for approximately 1 h after completion of the measurement due to the adiabatic protection of the calorimetric cells. This can be directly derived from inspection of the calorimetric transition curves, since aggregation phenomena involving heat effects would manifest themselves by strong changes in the slope of the final base line. No changes have been seen in the curves that would indicate aggregation reactions during the first heating.

Role of Disulfide Bonds and Extra Charges in BPTI. The significance of disulfide bridges is immediately illustrated by the drastic decrease in stabilization enthalpy and Gibbs energy resulting from modification of the cysteines in positions 14 and 38. At pH 2 absence of this disulfide bond reduces ΔH_{st} from approximately −290 kJ/mol to about −200 kJ/mol for both derivatives. This decrease in stabilization enthalpy is associated with a decrease of the Gibbs energy of stabilization at 25 °C, pH 2, by approximately 70%, as the values in Table I demonstrate. It should be noted that on the basis of laser Raman and NMR studies (Brunner et al., 1974; Snyder et al., 1976; Stassinopoulou et al., 1984; Wagner et al., 1979a; Wagner et al., 1984) BPTI-RCAM and BPTI-RCOM have no significantly altered overall solution structure in comparison to native

BPTI. Thus the residual two disulfide bonds and the noncovalent interactions are sufficient to maintain the overall three-dimensional structure as seen by these methods. However, thermal stability is obviously greatly reduced in comparison to that of native BPTI, which clearly proves that the interactions and concomitantly also the structure of the BPTI analogues must be different from that of native BPTI. However, as emphasized repeatedly (Hinz, 1983) in proteins, such energy changes can be distributed among a large number of weak interactions. Therefore, they need not manifest themselves in gross structural changes that would be visible in NMR or X-ray studies.

While native BPTI was characterized by an unusually small temperature dependence of the stabilization enthalpy, both BPTI-RCAM and BPTI-RCOM exhibit a larger ΔC_p value characteristic of small globular proteins (Privalov, 1979). However, an influence of the dissociable carboxyl groups in positions 14 and 38 on the energetics is still evident in the temperature dependence of the stabilization enthalpies (Figure 4). While BPTI-RCAM, having uncharged amide groups in positions 14 and 38, shows the large ΔC_p characteristic of small globular proteins (Privalov, 1979) that generally has been ascribed to hydrophobic hydration, BPTI-RCOM displays a lower variation of ΔH with temperature.

At present no rationalization of this result in molecular terms can be envisaged. The finding emphasizes, however, the need for a clearer picture of the contributions of electrostatic interactions to the overall stability of proteins.

As a consequence of the difference in ΔC_p, the variation with temperature of the Gibbs energy of stabilization is also different for BPTI-RCAM and BPTI-RCOM. Over the whole temperature range BPTI-RCOM is either equally or more stable than BPTI-RCAM.

The difference in ΔG_{st} at 25 °C, pH 5 (Table I) of −2.7 kJ/mol must be ascribed to the presence of the charged carboxyl groups. This is a surprising result, since at first glance one would expect a reduction of stability due to repulsion of the two contiguous negative charges. As a matter of fact, such a reduction of energetic interactions is manifest above 50 °C (Figure 6), where BPTI-RCOM requires a smaller enthalpy input for denaturation. Nevertheless, the Gibbs energy of stabilization of BPTI-RCOM is also more negative above 50 °C than that of BPTI-RCAM, due to the less positive entropy change on unfolding that BPTI-RCOM experiences (Table I).

The present result is in excellent agreement with results obtained by Wagner et al. (1979b) on the basis of NMR studies. Wagner et al. also observed a stabilizing effect per mole of charged group of approximately 1–2 kJ at 77 °C.

However, our studies provide additional information. As can be seen from Table I, ΔG_{st} at 25 °C, pH 2, for BPTI-RCOM is −4 kJ/mol more negative than the corresponding value for BPTI-RCAM. The comparison of the ΔG_{st} values of pH 2 and pH 5 suggests that the existence of negative charges of the carboxyl group at pH 5 is actually associated with a destabilizing effect when compared to the situation with uncharged carboxyl groups at pH 2.0. The creation of the two negative charges at pH 5 causes a decrease in the stability difference between BPTI-RCOM and BPTI-RCAM of 1.3 kJ/mol. However, the overall effect of the introduction of the two negative charges into positions 14 and 38 of BPTI is still a stabilization, as the comparison of the ΔG_{st} values at 25 °C, pH 5, for BPTI-RCOM and BPTI-RCAM shows.

In Table I equilibrium constants for the folding reaction have been given. These numbers refer to an all or none process and are overall equilibrium constants. It is, however, striking that the values given for BPTI-RCAM at 25 °C and pH 5 are surprisingly similar to equilibrium constants k_1/k_2 derived for local structure fluctuations on the basis of NMR studies (Wagner et al., 1984). Since the conditions under which the NMR studies were performed are rather similar, the coincidence of the overall and the local equilibrium constants implies that in BPTI-RCAM these processes may be correlated. In contrast, for native BPTI there is a large difference between the local and the overall equilibrium constants. Obviously the overall stability as afforded by the presence of all three disulfide bonds permits local fluctuations of single groups without allowing the global all or none transition to proceed.

It had been pointed out previously (Moses & Hinz, 1983) that BPTI is a good example for the verification of theoretical treatments (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965) which showed that in cross-linked chains a contribution to stability results from the lowering of the configurational entropy of the random state. It has been recently pointed out by Roder et al. (1985a) that on the basis of NMR studies thermally unfolded BPTI analogues are identical in their deuterium exchange pattern to thermally unfolded molecules to which 3 M Gdn·HCl had been added. This result puts a preliminary end to the old debate about whether there is a difference between thermally unfolded proteins and those unfolded by Gdn·HCl (at least up to 3 M). Thus one can assume that at 110 °C BPTI-RCAM and BPTI-RCOM exist in an unfolded state. A comparison of the corresponding specific entropy changes Δs_d at 110 °C (Δs_{st} values in Table I with opposite sign) provides important insight into the applicability of the theories of cross-links referred to before. The specific denaturational entropy change at 110 °C, pH 5, of BPTI-RCAM, Δs_d = 0.175 J/(g·K), is even larger than the average value observed with small globular proteins [Δs_d = 0.149 J/(g·K)] (Privalov, 1979). BPTI-RCOM, however, which differs from BPTI-RCAM only by the presence of the charges, has a smaller specific denaturational entropy change under identical conditions of Δs_d = 0.116 J/(g·K). These results illustrate quite clearly that the theoretical considerations are only valid for proteins that do not differ in their charge pattern. If they differ, as BPTI-RCAM and BPTI-RCOM do, quite unexpected effects may occur that alter the thermodynamic quantities in an unpredictable manner.

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Complementary DNA Cloning of Complement C8β and Its Sequence Homology to C9†

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ABSTRACT: The complete amino acid sequence of mature C8β has been derived from the DNA sequence of a cDNA clone identified by expression screening of a human liver cDNA library. Comparison with the amino acid sequence of C9 shows an overall homology with few deletions and insertions. In particular, the cysteine-rich domains and membrane-inserting regions of C9 are well conserved. These findings are discussed in relation to a possible mechanism of membrane attack complex formation.

Efficient lysis of biological membranes by complement is due to the sequential assembly of the terminal complement components into the pore-forming complex C5b·C6·C7·C8·$(C9)_n$. C5b-7 forms the first membrane inserting complex to which C8 must be added before polymerization of C9 at the site of the C5b-8 complex causes major membrane damage and cell lysis (Lachmann et al., 1970). C8 therefore plays an essential intermediary role.

Human C8 is a glycoprotein of M_r 150000 comprising three polypeptide chains organized into two subunits. The α-chain [molecular mass 64 kilodaltons (kDa)] is linked to the γ-chain (25 kDa) through a disulfide bridge, while the β-chain (64 kDa) is noncovalently bound to this complex (Kolb & Müller-Eberhard, 1976). Both α- and β-chains of C8 are essential for C8 activity, but no direct role for the γ-chain has so far been described. The β-chain mediates the binding of C8 to C5b-7 (Monahan & Sodetz, 1981; Brickner et al., 1985), while C8α appears to be more intimately integrated into the

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