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THERMAL STABILITY OF IMMOBILISED α-CHYMOTRYPSINOGEN

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Abstract

The unfolding of α-chymotrypsinogen covalently immobilized on silica beads has been studied by differential scanning calorimetry (DSC). The enzyme undergoes an unfolding transition which, unlike the free protein, cannot be approximated by a single two-state process. After immobilization, the unfolding is characterized by the presence of two partially overlapping transitions, both of them show two-state behavior. The two processes correspond to the separate unfolding of the two domains of the α-chymotrypsinogen molecule. The loss of cooperativity behavior is a consequence of the covalent immobilization. The two domains showed different thermal stability as functions of pH. One of them unfolded with a transition temperature T_{m_2} higher than T_m of the free enzyme, implying stabilization effect of immobilization. However, below pH 4.5, its native structure is lost. The other transition shows a remarkable pH-independent thermal stability from pH 2.5 to 7.0.

Keywords: α-chymotrypsinogen, microcalorimetry, protein DSC, protein immobilization, protein melting domains

Introduction

In aqueous solution small globular proteins of M_W<20–30.000 usually undergo a single cooperative unfolding transition where the entire protein molecule behaves as a single cooperative unit [1]. This means that only two conformations, i.e. the folded and unfolded states, are significantly populated during the unfolding (or folding) process. This behavior has been observed in cases where visual inspection of the X-ray diffraction data clearly suggests a bilobal structure of the protein molecule. Sufficient interactions occur between domains so that they unfold cooperatively. On the other extreme situation, where no interdomain interactions are present, two or more domains may unfold independently of each other. The origin of the cooperative behavior may be related to the solvent exposure of regions of the protein molecule complementary to those parts which undergo unfolding [2, 3]. The free energy of solvatation of these complementary parts drastically reduces the probability of partially-folded

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intermediates. This fact may be correlated to the interdomain contacts whose number and degree of matching define the magnitude of the energetic term. Protein domains can also be defined not only on a structural basis but also from a kinetic and dynamic point of view. From H-exchange kinetic experiments, it has been observed that some regions of protein, not necessarily continuous in space or sequence, are characterized by very low H-exchange rates: they form 'knots' around which the protein ties itself up [4, 5]. The knots contribute both to kinetic as well as thermodynamic stability and correspond to structural elements such as helix-strand portions of the protein molecule. More disordered regions of the protein, which exchange proton with faster rates, are the 'matrices' which regulate the cooperative behavior of the knots.

In the last few years, experimental conditions have been found where small globular proteins show a multicomponent unfolding transition and do not obey to the two-state law. In this case intermediate states of the unfolding pathway are present which are characterized by a domain in the folded state and another in the unfolded state. Decoupling of the protein domains may therefore be induced in appropriate conditions where changes of the domain intrinsic stability or the interdomain forces brings the probability of the intermediate partially unfolded state to its maximal value. This has been observed experimentally for ribonuclease A (RNase) in 50% ethanol [6], where progressive domain decoupling is observed upon lowering the pH. Phosphoglycerate kinase [2] and β-lactoglobulin [7] unfold in a single two-state process at high temperature as studied by DSC, but the transition splits into two parts due to domain decoupling during cold-induced denaturation.

It has also been observed that immobilization induces decoupling of the RNase structural domains [8] which otherwise unfold as a single cooperative unit (9). It is interesting to note that, after immobilization, enzymes such as RNase and α-chymotrypsin [10], retain their activity even if the unfolding mechanism is significantly altered [8]. These changes may be appreciated only when kinetic or activity information are based on structural energetic analysis. Therefore, immobilization may be considered a useful tool to gain insight on interdomain forces responsible for cooperative behavior and to stabilize intermediates of the unfolding pathway.

The interaction between part of the immobilized RNase molecule and the support surface is responsible for the observed decoupling of the DSC unfolding transition. This effect has been further investigated by studying the unfolding of the covalently immobilized α-chymotrypsinogen (CTG). As in the case of RNase, the unfolding is significantly altered with respect to the free protein. Two DSC transitions are observed, reflecting domain decoupling.

It was found that one of the two protein domains is stabilized with respect to the other after immobilization. The asymmetrical effect contributes to lower the cooperativity of the transition. The two domains have different pH dependence. One domain is in the melted state below pH 4.5. The disappearance of the domain unfolding can be correlated with the ionization of the negatively-charged aminoacid side chains.

Materials and methods

α-Chymotrypsinogen was purchased from Sigma Chemical Co. (USA) and used without further purification. Controlled Pore Carrier (CPC) silica beads aminopropyl derivative, pore size 375 Å, were obtained from Fluka, Chemical Co. (CH).

Immobilization of CTG on silica beads was carried out as follows: the beads were hydrated extensively and washed with 0.1 M phosphate buffer, pH 7.0. 10 g of beads (dry mass) were activated with 100 ml of glutaraldehyde (Fluka), 10% v/v for 45 min at 4±0.5°C and the excess of aldehyde was removed by washing the solid phase with the same buffer. 212 mg of CTG, dialysed in 36 ml of 0.1 M phosphate buffer, pH 7.2 were gently stirred with the activated CPC-silica beads at 4±0.5°C overnight. The amount of immobilized protein was 94%, corresponding to 19.9±0.1 mg per g of dry support, determined either by difference between the amount of protein in solution before and after immobilization either after hydrolysis of immobilized sample at 110°C in HCl by the Lowry–Folin method.

Differential scanning calorimetry experiments were performed with a MC1 calorimeter (MicroCal Inc, Northampton, MA, USA) equipped with solid samples cells. Usually 0.26 g of protein/CPC silica, corresponding to 5.17 mg of protein was introduced in the cell to perform a calorimetric experiment. Baseline measurements were performed with untreated or glutaraldehyde – activate CPC glass beads in absence of protein. T_m of the unfolding transition was independent on the scanning rate from 20–60°C h^{-1}.

Deconvolution and fitting analysis of DSC data was computed according to the procedure reported by Freire and Biltonen [11, 12] using the ORIGIN Software (MicroCal Inc. USA). According to this procedure the enthalpic contribution (the calorimetric contribution measured as ΔH_c as well as the van't Hoff enthalpy calculated from the curve, ΔH_{vH}) associated with the unfolding process were obtained.

The pH dependence of the CTG unfolding was studied by using different buffer systems as a function of pH. In the pH range 2.5–3.5, 4.0–5.0 and 5.5–7.0 HCl-glycil-glycine, Na-acetate and Na-phosphate buffers 0.1 M were used, respectively.

CTG structure was elaborated with Insight II/Discover version 2.0.0. (Biosym Technologies Inc., San Diego, USA).

Results

α-Chymotrypsinogen immobilized on silica beads undergoes a single cooperative unfolding transition as monitored by DSC. In Fig. 1 the actual DSC recording of the protein unfolding as well as the baseline are shown. A slight slope characterized the baseline as measured with the sample and reference cells filled with the silica beads without protein and with buffer, respectively. An unusual slight increase of the signal as a function of the temperature is observed which is not present in absence of the beads. The baseline slope increases slightly by lowering the pH of the buffer (not shown). The protein unfolding curve is characterized by a marked increase of the heat capacity of the protein in the unfolded state with respect to that of the folded form.

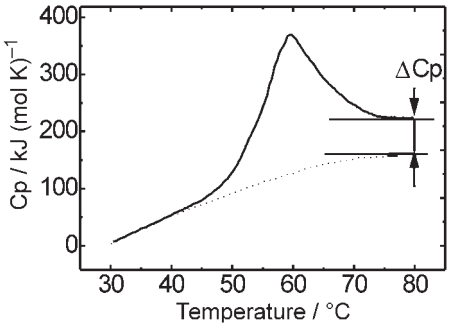

Fig. 1 Excess specific heat capacity, C_p, *vs.* temperature profile of α-chymotrypsinogen (CTG) immobilized on 3-aminopropyl-CPC-silica carrier (CPC-silica). Experimental curve (solid line); base line with CPC-silica without the protein (dashed line): 0.1 M acetate buffer; pH=5.5; 0.26 g of dry beads containing 5.17 mg of immobilized enzyme

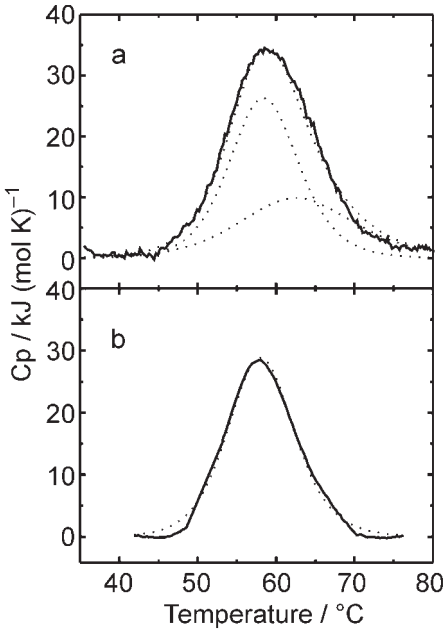

Fig. 2 Temperature dependence of the excess specific heat capacity of CTG immobilized on CPC-silica after subtraction of base line:
a) experimental curve (solid line); the dotted line is the best fit with two independent two-state transition: 0.1 M acetate buffer, pH=6.0;
b) only one transition fits the experimental curve: 0.1 M acetate buffer, pH=4.0

Therefore, a positive heat capacity change of unfolding, ΔC_p, accompanies the denaturation of the immobilized CTG as in the case of the free enzyme [13].

In Fig. 2a the excess molar heat capacity of immobilized CTG is shown as a function of the temperature at pH 6 after baseline subtraction (including the heat capacity change).

The deconvolution analysis of the DSC curve indicates that the transition has to be approximated by two independent processes in order to obtain a satisfactory fit of the experimental curve.

The presence of two processes accompanying the unfolding of CTG was observed in the pH range 5–7. Below pH 5, already at pH 4.5 the higher-temperature peak reduces significantly its thermal contributions. Consequently only one peak was sufficient to obtain the best fit of the unfolding curve. As an example, in Fig. 2b the excess molar heat capacity of CTG as a function of the temperature at pH 4 is shown. The presence of one peak was observed from pH 4.5 to 2.5. In Fig. 3 the unfolding transitions of the immobilized CTG are summarized from pH 7 to 2.5.

The thermodynamic parameters associated with the unfolding of the immobilized CTG are listed in Table 1. From the deconvolution analysis, the overall and peak calorimetric enthalpy changes of unfolding, ΔH_{tot} and ΔH_1, ΔH_2, respectively, and the transition temperatures T_{m_1} and T_{m_2} were calculated as a function of pH. Between pH 5 and 7, ΔH_{tot} is almost pH-independent, i.e. it has the same value within 6%.

Table 1 Thermodynamic parameters associated with the unfolding of the immobilised α-chymotrypsinogen

Chymotrypsinogen-CPC						
pH	T_{m_1}/°C	ΔH_1/kJ mol^{-1}	T_{m_2}/°C	ΔH_2/	ΔH_{tot}/	$\Delta C_{p_{tot}}$/
				kJ mol^{-1}		
7.0	58.3	317.0	62.9	198.5	515.0	7493.0
6.0	59.7	310.0	63.0	189.0	498.5	6764.0
5.5	59.0	310.0	65.1	183.0	493.0	6089.0
5.0	58.8	310.0	64.8	178.0	487.5	5436.0
4.5	58.9	325.0				4520.0
4.0	58.1	326.6				3653.0
3.5	57.8	320.5				2740.0
3.0	52.9	307.0				913.3
2.5	47.7	294.0				304.4

Errors: T_m=±0.4; ΔH=±1%; $\Delta C_{p_{tot}}$=±20%

At pH 7, ΔH of unfolding is 515±30 kJ mol^{-1}, slightly smaller than that associated with the unfolding of the free enzyme (ΔH_{tot}=590 kJ mol^{-1}). It should be noted that T_{m_1} and T_{m_2} in the case of the immobilized enzyme at pH 7 are 58.3°C and 62.9±0.4°C, respectively, whereas T_m for the free enzyme is 58.5±0.3°C. Clearly, part of the enzyme molecule has been thermally stabilized by immobilization.

In Fig. 4a ΔH_1, ΔH_2 and ΔH_{tot} are plotted as a function of pH. This figure shows an abrupt change of the pH dependence around pH 4.5–5. Below this pH the higher-

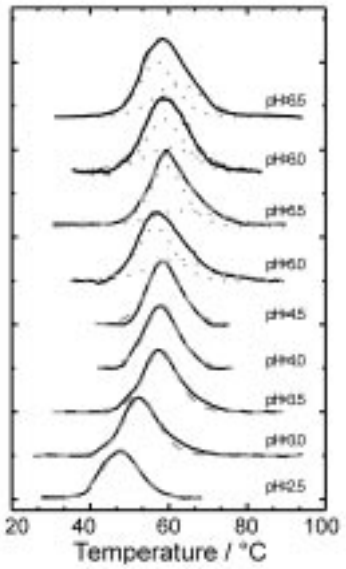

Fig. 3 Temperature dependence of the excess specific heat capacity of CTG immobilized on CPC-silica at several pH: experimental curve (solid line); the dashed line is the best fit with two independent two-state transition

temperature peak reduces its thermal content and the unfolding transition is characterized by the presence of the low-temperature process. The domain which unfolds at lower temperature has a thermal stability almost independent on pH (from pH 7 to 2.5). In Fig. 4b T_{m_1} and T_{m_2} are plotted as functions of pH. Both of them vary little with pH, except at very low pH, i.e. below pH 3. The values of T_{m_2} are higher than those associated with the unfolding transition of the free enzyme at a given pH [13]. T_{m_1} associated with the remaining peak below pH 4.5 has a less pronounced pH dependence than that of the free enzyme.

In Fig. 4c ΔH_1 and ΔH_2 are plotted as function of the respective T_m values. Within the experimental uncertainty, the data associated with the low-temperature process lie on a straight line with a positive slope. This implies that at least part of the heat capacity change of unfolding (about 1680±350 J mol^{-1} K^{-1}) is associated with the unfolding of the corresponding protein domain. In Fig. 4d ΔC_p is plotted as a function of the pH.

The fraction of the intermediate state molecule (I), formed by the first domain unfolded and the second one still in native state, can be calculated according the equation:

$$x_i = \frac{\exp\left(-\frac{\Delta^i_N G_i}{RT}\right)}{Q}$$

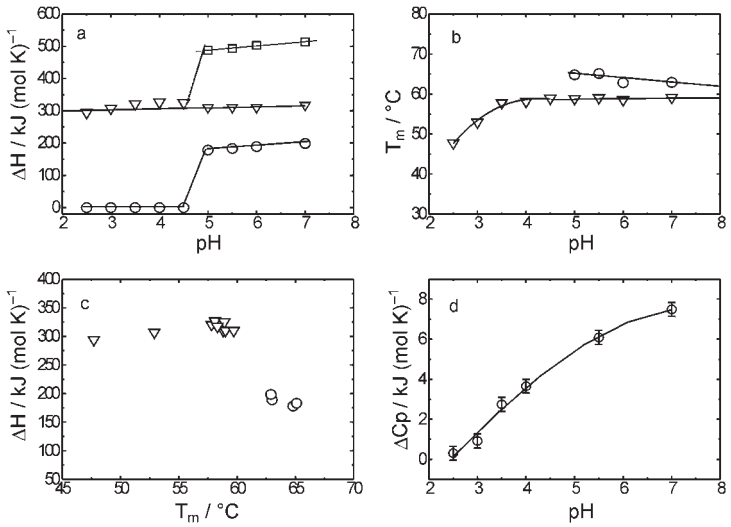

Fig. 4 Values calculate for the first transition: in this buffer conditions only one transition was necessary to fit the experimental curves (∇); two transitions postulated for the first fitting (O); second fitting (∇); total as sum of both transitions ( )
a) Enthalpy change of unfolding transition *vs.* pH
b) T_m *vs.* pH
c) Enthalpy change *vs.* T_m
d) Heat capacity change between folded and unfolded protein at different pH (O). Error bars is the average of 2–3 experimental curves

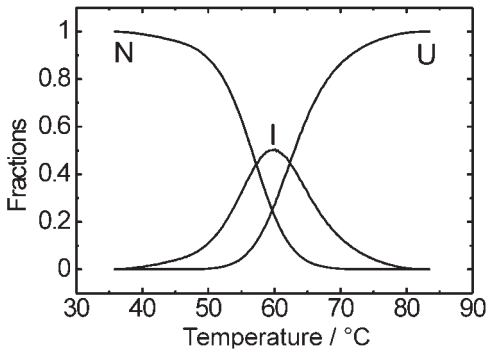

Fig. 5 Temperature dependence of the populations of native (N), unfolded (U) and intermediate (I) states for the DSC profile of the experiment carried out at pH=6.0

where $\Delta^{i}_{N}G_{i}$ is the Gibb's energy difference between the i^{th} intermediate and the reference states [11, 12, 26], and Q is the partition function:

$$Q=1+\sum_{i=1}^{n}\exp\left(-\frac{\Delta^{i}_{N}G_{i}}{RT}\right)$$

As showing in Fig. 5, two independent state transitions can be assumed because the presence of a significantly populated intermediate state (I) in the denaturation equilibrium.

Discussion

By examination of the X-ray diffraction data, it may be concluded that α-chymotrypsinogen polypeptide chain is folded into two structural domains.

The two regions may be defined by drawing an ideal plane through the catalytic triad of the active site cleft of the CTG molecule. The first region is formed by the polypeptide chain from residue 28–30 through residue 118–120. The second one includes the residues from 1 to 28–30 and from about 120 to 235–237, the last 8 residues forming an α-helix which may be considered an independent part (Fig. 6).

Both domains have a similar antiparallel β-barrel structure formed by six β-strands [15].

The thermal unfolding of CTG has been studied before by DSC in aqueous solution [14]. Analysis of the DSC data showed that the unfolding transition is well ap-

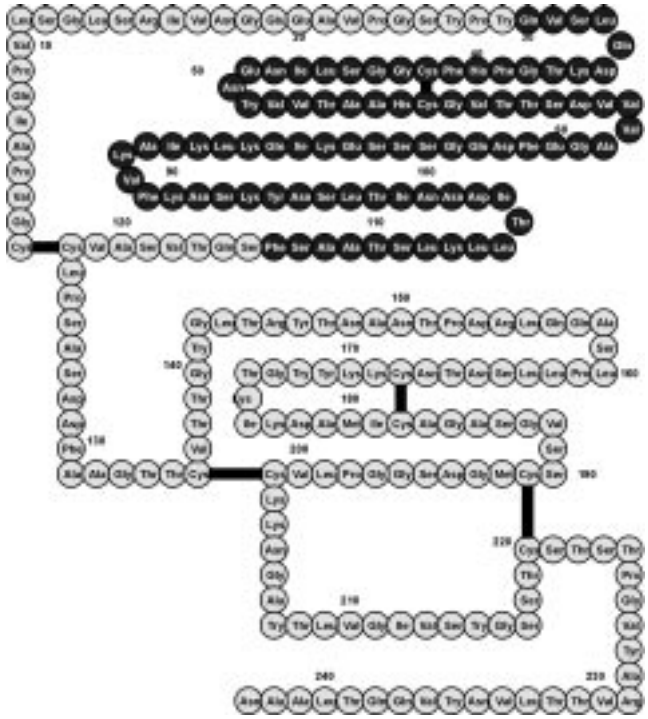

Fig. 6 The primary structure of CTG. The figure is derived from that reported by Fersht [25] by courtesy of Blow. The code used for amino acids is the same of the original figure where tryptophan is reported 'try' instead of 'trp'. The different colors (light and dark) represent the two thermodynamics domains of protein as described in the text

proximated by a single cooperative two-state process. In spite of the presence of two well defined structural regions, the unfolding of the molecule occurs as a single process due to the highly cooperative interaction of the two domains. The interdomain coupling energy is high enough to make the two domains behave as a single cooperative unit.

The two domains are not quite symmetrical and differences may be noted at structural level. The α-NH_2 terminal domain (domain 2) has 4 S–S bridges, whereas the domain 1 has 1 S–S bridge. Domain 1 has a higher density of charged amino acids than domain 2 (10 Lys and Arg residues instead of 8, 6 Glu and Asp groups instead of 5; 3 more acidic residues Asp-102, Glu-70 and Asp-194 may be considered as located at the interdomain interface). Moreover, residues involved in the oxyion stabilization, main chain substrate binding and substrate specificity pocket are in the domain 2, whereas two residues (His-57 and Asp-102) of the catalytic triad are provided by domain 1 [16].

After immobilization, CTG undergoes an unfolding transition which, when studied by DSC, appears as two partially overlapping processes. Each of them is a two-state transition. The splitting of the unfolding curve into two peaks may be attributed to decoupling of the two protein domains. This assumption is based on the analogy with the analysis of the RNase transition, which was not determined by the presence of heterogeneous populations of immobilized molecules nor dependent on the length of the coupling space arm [8]. Moreover, in the case of RNase no heterogeneity of the biological activity was found, as the binding studies with 3'-CMP have shown [8]. Furthermore, also the kinetics of the biological activity of α-chymotrypsin immobilized in the same conditions [10], is well approximated by a single exponential behavior [19]. As a consequence of immobilization, domain uncoupling of CTG domains occurs and the presence of a stable partially folded intermediate, containing part of the molecule in the folded state and the other in the melted state, becomes significant during the transition (Results, Fig. 5). One of the two domains is stabilized with respect to the other. In fact, at pH 7 T_{m_2} is 4.6°C higher than T_{m_1} (Table 1) and 4.4°C higher than T_m of the free enzyme unfolding transition.

This effect is related to the presence of the chemical link(s) between protein and support and not to the presence of the carrier *per se.* Control experiments showed that the unfolding of CTG in the presence of the support without any covalent linkage is superimposible on that of the free enzyme.

Besides the effect of the chemical link with the support, interactions between protein molecule and carrier are largely present and influence domain stability. In the case of RNase, which is a highly hydrophilic protein, there is evidence that supports the notion that both electrostatic and hydrophobic forces are involved [8]. The surface of the silica beads is negatively charged above pH 6.8, the *pK* of the siolic group [17], and may have favorable electrostatic interactions with the positively charged protein (*pK* 9.2). By lowering the pH from 7 to 3, the charge density of both protein and silica surface decreases and this effect is concurrent with significant changes of ΔC_p (Fig. 4d) and with the disappearance of the protein domain.

However, CTG is more hydrophobic than RNase: the polar to apolar residues ratio is 0.83 instead of 1.73 as for RNase [18] and the number of apolar contacts per molecular mass unit is 0.094 instead of 0.066 for RNase [13].

Therefore, it is reasonable to assume that hydrophobic interactions are expected to play a more significant role in CTG interactions with the carrier. In order to support this hypothesis the unfolding of immobilized CTG was studied in the presence of 50% ethanol, since ethanol lowers the dielectric constant of the medium and weakens hydrophobic interactions. The unfolding is still a two-state transition, although shifted to lower T_m. Moreover, unlike the transition in the absence of ethanol, it is fully reversible, in the sense that the calorimetric ΔH is recovered almost completely after a second temperature scan on the same sample [19]. This suggest that the hydrophobic groups of the protein interior, exposed to the solvent after unfolding, interact with the carrier surface making the protein refolding impossible in absence of ethanol.

Before the completion of the decoupling, the collapse of the domain structure occurs. This can be associated with changes in the ionization state of the negatively charged amino acids which have a *pK* of 4–5. As mentioned above, the density of the negatively charged amino acids is different in the two domains. Moreover, the two domains do not have the same thermal stability, even in the case of the free enzyme, as deduced from denaturation studies on α-chymotrypsin [20].

It is interesting to note that the samples of immobilized CTG can be brought from pH 7 to 3 and then back to pH 7 without any change in the DSC behavior, i.e. two peaks are still present. This implies that, in the case of the enzyme interactions are fully reversible as a function of pH.

The overall ΔH of unfolding of immobilized CTG is lower (75 kJ mol^{-1} at pH 7) than that associated with the unfolding of the free enzyme. This difference is a composite quantity since it includes the effects of the protein-support interactions as well as changes in the interdomain forces, i.e. structural changes following immobilization.

Below pH 5, free CTG (but not chymotrypsin) exhibits an abnormal heat of protonation [21, 22]. The anomalous excess heat change (about 130 kJ mol^{-1} from pH 5 to pH 1.5) has been associated with a conformational change which accompanies the protonation of the approximately 10 –COOH groups. More specifically, a significant conformational change has been associated with the protonation of Asp 194 which is buried in the unprotonated form inside the protein molecule [23]. Asp 194 is involved in the proteolytic activation of CTG; it is surrounded by 5 symmetrical, tightly-associated water molecules which hydrate the salt bridge formed with Ile 16 [24]. Below pH 5, the immobilized CTG undergoes a significant molecular rearrangement with a concurrent loss of a structural domain. It seems that immobilization 'amplifies' the conformational effect of the protonation also observed in the case of the free enzyme. It should be noted that, in the case of free CTG, the presence of a large excess heat of protonation does not alter the two states character of the unfolding process [22].

If Asp 194 is indeed involved in these structural changes, it is possible to suppose that domain 2 the $-NH_2$ terminal region, which includes Asp 194, is that part of the protein which is mostly destabilized at low pH. Moreover, domain 2 includes the

two sites where peptide cleavage occurs during the CTG activation, suggesting a proteolytic susceptibility greater than the highly compact domain 1.

Which domain is stabilized after immobilization? From the pH dependence of ΔH and T_m, it can be argued that the domain which has the higher negatively charged amino acid density is the most sensitive to their ionization state of carboxylates. This may be the α-NH_2 terminal domain.

Moreover, this domain has more S–S bridges and, by analogy with RNase, may be more reactive towards the support through its terminal α-NH_2.

If this hypothesis is correct the domain which collapses as the pH reaches the *pK* of the negatively charged amino acid is domain 2 which is the most stable at higher pH.

It is possible to give structural identity to a thermodynamic domain? Since domain 1 is stable at low pH and constitutes a calorimetric unfolding unit, which part of the enzyme molecule corresponds to calorimetric domain 1? Although admittedly quite speculative, we can nevertheless hypothesize that a given set of amino acids belonging to the structural domain 1 must correspond to the calorimetric unfolding unit, i.e. an actual, independent thermodynamic entity. This aminoacid assembly must have the thermodynamic properties of a normal small globular protein. First, it should have an unfolding transition with the two-state character, as actually it was observed in the immobilized protein. Second, it should have another typical property of small globular protein: a plot of ΔH *vs.* T_m should give a straight line. This line should predict at 110°C a value of ΔH common to most of small globular proteins, as observed by Privalov [11]. The experimental evidence of this plot is empirical and the correct interpretation is not still clearly justified theoretically [27]. Nevertheless it can be used to obtain some insights on the structural properties of the proteins. The condition of Privalov's plot (data in the acid pH range) has been verified by choosing a well defined set of amino acids belonging to domain 1. It has been selected from the X-ray crystallographic structure and includes the portion of the molecule from residue 30 to residue 114. It starts from the beginning of the β-barrel region of domain 2 and ends up with the last of the six β-strands of the barrel core of the domain: this part

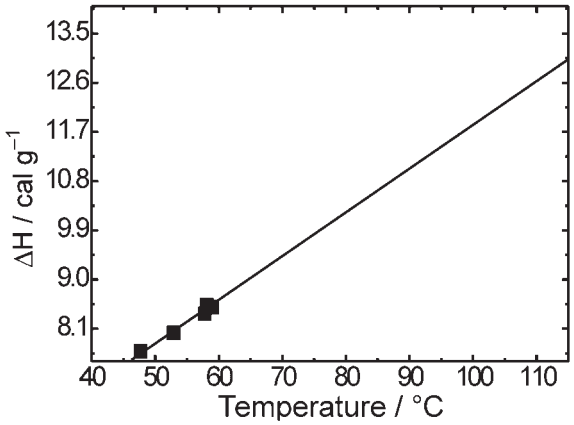

Fig. 7 Dependence of specific heat of unfolding on the temperature of transition

correspond to a relative molecular mass of M_r=9144 Daltons. Assuming that the thermodynamic properties of the domain remaining at acidic pH (Table 1) can be associated with the unfolding of this part of the molecule, DSC data can be normalized per g of the new unfolding unit and the enthalpy change can be plotted as a function of T_m. This plot, shown in Fig. 7, can be fitted by a straight line and a value of 12.6 cal g^{-1} (52.6 J g^{-1}) is found at 110°C, which agrees well with the value of 13.5 cal g^{-1} (56.4 J g^{-1}) found by Privalov for several globular proteins [13].

The slope of the curve is 3500 J mol^{-1} K^{-1}, which is in agreement with an average ΔC_p (Fig. 4d).

At pH 7, the overall ΔC_p is 7470 J mol^{-1} K^{-1}, about 60% of that reported in literature, i.e. 13300 J mol^{-1} K^{-1} (average value at pH 2.6) [14]. This observation suggests that the state of the immobilized CTG molecule may be different from that in the free enzyme. The difference involves subtle conformational differences, including domain decoupling and hydratation changes, which are induced by the interaction between protein molecule and support.

An estimate of the free energy of domain interaction ΔG_{12} may be obtained by using the following expression: $-\Delta G_{12}=\Delta H_1.(1-T_1/T_1^*)$ [6], where ΔH_1 is the enthalpy change of unfolding of domain 1 (310 kJ mol^{-1} at pH 6), T_{m_1} is the T_m in the uncoupled state of domain 1 (59.7°C) and $T^*_{m_1}$ is the T_m of domain 1 in the reference state (fully coupled as in the free enzyme, 58.5°C). A value of 6.3 kJ mol^{-1} is obtained, similar to that obtained with immobilized RNase [8].

In summary, it has been confirmed by studying the unfolding mechanism of CTG that covalent attachment to a support alters the thermal stability as well as the mechanism of unfolding of small globular proteins. Like RNase, CTG experiences the effect of immobilization as a change in the interdomain interactions and unmatching of interdomain contacts. These effects are strongly pH dependent: at low pH a partially folded intermediate is present, characterized by only one domain in the fully structured state.

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