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MELTING DOMAIN IN PROTEINS
Free and immobilized α-chymotrypsin

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Abstract

Recent progress in thermodynamic aspects of proteins, free or immobilized on solid support, are described. In agreement with results observed with Ribonuclease A [9], DSC analysis on α-chymotrypsin confirms a decoupling of melting domains with the immobilized protein in a large range of *pH*.

Keywords: enzymes, microcalorimetry, protein immobilization, protein melting domains

Introduction

The biotechnological applications of enzyme chemistry to industrial process often require the improvement of efficiency and stability of the biocatalysts. Industrial technology implies process conditions which are not conventional as far as the biocatalyst is concerned, including high substrate or reagents concentrations, presence of additives (either organic or inorganic) and temperatures as high as possible to speed up reactions. Therefore, the improvement of biocatalyst performance in such conditions has been attempted by means of *ad hoc* technologies, such as immobilization [1–3] or low-water systems [4] (organic solvents) and, in perspective, specific alteration of the polypeptide chain via genetic engineering [5] or chemical modifications [6]. Since immobilization often improves resistance to denaturation factors, it is a valid technological tool to optimize practical applications and performance. On the other hand, it can be also used to study protein unfolding mechanisms and energetics in order to shed more light on fundamentals concepts of protein chemistry and thermodynamics. Immobilized proteins change their energetics with respect to the solution state, as monitored by thermodynamic, activity and structural parameters [1, 2]. These changes as compared to aqueous solution may reveal new aspects of the folding properties of the protein architecture. In fact, after immobilization, not only enzymes are still active and often more thermostables than the free forms

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but also show a different unfolding behavior [7, 8]. In some case interdomain interactions are altered and domains decoupling has been observed in multidomain proteins: unlike the free enzyme, each domain unfolds as an independent unfolding unit [9].

The fundamental unit of the protein tertiary structure is the domain, defined as a polypeptide chain or a part of it that can independently fold into a stable structure. Domain is often the structural unit of a protein function, although different function can be espleted by a single domain. Domains are formed by different combinations of secondary structural elements or motifs, such as α-helices and β-strands, which are arranged in the three-dimension space in such a way to interact among each other. Another useful and, perhaps, more appropriate definition is based on thermodynamics: a domain is a contiguous region of a protein which independently undergoes all-or-none cooperative transition during the unfolding or folding process. This is an equilibrium condition, which allows the application of thermodynamics to a two-state process, i.e. from the folded to the unfolded state at a given set of external parameters (T, P, etc.), whatever is the structural state of the unfolded form insofar as it is well defined thermodynamically.

A domain is a highly dynamic assemble of atoms. Techniques, such as hydrogen exchange, sensitive to atomic motions and concerted oscillations, can be used to select parts of the protein architecture [10]. Solid-like regions which exchange the amide-bond protons slower than other parts of the protein (the 'matrices') are called 'knots', characterized by low motility and high packing density. They can correspond to the topological motifs that form domains. In fact, they do contribute to protein stability since when they are disrupted the protein unfolds by loosening the matrix and 'untieing' structural elements [11].

The transition of the protein between state is conveniently monitored by following some observables which have different values in the two states. The temperature dependence of variables such as the absorbance or fluorescence of aromatic aminoacids, quite sensitive to changes in local environment, or CD and NMR signals are used to study protein unfolding. The energetic terms involved in stability are calculated by means of the mathematical representation of the process and its approximation with the theoretical model. From these studies, the two-state model of the protein unfolding was proposed [12, 13]. The validity of the model was confirmed by differential scanning calorimetry (DSC) [14], through direct temperature scanning experiments in the region of protein denaturation. DSC confirmed the two-state hypothesis in many small globular proteins, but also showed that in the case of multidomain proteins the model cannot be applied to the overall process [15]. In this case, it was observed that each domain was able to unfold more or less independently, sometimes showing two-state behaviour.

Domain configuration of the protein molecule can be often readily deduced from topological inspection and pattern recognition of the structure [16] derived from X-ray diffraction data on crystals as well as from NMR of protein in solution. Small globular proteins (i.e. bovine pancreatic trypsin inhibitor or epidermal growth factor) consist of a single domain, either from structural and thermodynamic point of view. On the other hand, large protein (plasminogen) are formed by several domains. Sometimes, they can be physically separated by cleavage of the hinge or interdomain linking regions of the peptide chain and, once isolated, still retain their thermodynamic stability and original cooperativity [15]. As an intermediate situation, some small globular proteins, such as serine proteases, are formed by two domains, as it can be readily visualized by topological inspection of the X-ray diffraction data. However, as studied by DSC, they behave as a single cooperative unfolding unit, suggesting, unlike large multidomain protein, close interactions between domains or quite similar intrinsic domain stability.

Two-state behavior observed in the protein unfolding (or folding) process implies that the disruption of the protein domain(s) is a highly cooperative process, i.e. the number of partially folded intermediates is small. This may be the case also for two-domain proteins in certain experimental conditions where the two domains have the same intrinsic stability and unfold concurrently or the interdomain coupling is significant and the unfolding of one domain encourage the unfolding of the other. Many multidomain proteins are originated by gene duplication, therefore the similarity of denaturation behavior is not surprising. However, in other experimental conditions (different *pH*, presence of organic solvents, etc.) the same multidomain protein may not show a two-state unfolding transition. In this case, partially folded states become significantly populated. The probability of thermodynamically stable partially folded states depends on the magnitude and cooperativity of the interaction energy between domains [17, 18]. Enzymes or other proteins which depend for function on the relative motion of domains cannot have strong coupling between domains. Therefore, small changes in the interdomain interactions can alter significantly stability and unfolding behavior, leading to a failure of the description of unfolding by the two-state approximation.

When DSC is used to study protein unfolding, two-state approximation implies that the enthalpy change calculated by the temperature dependence of the observable through the van't Hoff plot, which assumes two-state behavior, must be equal to the true calorimetric enthalpy change, which depends on the mole of substance present and consequently on the molecular weight. The ratio of the two values of the enthalpy changes has to be one: in this case the cooperative unit corresponds to the size of the unfolding unit, i.e. corresponds to the size of the whole protein. The ratio, often called Cu, the cooperative unit, is considered a valid criterion to assess two-state behavior [13].

Most of the calorimetric studies on globular proteins were carried out in conditions where artifacts (such as aggregation, autolysis, etc.) could not be present because the breakdown of the two-state assumption would have made invalid any thermodynamically meaningful analysis of the data. These conditions are often close to neutrality or to a *pH* range were irreversible aggregation (after unfolding) or significant autolysis are observed [19]. In fact, detailed calorimetric study of protein unfolding at *pH* close to neutrality are difficult to find in literature [20]. By using immobilized enzymes it is possible to study protein unfolding even in the experimental conditions which are not suitable for the free enzyme. For example, in the case of an immobilized protein, aggregation cannot occur and unfolding can be studied at any *pH*.

When Cu is not equal to one, the apparent deviation from two-state behavior may be due to several reasons, including: a) non-equilibrium conditions during the calorimetric experiments [21, 22], b) the presence of a stable partially folded intermediate state along the denaturation pathway, c) changes in the protein aggregation state as a function of the temperature (during the calorimetric scan) or *pH*, d) changes of the extinction coefficient as a function of *pH* (if *pH* is used to perturbe the unfolding transition).

We suggest a further reason for the observed deviation from the two-state behavior of the unfolding process of two-domain small globular proteins, i.e. domain decoupling. This hypothesis is based mainly on two arguments: first, the change of the unfolding mechanism of proteins immobilized onto an inert support and, second, the heat of protonation of proteins as a function of *pH*. The second point relies on the observation of unusually large enthalpy change of protonation for some proteins in the acidic (α-chymotrypsin, cythochrome c [23, 24]) and alkaline (myoglobin [25]) *pH* range. The first two proteins have two-domain structures. These abnormal heat effects are related to conformational rearrangements of the protein molecule induced by the *pH* change. The first point is based on the study of immobilized proteins by DSC [8, 9]. Ribonuclease A (RNAase) is a two-domain protein which unfolds as a single cooperative unit in the acid *pH* range. After immobilization onto an inert support (silica beads) by means of a bifunctional reagent (glutaraldehyde), the unfolding transition is not a two-state process anymore and two partially overlapping processes have to be taken into account to fit the experimental curves. The overall enthalpy changes is quite to that of the free enzyme. The two transitions are associated with the independent unfolding of the two domains of the molecule. Therefore, as a consequence of immobilization, the two domains are not 'tied' anymore and decoupling is observed in the whole *pH* range studied (from 3 to 8). Decoupling of RNAase domains was also observed in the presence of ethanol in the case of the free enzyme [17].

Changes of the unfolding behavior after immobilization has been detected not only with RNAase but also with other 'multidomain' proteins, such as α-

chymotrypsin, chymotrypsinogen and papain. Chymotrysin is a typical two-domain protein. Its active site is located just at the domain interface and the enzymatic activity is closely dependent on interdomain movements and matching (the 'rack' mechanism [26]). In solution it unfolds as a single cooperative unit at acid *pH*, showing two-state behavior. At neutral *pH*, the unfolding is not a

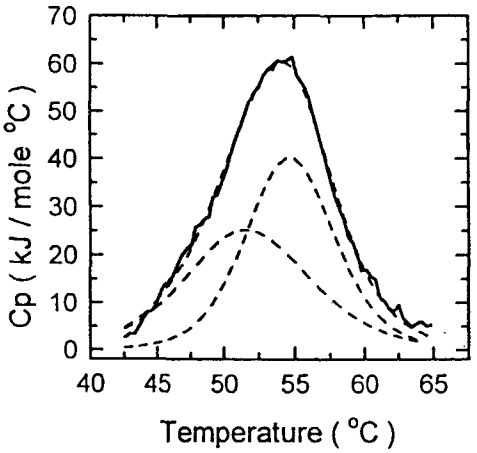

Fig. 1 Excess specific heat capacity, C_p, *vs.* temperature profile of α-chymothrypsin in 0.1 *M* phosphate buffer, *pH* 6.5. $T_{m1}=51.7$°C; $\Delta H1_{cal}=305$ kJ·mol^{-1}; $\Delta H1_{vH}=288$ kJ·mol^{-1}. $T_{m2}=54.7$°C; $\Delta H2_{cal}=338$ kJ·mol^{-1}; $\Delta H2_{vH}=422$ kJ·mol^{-1}. Calorimeter: MC1, Microcal Inc., MA, USA; scan rate 43 deg/h

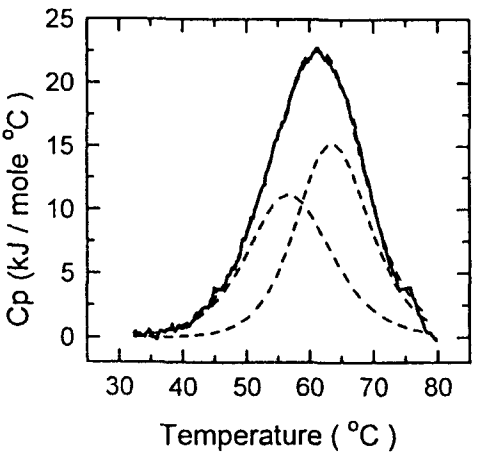

Fig. 2 Temperature dependence of the excess specific heat, C_p, of immobilized α-chymotrypsin on CPC-Silica in 0.05 *M* acetate buffer *pH* 5.5. Two overlapping transitions have to be considered in order to obtain the best fit of the overall experimental transition $T_{m1}=56.4$°C; $\Delta H1_{cal}=198$ kJ·mol^{-1}; $\Delta H1_{vH}=216$ kJ·mol^{-1}. $T_{m2}=64.5$°C; $\Delta H2_{cal}=213$ kJ·mol^{-1}; $\Delta H2_{vH}=218$ kJ·mol^{-1}. Calorimeter: MC1, Microcal Inc., MA, USA, equipped with solid sample vessels; scan rate 43 deg/h

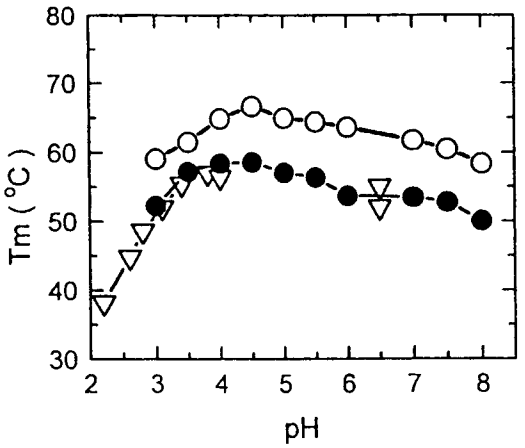

Fig. 3 Dependence on *pH* of the melting temperatures of molecular domains in α-chymotrypsin: (triangles): free enzyme; (filled circles): 1° transition; (open circles): 2° transition for immobilized enzyme

two-state process but two peaks are observed in the DSC transition, even at low concentration (Fig. 1). Conversely, after immobilization, chymothrypsin unfolds with two partially overlapping processes in the whole *pH* range studied, from *pH* 3 to *pH* 8 (Figs 2, 3): again, the overall transition is not a two-state process, although each subprocess, associated with the unfolding of a single uncoupled domain, has a two-state character (Cu equal to one). From these results it can be concluded that in the case of the free enzyme, the two domains actually 'coupled' as the *pH* is lowered from 7 to 3. At low *pH*, due to domain coupling, the protein unfolds as a single cooperative unit showing two-state behavior, which was not observed at *pH* close to neutrality. A conformational rearrangement of chymotrypsin molecule has been actually detected by measuring an abnormal heat of protonation by lowering the *pH* to *pH* 3 [23].

In conclusion, with both RNAase and chymotrypsin domain decoupling occurs upon immobilization: a domain is thermally stabilized (higher T_m) with respect to the other, which unfolds with the same T_m as in the free enzyme [8]. Moreover, immobilization prevents domain coupling at acid *pH*, unlike in the case of the free enzyme. The two domains remain independent even at low *pH*.

Furthermore low-energy forces regulate interdomain interactions since small shear and hinge movements among groups of atoms are necessary in order to obtain concerted motions [27]. In this sense, small perturbations of the interdomain interface (subtle changes often barely detectable by static conventional techniques) may be sufficient to change thermodynamic behavior. This is probably the case of the immobilized enzymes studied so far. It should be noted that interdomain interactions may be quite equivalent to intersubunit interactions

[28]. In the case of chymotrypsin, the aggregation state of the protein at neutral *pH* does not necessary implies the deviation from two-state behavior.

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Zusammenfassung — Die jüngsten Fortschritte bei thermodynamischen Aspekten von mobilen oder an Feststoffträgern immobilisierten Proteinen werden beschrieben. In Übereinstimmung mit den an Ribonuklease A [9] beobachteten Ergebnissen bestätigt eine DSC-Analyse an α-Chymotripsin eine Entkoppelung der Schmelzbereiche bei immobilisiertem Protein in einem weiten *pH*-Intervall.