ORIGINAL ARTICLE

Effects of the regulatory ligands calcium and GTP on the thermal stability of tissue transglutaminase

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Abstract Tissue transglutaminase undergoes thermal inactivation with first-order kinetics at moderate temperatures, in a process which is affected in opposite way by the regulatory ligands calcium and GTP, which stabilize different conformations. We have explored the processes of inactivation and of unfolding of transglutaminase and the effects of ligands thereon, combining approaches of differential scanning calorimetry (DSC) and of thermal analysis coupled to fluorescence spectroscopy and small angle scattering. At low temperature (38–45°C), calcium promotes and GTP protects from inactivation, which occurs without detectable disruption of the protein structure but only local perturbations at the active site. Only at higher temperatures (52–56°C), the protein structure undergoes major rearrangements with alterations in the interactions between the N- and C-terminal domain pairs. Experiments by DSC and fluorescence spectroscopy clearly indicate reinforced and weakened interactions of the domains in the presence of GTP and of calcium, and different patterns of unfolding. Small angle scattering experiments confirm different pathways of unfolding, with attainment of limiting values of gyration radius of 52, 60 and 90 Å in the absence of ligands and in the presence of GTP and calcium. Data by X-rays scattering indicate that ligands influence retention of a relatively compact structure in the protein even after denaturation at 70°C. These results suggest that the complex regulation of the enzyme by ligands involves both short- and long-range effects which might be relevant for understanding the turnover of the protein in vivo.

Keywords Tissue transglutaminase · Regulatory ligands · Thermal inactivation · Fluorescence spectroscopy · Differential scanning calorimetry · Small-angle scattering

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Introduction

With the possible exception of the so-called intrinsically disordered proteins, which are characterized by an unusually high content of unstructured regions (Uversky and Dunker 2010), biologic functions of native proteins depend on the maintenance of a folded structure through assembly of relatively rigid domains, joined by flexible mobile regions exposed to the environment. In soluble proteins, both hydrophobic interactions in the inner core and interactions with the solvent at the protein surface contribute to the thermodynamic stability, which is characterized by a minimum in potential energy (Jaenicke 2000).

Despite these constraints, even folded proteins usually display appreciable degrees of dynamic mobility and are sensitive to interaction with external ligands, which

eventually modulate biologic activity through conformational changes as it happens in allosteric proteins (Volkman et al. 2001). In extreme cases, the native state can be devoid of biologic activity, as it happens in "cryptic" or "latent" enzymes which depend, for activity, on interaction with essential cofactors or other forms of structural reorganization, e.g. covalent modification as it happens frequently in the case of proteinases (Boatright and Salvesen 2003). Because of these reasons, the catalytically competent state of "cryptic" enzymes can be formally distinct from the native one and described, from a thermodynamic point of view, as a "metastable" conformation. These particular features may become evident when the protein under investigation is subjected to thermal stress, to disrupt its regular structure in the latent and in ligand-activated state. Heat is particularly useful as an external probe because it is the only form of energy capable to promote unfolding through potentially reversible pathways, by increasing internal motions.

In the recent years, we have investigated the structure and function of tissue transglutaminase, which behaves as a "cryptic" enzyme catalyzing strictly calcium-dependent reactions of transfer of acyl-moieties from peptidyl glutamine residues to accepting primary amines. Either soluble amines (usually polyamines or alternatively histamine) or ε-aminogroups of protein-bound lysyl residues can serve as acyl acceptors releasing, as products, proteins either modified by covalent incorporation of amines or crosslinked through proteinase-resistant isopeptide bonds. Both reactions occur physiologically in relation to the cellular concentration of polyamines (Griffin et al. 2002; Lentini et al. 2004). Several studies indicate that tissue transglutaminase is a bifunctional enzyme displaying activities of GTP hydrolysis and of protein transamidation in an alternative switching-on pattern (Griffin et al. 2002). Interestingly, transamidation is usually inactive because it requires near-millimolar concentrations of calcium ions and breakdown of cell GTP, which is an inhibitor, conditions that are usually not met in the intracellular compartment (Bergamini et al. 2011).

Actually the physiologic function of tissue transglutaminase is a still unresolved issue and is also influenced by the enzyme cellular location (Gundemir and Johnson 2009; Bergamini et al. 2011). In relation to function within the intracellular space, it has been proposed that the enzyme is mainly involved in controlling the programs of cell death and proliferation (Fesus and Szondy 2005; Bergamini et al. 2010). Studies on permeabilized cells support these views (Smethurst and Griffin 1996) and indicate that ligands further modulate the turnover of the enzyme in situ (Zhang et al. 1998), opening completely new scenarios on the regulation of enzyme tissue levels (Bergamini 2007), which has been considered until now mainly in relation to enzyme induction. To understand the relevance of degradative effects in the biology of transglutaminase, we decided to study the effects of regulatory ligands on the in vitro thermal stability of tissue transglutaminase, complementing previous investigations on the sensitivity to proteinases (Casadio et al. 1999) and to chemical denaturants (Cervellati et al. 2009). The issue of thermal stability of tissue transglutaminase has been marginally dealt with previously, mostly in relation to exposition to shifts in pH (Lichti et al. 1985; Nury et al. 1989; Nury and Meunier 1990; Bergamini et al. 1999) but not in relation to effects of physiologically relevant ligands. We now discuss the new results we have obtained in relation to transglutaminase stability and unfolding by combined approaches aimed to correlate inactivation and structural perturbations of the protein.

Materials and methods

Materials

All biochemicals including buffers, calcium and GTP were analytical grade and were purchased from Sigma, Milan, Italy. Human erythrocyte TGase was purified by a slight modification of our standard procedure (Casadio et al. 1999), consisting of DEAE-cellulose chromatography, fractionation with PEG-8000 and chromatography on DEAE-Sepharose and Heparin-Sepharose. During the last steps in purification DTT 1 mM was included in the buffers to ensure reduction of the Cys370-Cys371 disulphide bond. The purity was checked by standard SDS-PAGE and the concentration of the purified protein was determined spectrophotometrically, assuming a coefficient of 1.38 at 280 nm for a solution 1 mg/ml. It was converted into molar concentration on the basis of a Mr of 77329, quoted in Swiss-Prot PDB (entry P21980). Activity of tissue transglutaminase was determined by a filter paper assay, measuring calcium dependent incorporation of radioactive putrescine into dimethylcasein as previously described (Casadio et al. 1999).

Thermal inactivation

To assess the enzyme thermal stability, we employed a two-steps procedure, submitting purified transglutaminase (0.1–0.2 mg/ml in 50 mM cacodylate buffer pH 7.5) to heat treatment at the temperature detailed in Figure legends, with the specified additions. Incubations were carried out in a high precision, electronically controlled Julabo Paratherm II water bath, with ±0.3°C temperature tolerance. At timed intervals, aliquots were withdrawn, diluted with cold buffer and tested for residual enzyme activity, at a standard temperature of 30°C (Casadio et al. 1999).

Differential scanning calorimetry

In the differential scanning calorimetry (DSC) experiments, the enzyme was dialysed against cacodylate buffer as above and submitted to progressive heating inside the sample cell of a VP-DSC Microcalorimeter, from Microcal Inc. (Northampton, MA, USA), using a temperature gradient of 0.8°C/min, instead of 1°C/min, as in previous studies, to minimize the precipitation effects we have reported in previous studies (Bergamini et al. 1999), at least in the absence of calcium. The reference cell contained buffer (and eventually ligands, as in the sample cell) and the extra heat capacity between the two cells was recorded continuously during thermal unfolding. Experiments were regularly carried out at a protein concentration between 0.7 and 1.3 mg/ml, after extensive de-aeration, omitting stirring, which leads to rapid protein denaturation under these conditions. Further operational and instrumental details have been described previously (Cervellati et al. 2009).

Analysis of calorimetric data was performed by means of the software Origin, provided by Microcal Inc., to obtain data on the effects of ligands on the thermal stability of the protein. The derived parameters cannot be considered as true thermodynamic constants because the enzyme undergoes irreversible denaturation during heating. Thus, downscanning or rescanning of the protein after the initial heating cycle did not reproduce the initial thermograms. In the case that appreciable posttransition exothermic heat exchange occurs, the posttransitional base line was evaluated by performing parallel experiments at acid pH, as suggested by Privalov et al. (1995).

Fluorescence spectroscopy

Fluorescence measurements were performed with a Perkin Elmer LS 55 spectrofluorimeter connected to a PC for data collection, recording emission spectra between 310 and 370 nm, with excitation at 295 nm, to ensure virtually pure tryptophan excitation. The instrument was connected with a thermostated circulating bath, programmed for constant temperature increments at a rate of 1°C/min to the cell compartment. Temperature changes were continuously recorded through a thermocouple inserted inside the cuvette. After subtraction of buffer blanks, the emission ratio was calculated from the fluorescence intensities at 350 and 330 nm, as in Kurochkin et al. (1995). The cuvette contained pure transglutaminase dissolved at pH 7.5 in different buffers (Tris, cacodylate or Hepes) each at 25 mM concentration, and the additions detailed in figure legends. Data are presented as single representative plots.

Small angle X-ray scattering

Experiments were performed using the SAXS beamline at the ELETTRA synchrotron (Trieste, Italy), essentially as previously described (Mariani et al. 2000). The use of this highly brilliant source allows by one side to shorten the time for data collection, by the other to perform rapidly thermal scanning programs employing a moderately concentrated (1.5–2.5 mg/ml) solution of transglutaminase.

The X-ray wavelength was 0.154 nm and the sample-to-detector distance was 2.5 m, so that the scattering vector Q range was 0.1–2.5 nm^{-1}. Protein samples were inserted into a sealed 1 mm glass capillary enclosed within a thermostated compartment connected to an external circulation bath and a thermic probe for temperature control. On each sample, heating cycles were performed stepwise with a 2–3°C increase in the temperature range from 25 up to 70°C, the highest temperature allowed on the instrument. No actual check for eventual GTP hydrolysis was performed on the analyzed samples, but in parallel experiments it was verified that maximal hydrolysis under the conditions of the experiments attained as maximum 12% of the initial concentration of the nucleotide, as determined by an enzymatic assay.

Experimental intensities were corrected for background, buffer contributions, detector inhomogeneities and sample transmission. Data analysis was performed in the frame of the so-called two-phase model, as previously described. According to Guinier approximation (Guinier and Fournet 1955), particle gyration radii (R_g), which are related to the particle size and shape, and scattering intensities at zero angle (I_0), which are a function of number of particles in solution and thus give an estimation of protein aggregation (Svergun et al. 2001), were derived as a function of sample temperature, in the absence of ligands and in the presence of 4 mM calcium or 0.5 mM GTP.

Results and discussion

Most recent evidences, that support a major role of tissue transglutaminase in the pathogenesis of autoimmune and neurodegenerative diseases (Iismaa et al. 2009), have greatly stimulated interest in this multifunctional enzyme, subjected to complex regulation by transcriptional and by allosteric mechanisms (Griffin et al. 2002; Bergamini et al. 2011). The pathologic relevance of transglutaminase is largely related to an increase in transamidation activity, which can arise through deregulated activity of a normal amount of cellular enzyme (Monsonego et al. 1998) or through an increased number of enzyme molecules inside the cells. Since both activity and turnover of

transglutaminase are submitted to control by the allosteric modulators calcium and GTP (Bergamini 2007), we have explored the intrinsic stability of the protein employing heat as the destabilizing agent, in the presence of the regulatory ligands. These studies are focused on activity and on folding of the protein, taking advantage of different experimental signals.

The data we have obtained are on line with the effects of ligands on the protein stability, we proved previously checking the sensitivity to proteolysis and to chemical denaturants (Casadio et al. 1999; Cervellati et al. 2009). These results can be explained in the frame of the protein structural model, assuming that the regulatory and the stability effects are both triggered by conformational changes which release/tether together the N-terminal and the C-terminal domain pairs. Activation/destabilization and inhibition/stabilization are linked together as two-faced aspects of the same process, and are triggered respectively by calcium and GTP.
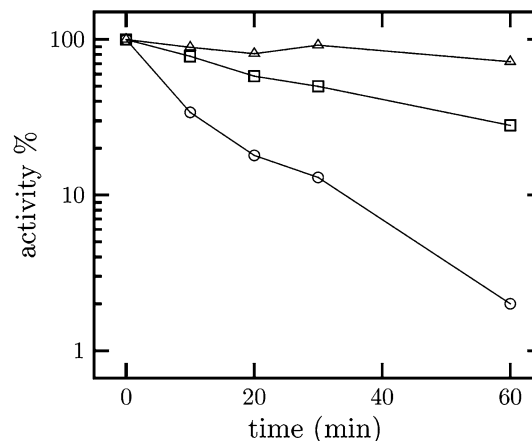
Thermal inactivation of tissue transglutaminase

In the initial experiments, we checked the time-dependent inactivation of tissue transglutaminase incubating the enzyme at different temperatures for increasing periods of time, before assaying residual activity at 30°C. At pH 7.5, the enzyme was stable even during relatively long incubation in the absence of ligands for temperatures up to 38°C, while progressive inactivation took place at slightly higher temperatures with pseudo-first order kinetics, as proved by the linear fitting of residual activity against time in semi-logarithmic plots. This suggests that changes in the aggregation state did not contribute to the kinetics of thermal enzyme inactivation in the explored temperature range between 38 and 45°C. The corresponding Arrhenius plots allowed to calculate Van't Hoff enthalpy for thermal stability of the protein, yielding an approximate value of 65 Kcal/mol.

Effects of ligands were checked at temperatures of intermediate protein stability, e.g. 40°C. As reported in Fig. 1, calcium ions at a concentration of 4 mM, which is saturating for activity, destabilized the enzyme at 40°C decreasing its half-life from about 30 min down to 8 min, while GTP was an effective protectant extending half-life to over 100 min, when added to a final concentration of 0.5 mM.

Thermal unfolding of transglutaminase

We have employed three experimental approaches (intrinsic protein fluorescence, DSC and small-angle X-rays scattering) to investigate thermal unfolding of transglutaminase, since each of them explores different aspects of the protein tertiary structure. The tryptophan intrinsic fluorescence (Bergamini


Fig. 1 Effects of ligands on heat inactivation at 40°C. Tissue transglutaminase (0.12 mg/ml in Tris buffer at pH 7.4) was incubated at 40°C in the absence of ligands (*squares*) and in the presence of calcium (*circles*) and of GTP (*triangles*). At the indicated time intervals, samples were withdrawn, diluted with cold buffer and assayed for residual activity by the amine incorporation procedure as described in the "Experimental" section

et al. 1999) gives information on the exposition of tryptophan residues to the solvent during protein unfolding, while DSC gives an indication of the strength of interaction among proteins domains and small-angle-scattering provides information on the overall three dimensional arrangement of the peptide chain.

Fluorescence studies

Intrinsic fluorescence is a sensitive probe of the folding of domains 1 and 2, which contain all tryptophan residues of tissue tranglutaminase. These properties are magnified when values are reported as ratio of fluorescence emission at the wavelengths of 350 and 330 nm (marked F350/F330).
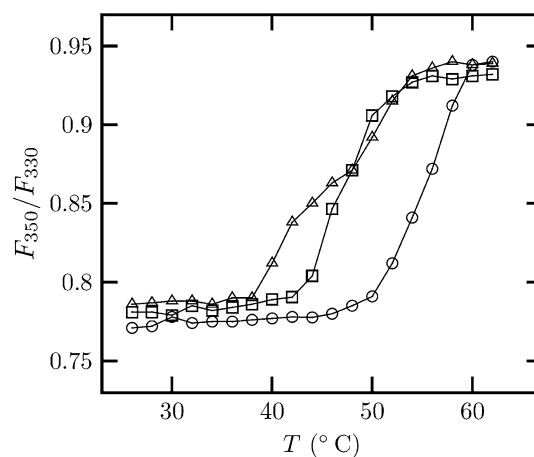
The tryptophan fluorescence of native tissue transglutaminase is characterized by a blue-shifted spectrum (λ_{max} 333 nm), which indicates that these residues in the native protein are embedded in mainly hydrophobic environments. The spectrum is progressively shifted to the red by heating, with maximum emission close to 355 nm in the unfolded state. At the initial temperature (25°C), identical F_{350}/F_{330} values (about 0.78–0.80 in individual experiments) were obtained in the absence and in the presence of ligands, indicating virtually identical tertiary structure in domains 1 and 2, despite ligands induce different conformational states, as proved previously (Mariani et al. 2000). In all instances therefore, tryptophan residues are prevalently embedded in apolar environments, at least when the protein does not assume the fully extended conformation, which has been reported for the enzyme locked in the catalytic transition state, by covalent labelling with a
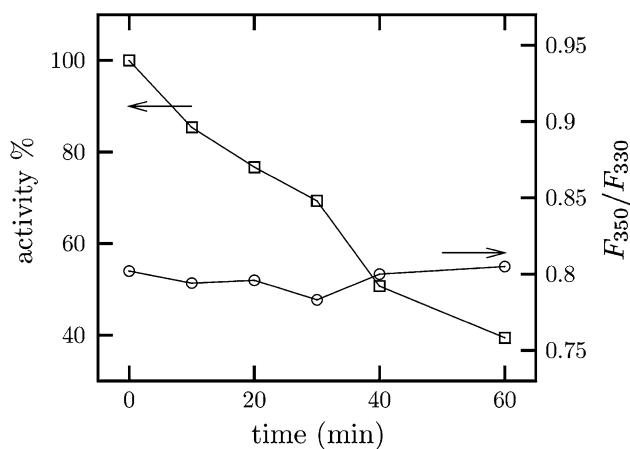
Fig. 2 Correlation between inactivation and changes in tryptophan fluorescence. Tissue transglutaminase was incubated at 41°C in the absence of ligands for prolonged time intervals, recording fluorescence emission spectra, upon excitation at 295 nm. From the spectra, the fluorescence emission ratio (F_{350}/F_{330}) was calculated after baseline subtraction. At the reported time intervals, 14 samples of protein were withdrawn from the fluorimeter cell to measure residual activity, after appropriate dilution


Fig. 3 Fluorescence melting profiles. Samples of purified transglutaminase (0.05 mg/ml in Tris buffer pH 7.4) were incubated in the sample compartment of a Perkin–Elmer fluorimeter, connected with a controlled recirculating heating bath. Fluorescence excitation wavelength was 295 nm and emission spectra were recorded between 310 and 380 nm, at the indicated temperature. After baseline subtraction, the fluorescence emission ratio at 350 and 330 nm was calculated as described in the "Experimental" section. In separate experiments on the same protein batch, we recorded fluorescence emission in the absence of ligands (*squares*) and in the presence of 4 mM calcium (*triangles*) and of 0.3 mM GTP (*circles*)

peptide substrate analogs (Pinkas et al. 2007). It is important to note that incubation of the enzyme at the temperatures which promote slow inactivation (e.g. 41°C as in Fig. 2) leads to time-dependent loss of activity, without spectral shifts which occur only at higher temperature. This indicates that inactivation at limit temperatures does not depend on protein unfolding but rather on minor localized changes, most likely in the active centre, to which tryptophan residues contribute (Iismaa et al. 2003). The active site region must therefore be characterized by a very sensitive and flexible structure as it happens in other enzyme proteins, e.g. creatine phosphokinase (Tsou 1995).

Conversely if heating is applied in a progressive way, the fluorescence emission of transglutaminase is altered to provide melting profiles which witness greater accessibility of tryptophan residues to the solvent because of protein unfolding. The fluorescence melting thermograms (Fig. 3) obtained plotting the F_{350}/F_{330} fluorescence ratio against temperature are typical of a two-states denaturation mechanism with a marked and sharp increase in fluorescence ratio from that typical of the native state to values very close to unity in an apparently cooperative way. Precise melting temperatures for the two states mechanism involving native and unfolded transglutaminase can be calculated for the protein in the absence of ligands ($T_m = 48°C$) and in the presence of GTP ($T_m = 55°C$). In the presence of calcium, the melting temperature is significantly decreased ($T_m = 44°C$), so that calcium and GTP display the opposite effects on the enzyme stability to thermal unfolding which had already been reported for chemical denaturation by guanidine (Di Venere et al. 2000;

Cervellati et al. 2009). Notably in the presence of calcium the melting profile does no longer fit a biphasic profile but rather follows a complex pattern suggesting the unfolding of a portion of domains 1 and 2 or alternatively the contemporaneous presence of at least three populations of molecules at temperatures close to the apparent T_m, through formation of distinct intermediates. Finally, the value of fluorescence emission ratio in the heated protein is always much lower than that observed with model compounds (e.g. *N*-acetyl-indole) suggesting that exposition of tryptophan residues to the solvent is not complete even in the denatured inactive protein.

Calorimetric investigations

Differential scanning calorimetry provided interesting information on the effects of ligands on stability of tissue transglutaminase, despite the limitation that thermal unfolding of the protein is irreversible, so that the enthalpy values we quote should be considered only as indicative ones, not as true thermodynamic constants (Cervellati et al. 2009). Notably the denatured enzyme tends to precipitate within the calorimetric cell probably because of aspecific interactions between hydrophobic regions exposed during protein unfolding.

In the absence of ligands, the thermograms display two partially fused transitions, which are effectively resolved by the deconvolution programme (Fig. 4). Transition I and
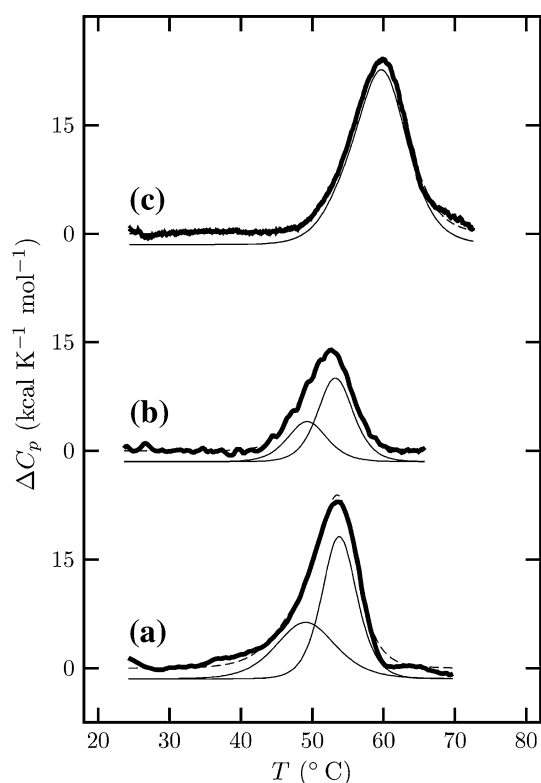
Fig. 4 DSC thermograms of tissue transglutaminase unfolding in the absence and in presence of ligands. Thermal scanning was performed at a constant gradient of 0.8°C/min in a Microcal VP-DSC apparatus at a protein concentration between 10 and 13 mM in different experiments. Traces *a*, *b* and *c* have been recorded in the absence of ligands and in the presence of 4 mm Calcium and of 0.5 mM GTP, respectively. Deconvolution was performed by the Origin tool, provided by the manufacturer, exactly as described in the "Experimental" section. Excess heat capacity ΔCp is presented after normalization for the concentration of the protein in the experimental cell

II were ascribed, respectively, to unfolding of the N-terminal domains (domain 1 and 2) and of the C-terminal domains (domain 3 and 4) by comparing DSC and thermal denaturation data monitored by fluorescence spectroscopy (Cervellati et al. 2009). Under the experimental conditions of the present study, transitions I and II are characterized by melting temperatures of 49 and 54°C, with a cumulative calorimetric unfolding enthalpy of 205 Kcal/mol.

This pattern is clearly altered by addition of ligands; in the presence of calcium ions, added at a concentration of 4 mM, which ensures attainment of maximal activity in kinetic experiments, the melting temperature of transition I tends slightly to decline, while transition II is unmodified (53.6°C). The most apparent effect is that of a the decrease in the ΔH value (110 Kcal/mol, when measured in the presence of ammonium salts to inhibit enzyme activity and self-crosslinkage) suggesting a smaller force of interaction between the N and the C-terminal domain pairs, as compared to experiments with the ligand-free protein. Appreciable precipitation of the protein with exothermic heat

exchange still occurs also with the enzyme inactivated by reaction with the inhibitor R283 (not shown). Even considering with great caution the determined ΔH value of unfolding, its relatively low value (110 Kcal/mol, cumulative of both transitions) is nevertheless indicative of a weakened interaction between the N- and the C-terminal domain pairs.

An opposite effect of protein stabilization was observed in thermograms recorded in the presence of saturating concentrations of GTP (0.5 mM), since transitions I and II fuse with each other, giving rise to an apparently single transition of higher unfolding enthalpy (ΔH = 245 Kcal/mol), at higher T_m (about 59.5°C), thus witnessing by one side an intrinsically higher thermal stability, by the other a tighter interaction between N- and C-terminal domains.

Small-angle X-ray scattering

We employed previously SAS to investigate transglutaminase conformation, recording changes in R_g and shape triggered by addition of ligands (Mariani et al. 2000). We have now repeated these experiments, obtaining slightly different values and we ascribe these discrepancies to methodological differences because of the use as solvents D_2O in the previous (SANS) and H_2O in the present experiments (SAXS), since D_2O and H_2O are known to affect differently protein compactness (Cioni and Strambini 2002). In the present occasion, at basal temperature (25°C), we obtained values of R_g of 34 Å in the absence of ligands, 32 Å and 45 Å in the presence of GTP and of calcium, respectively.

We have further utilized SAS to investigate transglutaminase thermal unfolding because the loss of protein native structure is usually reflected by an increase in the gyration radius (Cinelli et al. 2001; Millet et al. 2002). This occurs also in the case of denaturation of transglutaminase submitted to step-wise increase in temperature up to 70°C. The relevant data are summarized in Fig. 5, which displays changes of gyration radius (panel a) and I_0 intensity (panel b) at increasing temperature. In all experimental conditions, R_g increased progressively up to limit values of 52, 60 and 90 Å at 70°, respectively, in the absence of ligands and in the presence of GTP and of calcium. At this temperature, the enzyme should be present largely in an unfolded state, since it tends to aggregate and precipitate in the DSC experiments, as mentioned.

Ligands have different effects on the folding of transglutaminase as demonstrated by the changes in R_g (Fig. 5a), which increases from the basal to the limit value of 50 Å through an intermediate state with a R_g value of 42 Å, in the absence of ligands. In the presence of GTP, this biphasic pattern is maintained even if the changes in R_g values appear at different temperatures and the final value

Fig. 5 Dependence of gyration radius (R_g, **a**) and of zero angle intensity (I_0, **b**) as a function of temperature of a solution 2.5 mg/ml of transglutaminase in the absence of ligands (*squares*); in the presence of 4 mM calcium (*circles*) and in the presence of 0.5 mM GTP (*triangles*). *Solid lines* are eye-guide obtained by the Bezier smoothing method
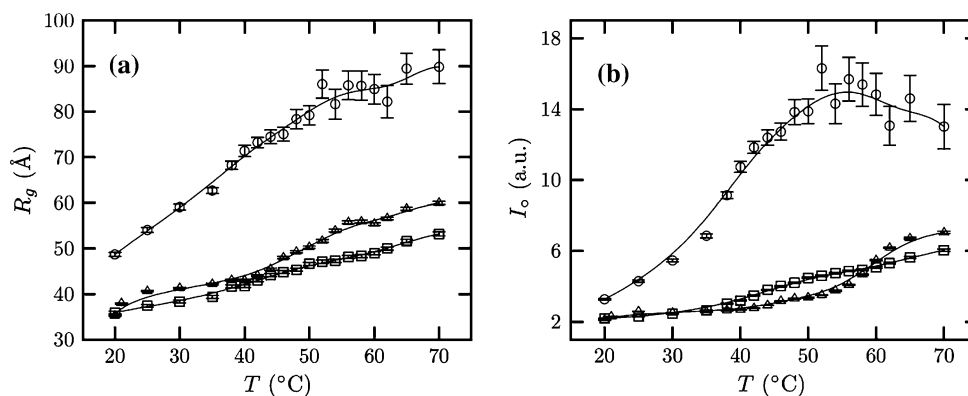
is larger, about 60 Å. The patterns of biphasic increase in these instances are suggestive of the presence of an unfolding intermediate, which should in any case involve the C-terminal domains, since no evidence of intermediates was obtained by the fluorimetric approach (compare Fig. 3). The intermediate is less evident in the presence of calcium, since in this case the increase in the R_g takes place with a constant progression to the final value of about 90 Å. Similarly the I_0 plot (Fig. 5b) confirms that heating induces an increase in the dimension of the scattering particle and that the transglutaminase protein remains rather compact, at least in the absence of calcium ions. The analysis of the intensity scattered at zero angle as a function of temperature confirms heating mainly induces an aggregation process, which begins at about 40°C in the absence of ligands, is hampered in the presence of GTP and is favoured in the presence of calcium. Further analysis of the SAXS data by a Singular Value Decomposition approach (not shown) indicated that the minimum number of analytical functions required to fit all the curves by linear combination is 3. Since this number of functions corresponds to the minimum number of particle species which are present in solution and account for the observed scattering, it is in agreement with the hypothesis of the occurrence of a folding intermediate.

The Kratky plot which is even more representative of the whole process (Fig. 6) indicates by one side definitively larger effects on the shape of transglutaminase by heating in the presence of calcium, when compared with results in the absence of the cation; by the other an enhanced sensitivity to temperature since disruption of the native conformation takes place at lower temperature in the presence than in the absence of calcium. We must underline that the melting temperatures which can be extracted from SAS experiments match closely those provided by the calorimetric investigations, confirming that by both approaches we are actually exploring the properties of a similarly denatured protein at nearly identical high concentrations. The extrapolated T_m values were 52, 47 and 58°C, respectively, in the absence of ligands and in the presence

of calcium and of GTP. From the curve of the Kratky plot, several features can be further derived since (i) the form factors obtained at all the considered experimental conditions indicate the presence of rather compact particles, characterized by a different aggregation state; and (ii) temperature mainly changes the amount of the different species in solution. In particular, the fraction of the monomeric particle (displaying the structure previously determined) decreases at high temperature. It is important to note at intermediate temperature a low aggregation state is formed, which disappears at higher temperature, generating larger aggregates. This effect is more evident in the presence of calcium and is dwelled in the presence of GTP.

Conclusion

The purpose of this study was to assess features of transglutaminase stability in relation to the contribution of degradative pathways to regulate its cellular levels. To our knowledge, the turnover of transglutaminase was measured in situ in a single occasion by Verderio et al. (1998), who reported a very short half-life (11 h) in 3T3 fibroblasts transfected with a transglutaminase cDNA clone under inducible control. Additional information on the stability of the enzyme would be important to understand cellular adaptation to hostile conditions, in which transglutaminase activation might be triggered as a defensive tool (Sohn et al. 2003). To this purpose, it is relevant to note that the enzyme half-life in human erythrocytes should be much longer since (i) erythrocytes stored under blood banking conditions even for longer than 3 weeks are an excellent source to prepare the human enzyme, and (ii) the enzyme content does not decrease significantly in senescent versus young erythrocytes (Bergamini, unpublished observations).

These likely discrepancies in enzyme turn-over can derive from alternative pathways of degradation depending on the proteinase estate in erythrocytes and in nucleated cells. Further differences might arise through metabolic regulatory events, which affect cell nucleotide and calcium
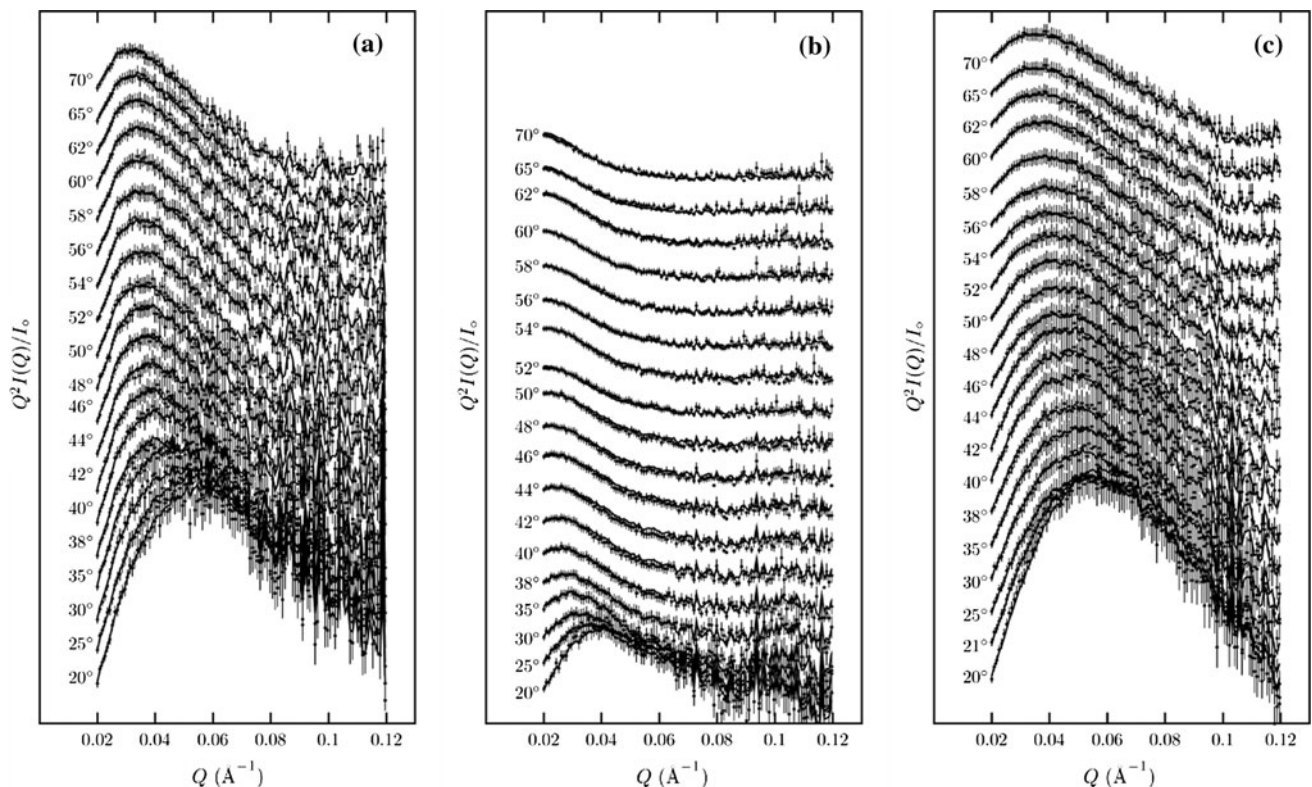
Fig. 6 SAXS profiles of transglutaminase at concentrations of 2.5 mg/ml at different temperatures. Curves are normalized by zero angle intensity (I_0) and shown in the form of Kratky plots. Solid lines are the form factors calculated by the single value decomposition method. The curves are scaled for clarity by a factor 10^{-4}. **a** Transglutaminase without ligands, **b** transglutaminase in the presence of 4 mM calcium, **c** transglutaminase in the presence of 0.5 mM GTP

levels as discussed elsewhere (Zhang et al. 1998; Bergamini 2007). To this purpose, it must be recalled that the main activities of transglutaminase (transamidation and GTP-signalling) are harboured by different protein regions since deletion of the C-terminal domain 4 and half of domain 3 abolish the transamidating activity, with retention (or even increase) in the GTPase activity which can be abolished only following deletion of the major portion of domain 2 (Lai et al. 1996). In contrast with the retention of the GTPase activity, the G-protein signalling is lost when the 4 domain is deleted (Feng et al. 1999). Thus breakdown of the transglutaminase protein by proteolytic cleavage at different regions can theoretically lead to accumulation of specific degradation fragments, which might still afford biologic functions.

In this perspective, it is worthy to recall that during apoptosis transglutaminase is cleaved by caspase 3 (Fabbi et al.1999), probably at a site close to the preferred site of cleavage by pancreatic proteinases in the loop 455–478 connecting domain 2 and domain 3 (Casadio et al. 1999). Conversely, it must be recalled that as for many other proteins, the sensitivity of transglutaminase to proteinase is strongly influenced by the maintenance of the native structure, as we checked previously employing cleavage by

V8 proteinase to probe the combined effects of pH and heat on the protein stability (Bergamini et al. 1999). Our present results move in this direction confirming the fundamental role of ligands in determining the protein stability, independently of the challenge to which the protein is submitted.

At the same time, it is apparent that preservation of catalytic activity and of native three dimensional structure are events uncoupled from each other. This is clearly demonstrated in the present experiments by the comparison of the time course of inactivation and fluorescence perturbations at intermediate temperature (Fig. 2), which are clearly independent of each other.

Tracing the thermal history of transglutaminase, it is possible to identify three significant steps, which are related respectively to the fine disruption of the active site integrity, the inter-domain interactions with unfolding of the N-terminal regions, and finally further unfolding steps which involve the C-terminal regions and mediate extensive protein aggregation. Each of them is influenced by the regulatory ligands. In detail, the first step is inactivation, which takes place at a temperature around 40°C, precedes unfolding, as suggested also by Nury and Meunier (1990) and is obviously modulated by the ligands calcium and GTP, as apparent from the data in Fig. 1. As already stated,

inactivation is an irreversible process which however does not involve covalent enzyme modification (Nury and Meunier 1990) as it might happen in other proteins (Jaenicke 2000), but is related to the three dimensional disorganization of the active site region and of domains 1 and 2. Details on these events are elusive but it is known that several factors contribute to regulate active site reactivity in the native protein, as the *cis*-geometry of a Pro–Pro peptide bond, the formation of disulfide and the interaction with Tyr510 (Bergamini et al. 2011). In addition, a single tryptophan residue (Trp 241) is essential for catalysis, stabilizing the transition state complex (Iismaa et al. 2003). In this perspective, the fact that we could not record any modification of spectral signals during low temperature enzyme inactivation, might itself mean that either the water accessibility of this Trp residue is not altered during inactivation, or—more likely—that our signals are not sufficiently sensitive, since they measure average fluorescence emission of the 13 Trp residues of the protein. In any case, the reactivity of the active site cysteine 277 is clearly under control by calcium (and by GTP) as it is known since long time (Folk and Cole 1966).

The second step is represented by unfolding of the domains, initially involving the N-terminal domains, as proved by the fluorescence spectroscopy investigations (Fig. 3). This is modulated by the ligands since they influence the strength of the interdomain interactions (see the calorimetric experiments in Fig. 4, but compare also results by Casadio et al. 1999), which decrease and augment in the presence of calcium and GTP, respectively. Thus, the melting temperatures are modified by the ligands. In addition, it is clear from Fig. 3 that the promotion of inactivation by calcium is related to the effects of the cation on structure of the N-terminal domains, which undergo alterations in folding so that the melting profile is no longer consistent with a two stage process. Indications in this perspective were also obtained in previous studies about the effects of ligands on proteolysis (Casadio et al. 1999). As expected ligands have opposite effects on protein stability towards thermal treatment as it is known in other models of protein perturbation (Casadio et al. 1999; Di Venere et al. 2000; Bergamini 2007; Cervellati et al. 2009). Ligands modify the melting temperature with appreciable agreement between calorimetric and spectroscopic determinations, with major protection by GTP through a tightened interaction between these domain pairs, so that the DSC melting thermograms are characterized by fused profiles in the deconvoluted components and much higher unfolding enthalpies. This obviously arises from the influence of ligands on the protein conformation.

The third step is related to disruption of additional structures at higher temperatures (in the range from 60 to 70°C) which should involve the C-terminal region, which

is relevant for the interaction of tranglutaminase with proteins targeted by its G-protein activity. During this process, the protein displays an enlargement in R_g as it is frequent for proteins undergoing progressive unfolding (Segel et al.1998; Millet et al. 2002) and extensive aggregation, while still maintaining a relatively compact structure, as judged by comparing the recorded limit R_g with the theoretical one for a fully denatured protein with the size of transglutaminase. The process of aggregation, which is best evident at the analysis by small-angle-scattering, is probably taking place through rather random interactions so that any indication of its structural basis is not yet possible.

The above discussion rests on determinants of intrinsic stability of transglutaminase as described under unnatural conditions combining the structural effects of the ligands and the destabilizing effects of heating at high temperatures. All conclusions are thus based on the differences in intrinsic stability of the enzyme in the ligand-stabilized conformational state, and this is consistent with the available *in situ* investigations in permeabilized cells (Zhang et al. 1998). The possible functional significance of these findings will be apparent when any residual biologic activity of the fragmented transglutaminase peptide chain will be investigated.

References

Bergamini CM, Dean M, Matteucci G, Hanau S, Tanfani F, Ferrari C, Boggian M, Scatturin A (1999) Conformational stability of human erythrocyte transglutaminase. Patterns of thermal unfolding at acid and alkaline pH. Eur J Biochem 266:575–582

Bergamini CM (2007) Effects of ligands on the stability of tissue transglutaminase: studies in vitro suggest possible modulation by ligands of protein turn-over in vivo. Amino Acids 33:415–421

Bergamini CM, Dondi A, Lanzara V, Squerzanti M, Cervellati C, Montin K, Mischiati C, Tasco G, Collighan R, Griffin M, Casadio R (2010) Thermodynamics of binding of regulatory ligands to tissue transglutaminase. Amino Acids 39:297–304

Bergamini CM, Collighan RJ, Wang Z, Griffin M (2011) Structure and regulation of type 2 transglutaminase in relation to its physiological functions and pathological roles. Adv Enzymol 78:1–46

Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. Curr Opin Cell Biol 15:725–731

Casadio R, Polverini E, Mariani P, Spinozzi F, Carsughi F, Fontana A, Polverino de Laureto P, Matteucci G, Bergamini CM (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions. Eur J Biochem 262:672–679

Cervellati C, Franzoni L, Squerzanti M, Bergamini CM, Spinozzi F, Mariani P, Lanzara V, Spisni A (2009) Unfolding studies of tissue transglutaminase. Amino Acids 36:633–641

Cinelli S, Spinozzi F, Itri R, Finet S, Carsughi F, Onori G, Mariani P (2001) Structural characterization of the pH-denatured states of ferricytochrome-c by synchrotron small angle X ray scattering. Biophys J 81:3522–3533

Cioni P, Strambini GB (2002) Effect of heavy water on protein flexibility. Biophys J 82:3246–3253

Di Venere A, Rossi A, De Matteis F, Rosato N, Finazzi-Agrò A, Mei G (2000) Opposite effects of Ca^{2+} and GTP binding on tissue transglutaminase tertiary structure. J Biol Chem 275:3915–3921

Fabbi M, Marinpietri D, Martini S, Brancolini C, Amoresano A, Scaloni A, Bargellesi A, Cosulich E (1999) Tissue transglutaminase is a caspase substrate during apoptosis. Cleavage causes loss of transamidating function and is a biochemical marker of caspase 3 activation. Cell Death Differ 6:992–1001

Feng JF, Gray CD, Im MJ (1999) Alpha 1B-adrenoceptor interacts with multiple sites of transglutaminase II: characteristics of the interaction in binding and activation. Biochemistry 38:2224–2232

Fesüs L, Szondy Z (2005) Transglutaminase 2 in the balance of cell death and survival. FEBS Lett 579:3297–3302

Folk JE, Cole PW (1966) Transglutaminase: mechanistic features of the active site as determined by kinetic and inhibitor studies. Biochim Biophys Acta 122:244–264

Griffin M, Casadio R, Bergamini CM (2002) Transglutaminases: nature's biological glues. Biochem J 368:377–396

Guinier A, Fournet G (1955) Small angle scattering of X-rays. Wiley, New York

Gundemir S, Johnson GV (2009) Intracellular localization and conformational state of transglutaminase 2: implications for cell death. PLoS One 4:e6123

Iismaa SE, Holman S, Wouters MA, Lorand L, Graham RM, Husain A (2003) Evolutionary specialization of a tryptophan indole group for transition-state stabilization by eukaryotic transglutaminases. Proc Natl Acad Sci USA 100:12636–12641

Iismaa SE, Mearns BM, Lorand L, Graham RM (2009) Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. Physiol Rev 89:991–1023

Jaenicke R (2000) Stability and stabilization of globular proteins in solution. J Biotechnol 79:193–203

Kurochkin IV, Procyk R, Bishop PD, Yee VC, Teller DC, Ingham KC, Medved LV (1995) Domain structure, stability and domain-domain interactions in recombinant factor XIII. J Mol Biol 248:414–430

Lai TS, Slaughter TF, Koropchak CM, Haroon ZA, Greenberg CS (1996) C-terminal deletion of human tissue transglutaminase enhances magnesium-dependent GTP/ATPase activity. J Biol Chem 271:31191–31195

Lentini A, Abbruzzese A, Caraglia M, Marra M, Beninati S (2004) Protein-polyamine conjugation by transglutaminase in cancer cell differentiation: review article. Amino Acids 26:331–337

Lichti U, Ben T, Yuspa SH (1985) Retinoic acid-induced transglutaminase in mouse epidermal cells is distinct from epidermal transglutaminase. J Biol Chem 260:1422–1426

Mariani P, Carsughi F, Spinozzi F, Romanzetti S, Meier G, Casadio R, Bergamini CM (2000) Ligand-induced conformational changes in tissue transglutaminase: Monte Carlo analysis of small-angle scattering data. Biophys J 78:3240–3251

Millett IS, Doniach S, Plaxco KW (2002) Towards a taxonomy of the denatured state: small angle scattering studies of unfolded proteins. In: Rose GD (ed) Advances in protein chemistry, vol. 62. Academic Press, San Diego, pp 241–262

Monsonego A, Friedmann I, Shani Y, Eisenstein M, Schwartz M (1998) GTP-dependent conformational changes associated with the functional switch between Galpha and cross-linking activities in brain-derived tissue transglutaminase. J Mol Biol 282:713–720

Nury S, Meunier JC, Mouranche A (1989) The kinetics of the thermal deactivation of transglutaminase from guinea-pig liver. Eur J Biochem 180:161–166

Nury S, Meunier JC (1990) Molecular mechanisms of the irreversible thermal denaturation of guinea-pig liver transglutaminase. Biochem J 266:487–490

Pinkas DM, Strop P, Brunger AT, Khosla C (2007) Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol 5:e327

Privalov G, Kavina V, Freire E, Privalov PL (1995) Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution. Anal Biochem 232:79–85

Segel DJ, Fink AL, Hodgson KO, Doniach S (1998) Protein denaturation: a small-angle X-ray scattering study of the ensemble of unfolded states of cytochrome c. Biochemistry 37:12443–12451

Smethurst PA, Griffin M (1996) Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca^{2+} and nucleotides. Biochem J 313:803–818

Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY (2003) Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. J Clin Invest 111:121–128

Svergun DI, Petoukhov MV, Koch MH (2001) Determination of domain structure of proteins from X-ray solution scattering. Biophys J 80:2946–2953

Tsou CL (1995) Inactivation precedes overall molecular conformation changes during enzyme denaturation. Biochim Biophys Acta 1253:151–162

Uversky VN, Dunker AK (2010) Understanding protein non-folding. Biochim Biophys Acta 1804:1231–1264

Verderio E, Nicholas B, Gross S, Griffin M (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachement and cell death. Exp Cell Res 239:119–138

Volkman BF, Lipson D, Wemmer DE, Kem D (2001) Two-state allosteric behavior in a single-domain signaling protein. Science 291:2429–2433

Zhang J, Lesort M, Guttmann RP, Johnson GV (1998) Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. J Biol Chem 273:2288–2295