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ORIGINAL ARTICLE

Unfolding studies of tissue transglutaminase

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Abstract Activation of tissue transglutaminase by calcium involves a conformational change which allows exposition of the active site to the substrate via movements of domains 3 and 4 that lead to an increase of the inter-domain distance. The inhibitor GTP counteracts these changes. Here we investigate the possible existence of non-native conformational states still compatible with the enzyme activity produced by chemical and thermal perturbations. The results indicate that chemical denaturation is reversible at low guanidine concentrations but irreversible at high concentrations of guanidine. Indeed, at low guanidine concentrations tissue TG-ase exists in a non-native state which is still affected by the ligands as in the native form. In contrast, thermal unfolding is always irreversible, with aggregation and protein self-crosslinkage in the presence of calcium. DSC thermograms of the native protein in the absence of ligands consist of two partly overlapped transitions, which weaken in the presence of calcium and merge together and strengthen in the presence of GTP. Overall, the present work shows, for the first time, the reversible denaturation of a TG-ase isoenzyme and suggests the possibility that also in in vivo, the enzyme may acquire non-native conformations relevant to its patho-physiological functions.

Keywords Tissue transglutaminase · Denaturation · Guanidine hydrochloride · Thermal unfolding · Differential scanning calorimetry · Ligands

Abbreviations

TG-ase	Transglutaminase
DSC	Differential scanning calorimetry
CD	Circular dichroism
Tm	Melting temperature
SAS	Small-angle-scattering
GdmHCl	Guanidinium hydrochloride

Introduction

Animal transglutaminases (TG-ases) catalyze the calcium dependent irreversible crosslinkage of selected substrate proteins through glutamyl-lysine isopeptide bonds (Griffin et al. 2002). Among the several TG-ase isoenzymes known, much attention has focused on the widespread tissue TG-ase. Its regulation is complex since the cross-linking activity has absolute requirement for calcium ions, it is inhibited by GTP (Achyuthan and Greenberg 1987; Bergamini 1988) and it is also regulated by sphingosylphosphorylcholine, a rare membrane phospholipid which enhances calcium activation (Lai et al. 1997). However, the initial observations that tissue TG-ase hydrolyzes GTP (Lee et al. 1989; Nakaoka et al. 1994) led to the suggestion that it is a bi-functional enzyme, capable to act alternatively as a protein crosslinker or as a G-protein, depending upon the different conformations induced by the effectors (reviewed by Griffin et al. 2002).

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The switch between the G-protein and the transamidating activity seems to be related to cell survival or to cell death, and this behaviour is apparent also in situ, in cells submitted to pharmacologic modulation of the cellular concentration of calcium and GTP (Zhang et al. 1998).

Tissue TG-ase is a monomeric protein, composed of four domains functionally arranged in pairs. Domains 1 and 2 contain the active and the regulatory sites, while domains 3 and 4 have essentially an inhibitory effect on the transamidating activity, as they hinder the substrate access to the active site (Casadio et al. 1999). The molecular bases of TG-ase activity regulation (Monsonego et al. 1998; Casadio et al. 1999; Mariani et al. 2000) imply movements of the protein domains (Griffin et al. 2002) which probably affect also its stability both in vitro and in vivo (Bergamini 2007). To further our knowledge on the enzyme regulatory mechanisms is critical for a better understanding of the involvement of TG-ase in human diseases, particularly in relation to its ability to act as self-antigen in celiac disease (Sollid 2000; Pinkas et al. 2007), and for the development of human therapies. In this respect, a description of the molecular dynamic features of tissue TG-ase is an essential step to unravel the conformational pathways that allow the protein either to express its complex and diversified enzymatic functions when in physiological conditions, or to act as an autoantigen in pathologic conditions. Based on these considerations we believe that a knowledge of the protein unfolding pathways is quite useful.

So far, data on TG-ases are largely restricted to plasma Factor XIII, available in large amounts as a recombinant protein (Rinas et al. 1990; Kurochkin et al. 1995; Dong et al. 1997). At best of our knowledge, three reports consider in some details unfolding of tissue TG-ase, taking into account thermal (Nury et al. 1989; Bergamini et al. 1999) and guanidine denaturation (Di Venere et al. 2000). Here we present data that complement those studies.

Experimental procedures

Materials

Biochemical reagents employed in most experiments, including calcium, GTP and the buffers Tris and cacodylate, were from Sigma. In circular dichroism measurements, we utilized Microselect grade reagents from Fluka and guanidine (ultrapure) from Mann. For seek of uniformity, experiments have been performed employing Tris as buffer substance, despite the possibility that pH might be modified during heating in the DSC experiments, because of the relatively high temperature-dependence of pK for amine buffers. However, a direct check proved only moderate changes in pH in the temperature range we have explored.

Transglutaminase preparation and assay of activity

Transglutaminase was prepared from human erythrocytes by a procedure consisting of chromatography on DEAE-cellulose, PEG fractionation and chromatography on DEAE–sepharose and heparin–sepharose (Bergamini et al. 1999). Purified TG-ase was homogeneous by SDS-polyacrylamide gel electrophoresis, in contrast to the commercial enzyme from guinea-pig liver, utilized in other studies (Di Venere et al. 2000), which is only 60% pure (Lee et al. 1989). The enzyme was utilized within 3–4 days from preparation because of progressive non-enzymatic degradation during storage. The concentration was determined from the UV absorbance, using the specific coefficient $A_{280\ nm,\ 0.1\%,\ 1\ cm}$ of 1.38, and activity was assayed by a filter paper procedure, measuring the rate of incorporation of radioactive putrescine into dimethylated casein in the presence of calcium ions. All procedures were described in previous publications from our laboratory (Bergamini et al. 1999).

Inactivation by guanidine and reactivation experiments

Inactivation by guanidine was investigated by incubating TG-ase for increasing periods of time with the reported concentrations of the denaturant, before dilution in guanidine-free buffer and immediate assay of residual activity. Care was taken to ensure that final concentration of guanidine in the assay mixture never exceeded 6 mM. In any case, results were corrected for the direct inhibition of activity by guanidine carried over into the assay as compared to controls containing TG-ase in the absence of guanidine. In experiments of recovery of activity, the enzyme was incubated with the denaturant until attaining loss of about 90% of activity and it was then either diluted into guanidine-free buffer or submitted to size-exclusion chromatography. When appropriate, ligands were added as detailed in the text.

Circular dichroism

Far-UV CD spectra were recorded at 25°C on a Jasco J-715 spectropolarimeter with a 0.2 cm cell path length, with spectral acquisition between 190 and 260 nm. In the case of high concentrations of guanidine the spectral range was necessarily restricted because of the high absorbance of the denaturant solution. A total of ten spectra were recorded and averaged after baseline subtraction. Data are presented as the mean residue molar ellipticities (θ, degrees cm^2/dmole), calculated on the basis of the amino acid composition of the protein. Deconvolution of the spectra to calculate secondary structure composition was performed by the Convex Constraints Algorithm (Perczel et al. 1992).

Fluorescence measurements

For steady state tryptophan fluorescence measurements the excitation wavelength was set at 292 nm and the emission was recorded at 330 and 350 nm employing either a MPF3 or a LS55 Perkin Elmer spectrofluorimeter. Ratio of fluorescence emission at these wavelengths was calculated from the emission intensities after subtraction of baseline from continuously recorded traces. Data are presented from two types of experiments performed at either variable concentrations of guanidine and constant temperature (25°C) or at fixed concentrations of guanidine and increasing temperature (between 25 and 65°C). In this last instance the temperature in the cell compartment of the fluorimeter was increased steadily with a thermal gradient of 1°C/min, by means of an external circulating bath, connected with the instrument. The temperature in the compartment was measured through a thermocouple inserted within a control cell.

Microcalorimetry

Microcalorimetric investigations of protein unfolding were carried out by differential scanning calorimetry (DSC) in a VP-DSC from Microcal Inc. The sample cell contained 0.7 ml of degassed TG-ase (0.7 mg/ml) equilibrated in Tris buffer by extensive dialysis, while the reference cell contained the solvent, without enzyme. Ligands were added when appropriate. Scanning was performed between 25 and 65°C, with a thermal gradient of 1°C/min. The resulting thermograms were analysed by the software Origin, supplied with the instrument, to determine T_m of the transitions and the associated enthalpies of unfolding. As discussed in the text, thermal unfolding of TG-ase is an irreversible process which leads also to protein aggregation; this prevents a rigorous application of thermodynamics to analysis of protein since this is restricted to proteins which undergo reversible unfolding/folding cycling (Privalov and Dragan 2007). Therefore, the reported values of unfolding enthalpies cannot be considered true thermodynamic constants but only indicative values.

Results

Inactivation and unfolding of TG-ase using GdmHCl

Incubation of erythrocyte TG-ase with GdmHCl leads to guanidine concentration- and time-dependent inactivation. Figure 1a shows that, while there is a marginal loss of activity at low concentration of denaturant (0.2–0.3 M), at slightly higher concentrations of GdmHCl (0.5–0.75 M) we observe a net increase of the inactivation rate. The loss of activity is virtually complete within 60 min of incubation with moderately high concentrations of GdmHCl (1–1.2 M, data not shown). The diverse perturbations of the enzyme activity produced by guanidine at concentrations lower than 0.8 M (Fig. 1a) could be due either to a threshold of the denaturant concentration able to induce TG-ase unfolding, or to the capability of the enzyme to recover its activity during the time of the assay, after

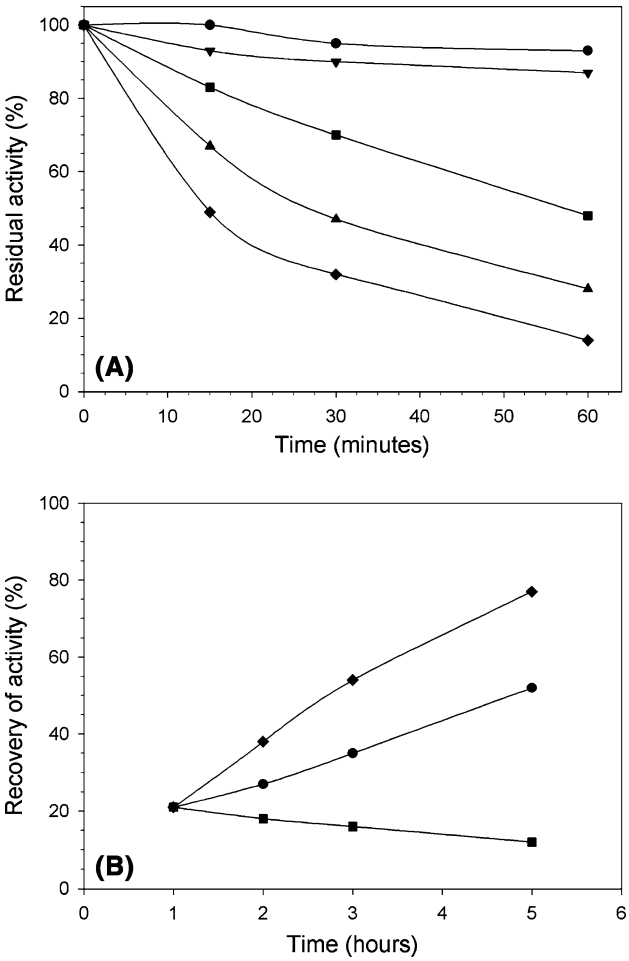

Fig. 1 Reversible inactivation of tissue TG-ase by guanidine (**a**): time course for inactivation of TG-ase by increasing concentrations of GdmHCl. The enzyme equilibrated in 25 mM Tris–HCl, 1 mM mercaptoethanol pH 7.5 was incubated with GdmHCl at final concentrations of 0.2 (*filled circle*), 0.3 (*filled inverted triangle*), 0.5 (*filled square*), 0.6 (*filled triangle*) and 0.75 M (*filled diamond*). At the indicated time intervals samples were withdrawn, diluted with buffer and assayed for residual activity as detailed in the Experimental procedures (**b**): effects of ligands on the recovery of catalytic activity. The enzyme was incubated with 0.4 M guanidine for 1 h, to achieve a residual specific activity of 20% of the original one. It was further desalted by chromatography on a small column of Sephadex G-25 and the eluate was divided into aliquots which were incubated at room temperature for increasing periods of time without any addition (*filled circle*) or after supplementation with either 0.5 mM GTP (*filled diamond*) or 3 mM calcium (*filled square*)

dilution of GdmHCl in the incubation mixture. To check these hypotheses, we have inactivated TG-ase to a 20% residual activity through incubation with 0.4 M GdmHCl, and the recovery of the enzyme activity has been monitored after removal of the denaturant by size-exclusion chromatography (Fig. 1b). The observed significant recovery of activity clearly indicates that the inactivation by guanidine is essentially a reversible process; conversely, the reversibility of the inactivation is completely lost when the enzyme is treated with GdmHCl at concentration higher than 0.8 M (data not shown). Notably, Fig. 1b shows that the regain of catalytic activity is affected by the ligands in a complete different manner. In fact, addition of GTP to the medium increases the extent and the rate of recovery, while in the presence of Ca^{2+} ions, not only no reactivation is observed, but also we observed an enhancement of deactivation with time. Similarly, when the effect of the ligands was tested on the rate of inactivation of TG-ase by guanidine, we found that Ca^{2+} ions enhanced the rate of enzyme inactivation while GTP exhibited a protective effect (data not shown); these findings are consistent with those reported by Di Venere et al. (2000).

Using CD spectroscopy, we investigated the dependence of the TG-ase secondary structure by GdmHCl. The far-UV CD spectrum of the native protein is reported in Fig. 2. Its deconvolution by the Convex Constraint Algorithm procedure gives approximately 20% α-helix, 30% β-sheets and turns, and 35% of extended structure. The remaining 15% is due to contributions from the aromatic residues. These values match closely those determined by both FT-IR spectroscopy (Tanfani et al. 1993) and molecular modelling (Casadio et al. 1999). Interestingly, they are not modified by addition of either Ca^{2+} ions or GTP, confirming that binding of these ligands does not affect appreciably the secondary structure of TG-ase, as previously suggested (Tanfani et al. 1993). In Fig. 2, we also report the CD spectra obtained in the presence of GdmHCl in the concentration range in which the activity is affected in a reversible manner. Indeed, we observe only partial modifications of the CD profiles, indicating that the protein is still folded and mainly preserves its secondary structure. At higher concentrations of GdmHCl (1.0–3.0 M) the spectra indicate a progressive unfolding of the protein (data not shown), in accordance with the finding that it becomes irreversibly inactive (as reported above).

The analysis of the protein tertiary structure by fluorescence spectroscopy provided specific information about domains 1 and 2 which contain the whole set of the 13 tryptophan residues. The emission spectrum of TG-ase, upon excitation at 292 nm, is characterized by an intense wide band with a maximum centred at ~334 nm (data not shown), suggesting that the Trp residues are embedded in a heterogeneous, mainly hydrophobic environment. Even low concentrations of guanidine alter the fluorescence emission spectrum, with a significant red shift to 341 nm and to 350 nm, for the enzyme equilibrated in 1.2 and in 1.9 M GdmHCl, respectively (Fig. 3, right ordinate). These changes reflect the high sensitivity to the denaturant of the spatial arrangement of domains 1 and 2 and the fact that the induced conformational changes promote the exposition to the solvent of the Trp residues.

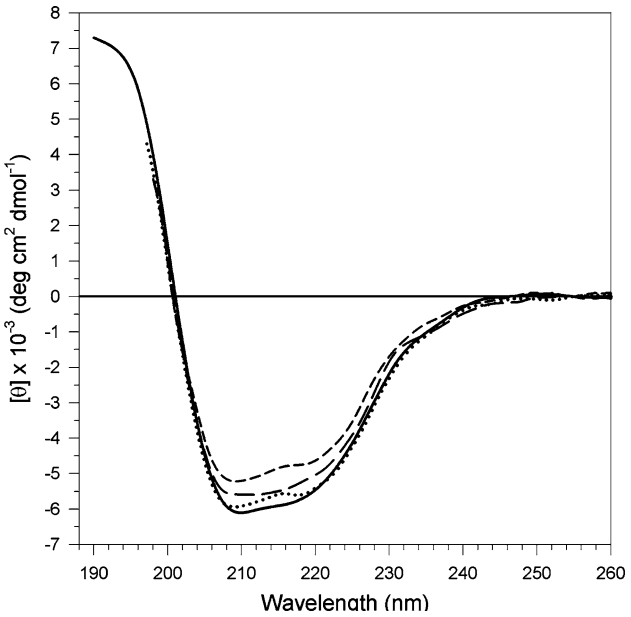

Fig. 2 Far-UV CD spectra of TG-ase in the native state (*solid line*) and as a function of increasing concentration of GdmHCl. The enzyme was incubated in the presence of 0.2 M (*dotted line*), 0.4 M (*long dashed line*) and 0.6 M (*short dashed line*) GdmHCl and spectra were recorded after 30 min of incubation

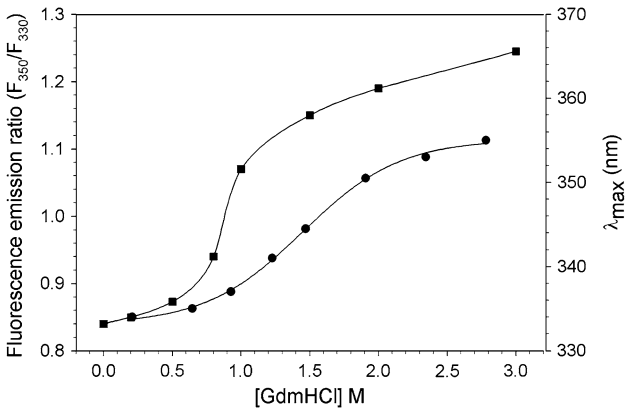

Fig. 3 Influence of increasing concentrations of GdmHCl on the fluorescence emission of TG-ase. Data were recorded either as the ratio of fluorescence emission intensities at 350 and 330 nm (*filled square*, left ordinate) or as the wavelength of maximal emission (*filled circle*, right ordinate)

Particularly enlightening is the comparison of those data with the behaviour of the ratio of the fluorescence emission at 350 and 330 nm as a function of GdmHCl (Fig. 3, left ordinate). In this case, a net transition takes place at about 0.85 M GdmHCl, that is a concentration of denaturant significantly lower with respect to the mid point of the transition reported by the change in the fluorescence emission maximum, ~1.35 M GdmHCl. We interpret these data as indication that, in the guanidine low concentration range, TG-ase undergoes mild conformational changes of the tertiary structure that, however, lead to a quenching of the tryptophan fluorescence possibly due to interactions with others TG-ase side-chains. Nonetheless, domains 1 and 2 preserve their protection from the solvent. These changes in the protein tertiary structure produce a partial loss of activity and are reversible, thus allowing recovery of the protein function upon removal of the denaturant. At concentration of GdmHCl higher than ~1 M we observe a more pronounced unfolding that leads to water accessibility, as indicated by the pronounced red shift of the Trp emission maximum. At this stage the protein becomes irreversibly inactivated. Indeed, we may interpret these results as a confirmation of the functional data reported above.

Unfolding and inactivation induced by heating

The hypothesis that TG-ase unfolding implies transitions among intermediate functional conformational states, as suggested by the GdmHCl experiments, is supported by the combined use of the denaturant with heat. Figure 4 shows the behaviour of the fluorescence emission intensity ratio, 350/330 nm, as a function of temperature, for the enzyme equilibrated at various concentration of GdmHCl. In the presence of 0.1 M GdmHCl the thermal denaturation profile is very similar to that of the native protein: the main difference is a lower temperature transition, about 46 versus 50°C. A significantly diverse profile, though still indicative of a two-state transition, is observed in the presence of 0.4 M GdmHCl. In this case the data suggest the protein exists in a non-native conformational state characterized by a even lower transition temperature, approximately 36°C. At higher GdmHCl concentration the denaturation profile loses its sigmoid shape indicating that, in these experimental conditions, the protein does not exist anymore in a well-defined conformation and thus the unfolding process involves a multistate equilibrium.

Previous studies from our laboratory showed that thermal inactivation of TG-ase is irreversible (Bergamini et al. 1999), thus suggesting that heat perturbs the protein structure with a different mechanism with respect to the one associated to GdmHCl denaturation. The thermal denaturation profile of native TG-ase, reported in Fig. 4, indicates that it covers a temperature range from ~40 to ~58°C. Aiming to complement the data on the role of ligands in modulating the protein structure and function in non-native conditions, we devised an experiment where the protein architecture was destabilized by heating at 42°C (Fig. 5). We chose to equilibrate the protein at this temperature because, based on the thermal unfolding profile (Fig. 4) and previous data (Bergamini et al. 1999), we expect the enzyme should be in structural and functional conditions comparable to the ones described in Fig. 1b when using 0.4 M GdmHCl. Recognizing that heat perturbs the protein in an irreversible fashion here we measured the rate of inactivation: Fig. 5 clearly shows that

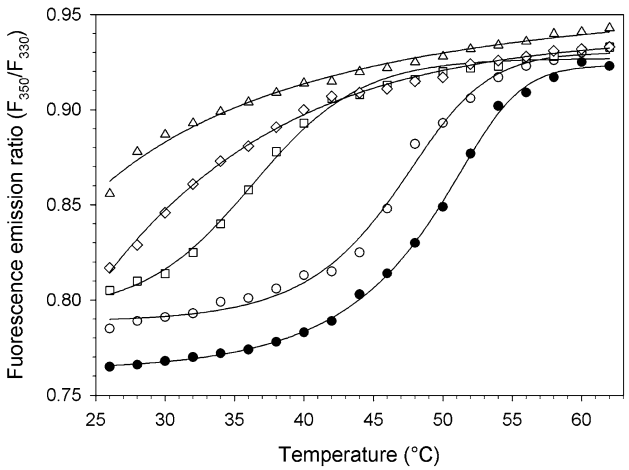

Fig. 4 Ratio of fluorescence emission intensities at 350 and 330 nm of TG-ase (*filled circle*) and in the presence of various concentrations of GdmHCl: 0.1 M (*open circle*), 0.4 M (*open square*), 0.65 M (*open diamond*) and 1.0 M (*open triangle*), at increasing temperature

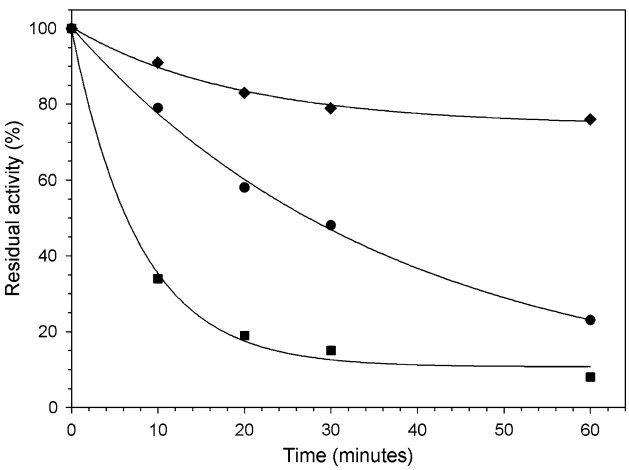

Fig. 5 Effects of ligands on the time course of thermal inactivation of TG-ase at 42°C. The enzyme (0.15 mg/ml) was incubated for the reported time intervals in the absence of ligands (*filled circle*), and in the presence of either 0.5 mM GTP (*filled diamond*) or 4 mM Ca^{2+} (*filled square*)

while GTP has a protective effect, the addition of Ca^{2+} ions results in a net increase of the inactivation rate.

Figure 6 reports the DSC thermograms of TG-ase unfolding in the absence (trace *a*) and in the presence of ligands (trace *b* and *c*). In the case of the pure protein (trace *a*) the thermogram indicates a large excess in heat capacity (ΔCp) which can be resolved into two partly overlapping transitions, with T_{m1} and T_{m2} of 49.5 and 53.5°C, respectively, which have been ascribed previously to the unfolding of the domains representing respectively the TG-ase N-terminal and the C-terminal regions (Bergamini et al. 1999). These melting temperatures are also maintained during unfolding in the presence of 4 mM calcium ions (trace *b*). Notably, however, the apparent unfolding enthalpies are quite different, being 204 and 110 kcal/mole in the absence and in the presence of Ca^{2+}, respectively. The data suggest that the protein domains, while preserving their basic structural features, interact less tightly in the presence of calcium (see also Mariani et al. 2000).

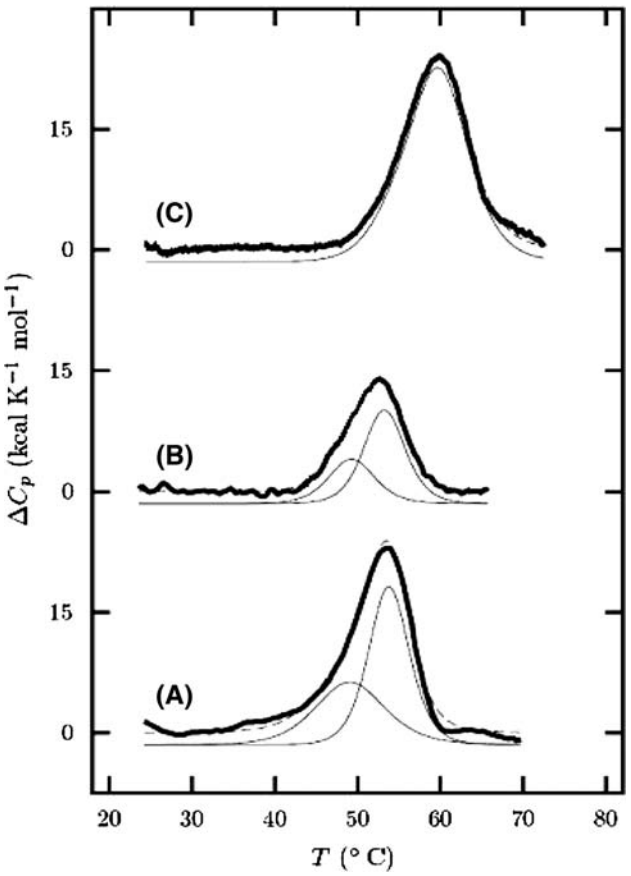

Fig. 6 Thermograms of unfolding of TG-ase during heating in the absence of ligand (trace *a*) and in the presence of either 4 mM calcium (trace *b*) or 0.5 mM GTP (trace *c*). The *thick lines* represent original data after subtraction of baseline and normalization for protein concentration, while the *thin lines* show the deconvolution into component transitions, obtained by the standard Origin procedure. For additional details, see "Experimental procedures"

Differently, when the experiments are carried out in the presence of GTP (trace *c*), the thermogram is characterized by a single stronger transition shifted at higher temperature, 60°C, with an apparent unfolding enthalpy of 245 kcal/mole, which we interpret to reflect the contemporaneous unfolding of the N- and the C-terminal domains that, in this case, behave as a single unfolding unit.

Discussion

Reversibility is not a general property of partly unfolded state, depending strongly on the nature of the protein and of the denaturing agent. For instance it is frequently limited following thermal denaturation in multidomain proteins (Strucksberg et al. 2007). Uncoupling is observed frequently during denaturation by guanidine. In this case inactivation occurs without detectable structural derangement, because the denaturant may act in complex multistep processes involving both local and long-distance effects eventually modifying protein flexibility (Tsou 1995; Bai et al. 1999). Under these conditions the apparent maintenance of structural integrity can allow reversibility to occur, usually favoured by the accumulation of populated intermediates during the unfolding/refolding process (Bai et al. 1999) and further aided by the presence of protein (HSP) or molecular chaperons (e.g. TMNO), which assist refolding in vitro as well as in vivo.

A recent reviewing of the literature led to the conclusion that ligands affect the stability of tissue transglutaminase both in vitro and in vivo and they potentially participate in control of the protein turn-over in vivo (Bergamini 2007). To expand and complement the data available so far, in this report, we have investigated the effects of ligands on inactivation and unfolding of tissue TG-ase employing both guanidine and heat as denaturing agents.

The enzyme, upon incubation with GdmHCl, reveals a different behaviour depending upon the denaturant concentration. The recovery of catalytic activity is significant only when GdmHCl is lower than ~0.8 M. In these experimental conditions the denaturant induces moderate conformational changes in domains 1 and 2, with retention of the secondary structure and some possible reorientations of the aromatic side chains. We envisage this protein state as an intermediate in the unfolding process that, notably, maintains the ability to interact with its own regulatory ligands. These features, typical of proteins in the "molten globule state" (Haynie and Freire 1993), disappear at higher concentrations of guanidine (>1 M) where, in fact, we observe a progressive increase of the unfolding associated to the onset of multiple conformational states and to an irreversible loss of activity. Overall, the results may be summarized by a scheme of the type:

$$\mathbf{N} \overset{K}{\leftrightarrows} \mathbf{I} \overset{k}{\longrightarrow} \mathbf{U}$$

where K is the equilibrium constant for the accumulation of the intermediate state (**I**) in equilibrium with the native one (**N**) and k is the rate constant for the irreversible conversion of I into the denatured unfolded state (**U**). In this perspective, according to our data, the accumulation of the intermediate state is a reversible process sensible to the presence of ligands, while the last step is irreversible and related to the concentration of the denaturant. The addition of either calcium ions or GTP shifts the unfolding process to lower and to higher concentrations of guanidine, respectively.

A different situation emerges when taking into account the inactivation of tissue TG-ase by thermal treatment since an irreversible pattern of inactivation/unfolding is constantly observed with massive protein aggregation. Interestingly, no recovery of enzyme activity has ever been measured by cooling the enzyme, inactivated ~80% of its initial activity by heating at 45°C, also in solutions supplemented with ligands. In contrast, the ligands themselves can enhance or inhibit the rate of inactivation, when the incubation is carried out in the presence of calcium and GTP, respectively. The irreversibility of the thermal inactivation is also consistent with the DSC thermograms which display large post-transition excess in thermal capacity which are typical of proteins undergoing massive aggregation in the unfolded state. This phenomenon takes place at high temperature at the end of the calorimetric experiments, where the sample presents always a high turbidity (data not shown). By SDS-PAGE we have detected stable protein aggregates (dimers and even larger polymers) in samples heated in the presence of calcium as if the enzyme had underwent self-crosslinkage (data not shown).

In scans in the absence of ligands or in the presence of calcium the calorimetric transition can be resolved into two components which are partly overlapped. In contrast, scans in the presence of GTP are characterized by a single transition of higher heat capacity, as it would happen in the case of a tighter interaction in the protein regions which generate the two transitions observed in the previous cases.

From a "cellular" point of view, it must be kept in mind that the effects of ligands in controlling the overall dynamics of tissue TG-ase and its catalytic activity are observed in vitro at high concentrations of calcium and GTP (low millimolar range) but at more "physiologic" concentrations in permeabilized cells in situ (see also Smethurst and Griffin 1996). In addition, the ligands contribute to modulate TG-ase stability in vitro, and probably in vivo (Bergamini 2007), by acting on the protein structure. In vitro, the influence of calcium and GTP on protein stability, as observed in the experiments of unfolding by guanidine (present study; Di Venere et al. 2000) or following thermal treatment during DSC experiments, reflects the diverse unfolding process of the individual domains, induced by the binding of the ligands to specific protein sites. In the case of calcium ions, while they disclose the access to the active centre by moving away the inhibitory domains 3 and 4, it is also worth noting that by "opening" the protein structure, they allow interactions with inhibitory chemicals and favour the disruption of the native folding. In addition, because the loosening of interdomain interactions may alter the rigidity of the interdomain regions, as we proved in a previous study (Casadio et al. 1999), we expect it may modulate the sensitivity of the enzyme to proteolytic degradation.

The present work documents for the first time the possibility of reversible denaturation of a TG-ase isoenzyme. This is a novel finding, since most of the previous experiments were performed on the homologous dimeric transglutaminase Factor XIIIa (Rinas et al. 1990) whose denaturation proceeds irreversibly, because it involves the dissociation of the dimer into single subunits, an irreversible step dependent on the interaction between the pairs of domains 1 and 2 in each monomer (Kurochkin et al. 1995). As suggested above, the reversibility of denaturation of tissue TG-ase seems to be related to the formation of a non-native intermediate state, sensitive to ligands, being able to recover enzymatic activity in different ways depending on their presence or absence. Thus, we suggest that calcium and GTP bind to that intermediate state producing an unfavourable or favourable state, respectively, that will lead either to misfolding or to the correct refolding, upon removal of guanidine. The possibility that this phenomenon of limited unfolding may take place in vivo is obviously hypothetical. However, the relevance of the results obtained by in vitro folding/unfolding experiments to the in vivo conditions is a general issue. In fact, "chaotropic" nitrogen compounds promoting protein unfolding (e.g. urea, guanidine and methylguanidine) can accumulate at potentially relevant concentrations in normal renal medulla and in other tissues following the onset of pathological states such as chronic renal failure. Moreover, it is known that animal tissues may contain appreciable concentrations either of refolding osmolytes such as betaine or trimethylamine-*N*-oxide (Baskakov et al. 1998). We are presently reconsidering this topics on transglutaminase from porcine kidney, since this tissue is (or may be) exposed, in vivo, to high concentrations of urea or of guanidine derivatives (notably methylguanidine and guanidinosuccinic acid), which are well-known uremic toxins (Kishore et al. 1989).

In vitro and in vivo, the relevance of domains 3 and 4, and of their interaction with the ligands in dictating both the expression of catalytic activity and the stability of the

enzyme is further supported by observations on Factor XIII, in which binding of antibodies has major effects on activity through an interference on the function of domains 3 and 4 (Mitkevich et al. 1998).

In conclusion, understanding these issues is quite important in the light of the increasing number of autoimmune diseases correlating with raised titers of antibodies directed against the N- or the C-terminal domains of native TG-ase, including coeliac disease (Sollid 2000), type 1 diabetes mellitus, rheumatic diseases and possibly autoimmune myocarditis (Schuppan and Hahn 2001; Frustaci et al. 2002; Luft et al. 2003). In this respect it is not always clear whether these associations are just epidemiologic events or depend on common pathogenetic mechanisms, such as the large conformational changes tissue TG-ase undergoes during interaction with the substrates (Pinkas et al. 2007). A clearer understanding of the biology of tissue transglutaminase, including its structural and dynamic features, will be relevant for molecular medicine, also taking into account the demonstration of tissular accumulation of proteolytic fragments of the enzyme (Fabbi et al. 1999).

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