

Thermodynamics of binding of regulatory ligands to tissue transglutaminase

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Abstract The transamidating activity of tissue transglutaminase is regulated by the ligands calcium and GTP, via conformational changes which facilitate or interfere with interaction with the peptidyl-glutamine substrate. We have analysed binding of these ligands by calorimetric and computational approaches. In the case of GTP we have detected a single high affinity site ($K_D \approx 1 \mu\text{M}$), with moderate thermal effects suggestive that binding GTP involves replacement of GDP, normally bound to the protein. On line with this possibility no significant binding was observed during titration with GDP and computational studies support this view. Titration with calcium at a high cation molar excess yielded a complex binding isotherm with a number of “apparent binding sites” in large excess over those detectable by equilibrium dialysis (6 sites). This binding pattern is ascribed to occurrence of additional thermal contributions, beyond those of binding, due to the occurrence of conformational changes and to catalysis itself (with protein self-crosslinking). In contrast only

one site for binding calcium with high affinity ($K_D \approx 0.15 \mu\text{M}$) is observed with samples of enzyme inactivated by alkylation at the active site (to prevent enzyme cross-linkage and thermal effects of catalysis). These results indicate an intrinsic ability of tissue transglutaminase to bind calcium with high affinity and the necessity of careful reassessment of the enzyme regulatory pattern in relation to the concentrations of ligands in living cells, taking also in account effects of ligands on protein subcellular compartmentation.

Keywords Transglutaminase · Binding of ligands · Calcium · GTP · Isothermal titration calorimetry

Introduction

Type 2 transglutaminase (TGase 2, also called tissue transglutaminase) is a member of a wide family of enzymes which catalyze the crosslinkage of substrate proteins through formation of isopeptide bonds between peptidyl-bound γ -glutamyl moieties of glutamine and ε -amino groups of lysine residues. This reaction is designed as transamidation because it involves transfer of acyl-groups from primary (glutamine) to secondary amides. Transglutaminases are widely distributed in nature, from bacteria to plants and animals. Activity of the enzymes from animals is apparently tightly regulated by modulatory ligands, via conformational changes (reviewed by Griffin et al. 2002).

In the case of TGase 2, regulation is achieved through the interaction with calcium (an essential cofactor) and with GTP (an inhibitor at subsaturating concentrations of calcium). Observations from in vitro studies indicate that the transamidation reaction is dependent on relatively high concentrations (millimolar) of the calcium ions and GTP at

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physiologic concentration is an effective inhibitor giving rise to a sigmoid profile of activity in calcium saturation plot in the presence of both ligands. These results lead to the conclusion that TGase 2 is a low-affinity calcium-binding protein and is a latent enzyme under the conditions prevailing in the intracellular compartment (Griffin et al. 2002), because of the powerful inhibition by GTP. Over years it became clear that TGase 2, along with the ability to polymerize protein substrates, can also transduce extracellular signals acting as G-protein upon association with the plasma membrane (Park et al. 2001). The activities of protein transamidation and of signalling GTPase are switched on–off in a mutually exclusive way, since the GTPase is obviously dependent on GTP, which is the inhibitor of transamidating reaction. We have developed a conformational model to explain these effects (Casadio et al. 1999).

More recent studies indicate that the situation *in vivo* is likely to be more complicated than assumed from the studies *in vitro*, because of additional regulatory effects which include translocation of the enzyme to the plasma membrane. A considerable fraction of the protein is exposed on the external cell surface and behaves as an ectoenzyme to crosslink ECM proteins and stabilize cell–matrix interactions (Bergamini et al. 2005), which can be regulated by membrane phospholipids (Lai et al. 1997) and by thiol-nitrosylation of the enzyme itself (Lai et al. 2001). Investigations were also performed *in situ* on cultured cells, to fully appreciate regulation under physiologic conditions. In these series of studies unexpected results were obtained since transglutaminases proved to be sensitive also to interaction with substrate proteins (Mitkevich et al. 1998; Pinkas et al. 2007) and TGase 2 *in situ* is active even at very low concentrations of calcium, thus behaving as a high-affinity calcium-binding protein (Smethurst and Griffin 1996; Zhang et al. 1998). These findings underline the necessity to reinvestigate the effects of ligands by careful analysis of ligand–protein interaction. We now face this issue by combining isothermal titration calorimetry (ITC) and protein modelling approaches, taking into account the main regulatory ligands calcium and GTP.

Materials and methods

Materials

Human erythrocyte TGase was purified by a slight modification of our standard procedure (Casadio et al. 1999). The changes include fractionation with ammonium sulphate (55% saturation) of the enzyme eluted from the DEAE–Sepharose resin followed by size exclusion chromatography onto AcA 34, before the final affinity chromatography on Heparin–Sepharose. 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). The TGase inhibitor R283 (1,3, dimethyl-2-[(2-oxopropyl) thio]imidazolium chloride) was prepared as previously defined (Freund et al. 1994).

Calorimetric analysis of binding of ligands

Binding of ligands to TGase 2 was explored by isothermal titration calorimetry (ITC) employing a VP-ITC apparatus from Microcal Inc. (Northampton, MA, USA). In typical experiments the enzyme (dialysed against 25 mM Tris, 0.5 mM mercaptoethanol, pH 7.5) was placed inside the sample cell thermostated at 25°C at a concentration of 10 µM and the ligands, either GTP or calcium dissolved in the dialysis buffer, were injected from the syringe at amounts suitable to achieve progressive saturation of binding sites. The concentration of ligands in the titrant stock solution was checked by careful weighing of dry salt in the case of calcium and by UV spectroscopy in the case of GTP. Experimental data were analyzed by means of the software Origin provided by the manufacturer. Calcium titration experiments were performed on the native protein as well as on the enzyme inactivated by irreversible alkylation of the active site by reaction with R283 (Freund et al. 1994), for the reasons explained below in “[Results and discussion](#)”. For complete inactivation the enzyme (1.5 mg/ml) was incubated for 20 min at room temperature with 0.5 mM R283, and verified by activity assay following incubation with the inhibitor and subsequent dialysis to remove excess inhibitor. Three independent experiments with GTP and calcium were analyzed separately.

Computational studies of interaction of guanine nucleotides and transglutaminase

Docking for binding of GDP/GTP was performed on the structure of tissue TGase 2 crystallized in the presence of GDP (PDB code 1KV3) (Liu et al. 2002), by recognition of the binding pocket surface. We have chosen this structure rather than that reported recently by Pinkas et al. (2007) (PDB code 2Q3Z) because these authors investigated TGase 2 blocked at the active site by a reactive substrate-homolog peptide and is apparently devoid of nucleotides which are commonly associated with TGase 2 under native conditions.

After a first performance of blind docking on TGase 2/ GDP with satisfactory results (see Fig. 4d) employing the basic crystal structure as a ligand free protein, further docking were generated against GTP, employing the

AutoDock v3 tool (Morris et al. 1998). Briefly, box size was set in order to cover all the residues involved in TGase binding site for GDP, as identified in 1KV3 crystal. Energy minimization for binding was performed by using LGA (Lamarckian Genetic Algorithm) with a number of individuals in population of 50, a maximum number of energy evaluations of 250,000 and an elitism parameter set to 1. Gene mutation and crossing over were set to 0.02 and 0.80, respectively. Flexible docking was performed and 50 LGA runs were launched. All the energetically favourable results were analyzed and validated according to literature. Fig. 4, generated with LIGPLOT tool (<http://www.biochem.ucl.ac.uk/bsm/ligplot/ligplot.html>), shows the accommodation of the GDP and GTP ligands inside the guanine nucleotide binding site of TGase 2.

Molecular dynamics of ligand free TGase 2 and of TGase 2 docked with GDP or with GTP were performed by Gromacs v.3.3 (<http://www.gromacs.org>). The simulations were carried out in water, applying a G43A1 forcefield for 1 ns (about 80 h on P4 3.0, 2 Gb RAM for each simulation) at the constant temperature of 298 K.

Results and discussion

Background on ligand binding

In previous investigations we explored the effects of ligands on TGase 2, taking into account stoichiometry of binding as well as effects on stability of the protein against chemical or thermal denaturation, and degradation by proteinases. Data on binding were obtained by affinity labelling with dial-GTP and by equilibrium dialysis of calcium. In this way we detected a single binding site for GTP (Bergamini and Signorini 1993) and six binding sites for calcium (Bergamini 1988), in agreement with data by other authors for binding of GTP to TGase 2 (Iismaa et al. 2000) and of calcium to the homologous TGase 3 (Ahvazi et al. 2002). Data on calcium binding to TGase 5, which is also regulated by GTP (Candi et al. 2004) are not available. In relation to the effects of ligands on protein structure and stability, previous results demonstrated a stabilizing action of GTP and a destabilizing action of calcium towards all challenges tested, and this behaviour is displayed even in situ (Bergamini 2007). This is in agreement with the structural–functional model we have developed (Casadio et al. 1999) assuming that the stability of the enzyme is highly dependent on the strength of interaction between domain 2 and 3, as confirmed by recent evidence provided by differential scanning calorimetry and small-angle scattering experiments (Bergamini et al., in preparation). However, complete understanding of the interactions between TGase 2 and ligands and of their structural–

functional correlates require precise thermodynamic data on affinity and energetic of binding by ITC, which is presently the procedure of election for these investigations (Velazquez Campoy and Freire 2005).

Thermodynamics of interaction with ligands by ITC

In the present study we have determined the thermodynamic constants for binding of the ligands to TGase 2 by ITC. In the specific case of calcium, titrations were performed on native TGase and on the enzyme inactivated by reaction with the inhibitor R283.

Data on binding of GTP and GDP were evaluated in experiments performed by injecting nucleotides from the titration syringe into the solution of the stirred enzyme. The data obtained at a standard temperature of 25°C are presented in Fig. 1. They nicely document the tight binding of 1 mol of GTP per mole of enzyme, with a dissociation constant of 1 μ M, in an enthalpy driven process ($\Delta G = -3.1$ kcal/mol). Notably, no net heat exchange was observed during titration with GDP. In relation to this finding it is worth to point out that available evidence indicates from one side the permanent binding to the enzyme of a guanine nucleotide (Liu et al. 2002) (either GDP or GTP, which can substitute for each other by exchange), and from the other side a protective effect of this interaction, since the native conformation is lost rapidly upon stripping the nucleotide (Murthy and Lorand 2000), with concomitant inactivation. In this perspective, binding of GTP should actually represent a

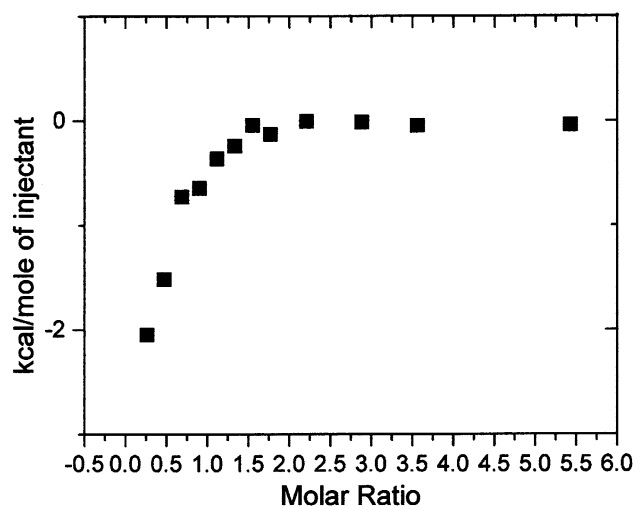


Fig. 1 Binding of GTP to TGase 2 by isothermal titration calorimetry. The enzyme was dissolved in buffer (25 mM Tris–HCl, 50 mM NaCl, 1 mM mercaptoethanol, pH 7.5) at a concentration of 14 μ M and titrated with a solution of GTP (323 μ M) in the same solvent, with a sequence of 18 injections of 12 μ L each, after a first injection of 5 μ L, which was discarded as usual in titration studies. The experiment was performed at 25°C

GDP–GTP exchange rather than a true binding process, since stoichiometric amounts of GDP are present in the purified protein (Liu et al. 2002). Thus, the lack of any net heat exchange during titration with GDP should represent the on–off exchange of GDP itself, which obviously should take place without any thermal effect, while the energetics of GTP binding is indeed the difference in enthalpy ΔH_b between GDP and GTP binding.

In the case of calcium, results from titration data of the native enzyme within a wide range of cation up to a large molar excess (Fig. 2) indicate the occurrence of several thermal effects (interpreted by the deconvolution programme as “apparent binding sites”) with a stoichiometry of up to 20 apparent sites clearly exceeding the stoichiometry of 6 sites determined by equilibrium dialysis.

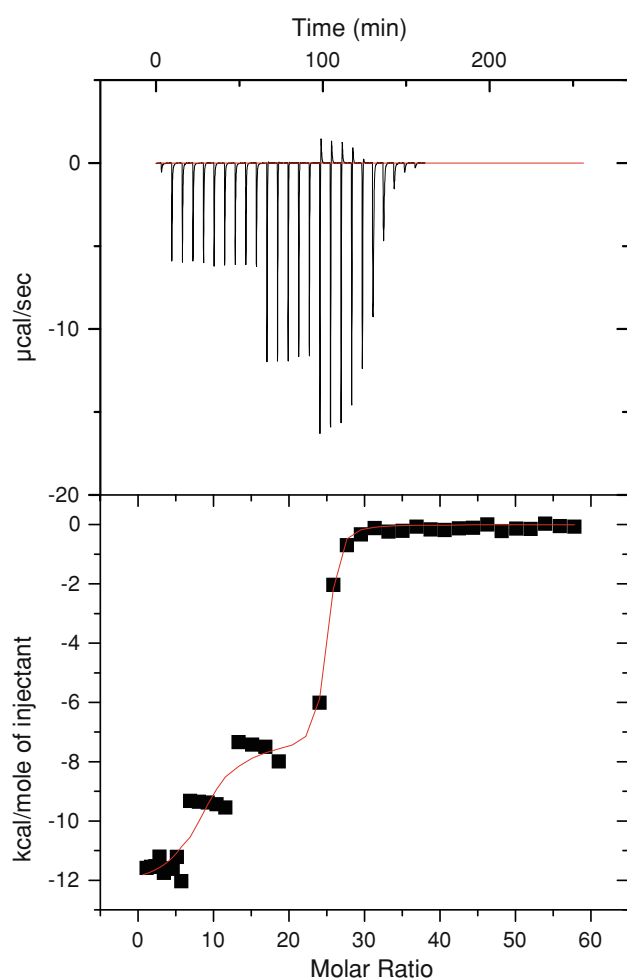


Fig. 2 Binding of calcium to native TGase 2 by isothermal titration calorimetry. This experiment was performed at 25°C on a solution of TGase 2 (15 μM dissolved in 25 mM Tris–HCl, 1 mM mercaptoethanol, pH 7.5), which was titrated with a solution of CaCl_2 (6 mM) in the same buffer. The injection scheme was 10 injections of 2 μL each, 5 injections of 4 μL each, followed by 25 injections of 6 μL each. *Upper panel* raw binding data, *lower panel* deconvoluted binding curve

During titration the native enzyme constantly developed turbidity, which we ascribe to self-crosslinkage, following activation of transamidation by the calcium added during titration. This effect was confirmed by SDS–PAGE which demonstrated progressive decline in the intensity of the monomer protein band and appearance of HMW aggregates which resist boiling in the presence of SDS and mercaptoethanol (not shown). Following these findings, we decided to explore the binding of calcium to the enzyme inactivated by incubation with the site directed inhibitor R283. Relevant data are presented in Fig. 3. In this case only one high affinity site remains available to bind the cation ($K_D \approx 0.15 \mu\text{M}$), suggesting on one side that occupancy of the additional low-affinity sites is dependent on some sort of molecular rearrangement of the protein, and on the other that heat exchange due to catalysis and to

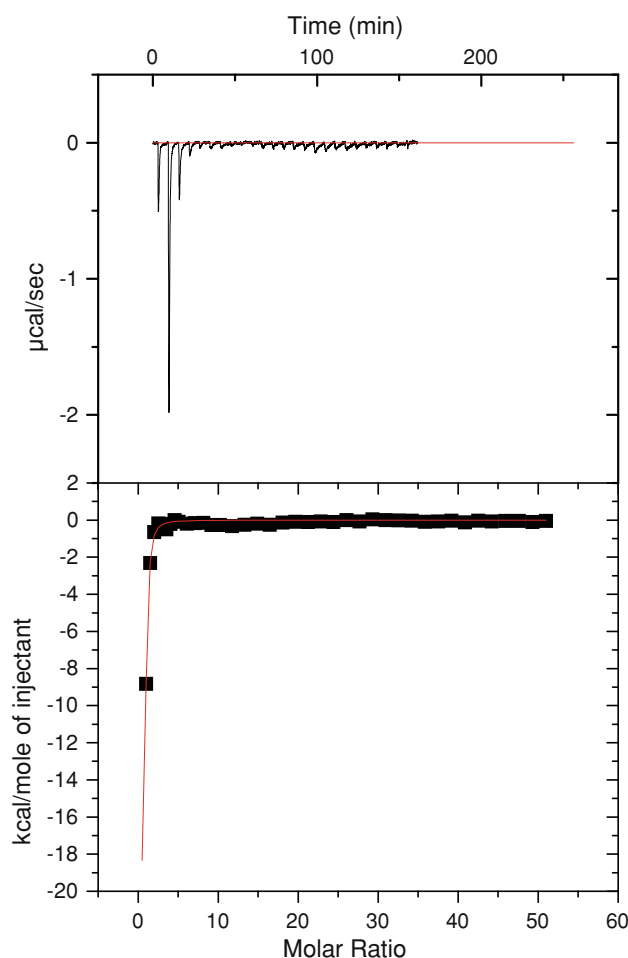


Fig. 3 Binding of calcium to inactivated TGase 2 by isothermal titration calorimetry. This titration was performed employing the same injection scheme as in Fig. 2 on a sample of TGase 2 (16.6 μM in the same buffer), alkylated to complete inactivation, as described in “Materials and methods” section. *Upper panel* raw binding data, *lower panel* deconvoluted binding curve

conformational changes might contribute to the thermal effects observed in the case of the native enzyme. Clearly inactivation of the enzyme blocks these processes.

Molecular docking and dynamics of guanine nucleotide binding

We employed computational procedures to further understand interaction of TGase 2 with GXP ligands. For this purpose we must underline that the structure of recombinant TGase 2 in the GDP-binding state in the crystals is described as that of a dimeric protein (Liu et al. 2002) and that, at variance with the crystallographic data, all evidences collected with TGase 2 in solution (by size exclusion chromatography and analytical ultracentrifugation, C.M.B. unpublished observations) and small angle scattering are indicative of a monomeric structure, although aggregates are formed in the presence of calcium (Mariani et al. 2000). We imagine therefore that the discrepancies between the results of the crystallographic diffractions and

the usual observations in solution are due to protein assembly during crystallography.

We have analyzed in detail the site of interaction with guanine nucleotides (see Fig. 4) by comparing the results of “blind” docking for TGase 2/GDP with the original crystallographic coordinates in the complete PDB file 1KV3, for the reasons discussed in the [Materials and methods](#) section. As expected, structures of the two GDP complexes are not perfectly superimposable; however, the majority of the stabilizing interactions with the protein residues are preserved (compare Fig. 4a, b). More specifically, the binding of GDP to α - and β -phosphate moieties is stabilised by three hydrogen bonds with Arg 478, Val 479 and Arg 580, respectively. The guanosine moiety in turn is hydrogen bonded to Ser 482 and Tyr 583. Moreover, further stabilisation of the aromatic ring is provided by the presence of Phe 174. To comment on the GDP binding after the “blind” docking procedure, these observations demonstrate (see panel 4b) that the involvement of these residues is also confirmed after computing and furthermore there is formation of more hydrogen

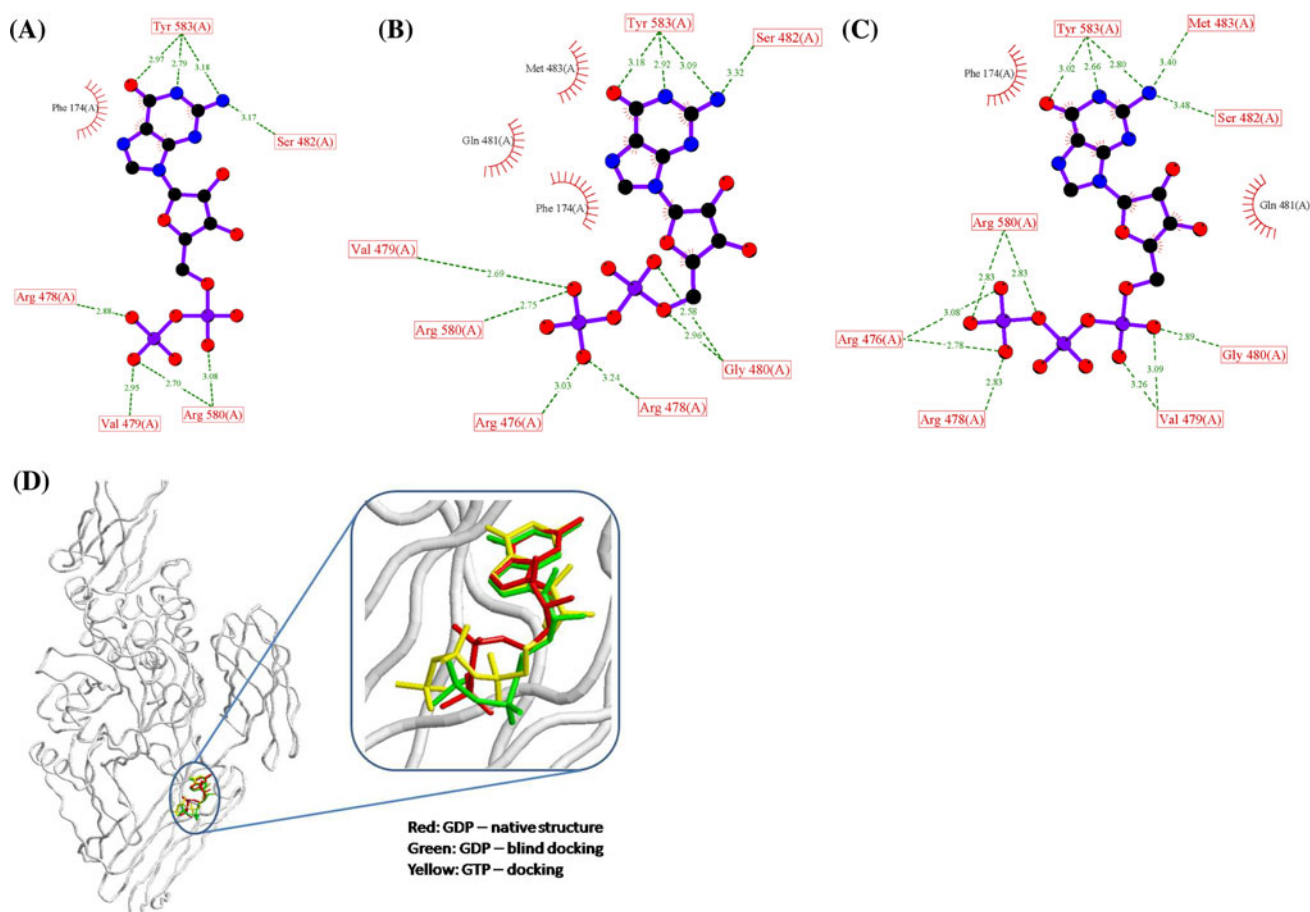


Fig. 4 Native crystal structure of TGase 2/GDP, PDB code 1KV3 (a) compared with blind docking of TGase 2/GDP (b) and docking of TGase 2/GTP (c) and the detail of the conformation of nucleotides in

the binding pocket (d). Red colour in d refer to GDP in 1KV3, green GDP in the blind docking and yellow to GTP docking

bonds in the phosphate portion (Gly 480 and Arg 476). “Blind” docking is therefore producing even more stable ligand–protein interactions than observed in the original structure suggesting that our procedure is valuable.

When GTP is considered as a ligand, the analysis of GTP binding indicates that both GDP and GTP occupy the same binding pocket of the TGase 2 with perfect superposition of the guanine ring but slight variations in the polyphosphate chain (compare Fig. 4d). The structure in Fig. 4c is that of the docked TGase 2/GTP complex and it shows a further increase in the number of hydrogen bonds between the protein and the GTP moieties. Indeed the γ -phosphate moiety is stabilised by Arg 476, Arg 478 and Arg 580 in agreement with previously reported data (Begg et al. 2006b), while Val 479 and Gly 480 produce the stabilisation of the α -Phosphate moiety. Finally, Phe 174, Ser 482, Met 483 and Tyr 583 participate in the guanosine moiety binding.

Molecular dynamic simulations confirmed results of the docking procedures, suggesting stronger stabilization in the case of TGase 2/GTP through the formation of more hydrogen bonds and saline bridges (see Table 1). In fact, the reduced distance between oppositely charged groups (they are closer by 0.05 nm than in the case of TGase 2 without GTP) hints a favourable condition for the formation of more salt bridges. In the same way, the distance among negative charges is higher in TGase 2/GTP complex than in TGase 2 (Δ 0.45 nm) and in TGase 2/GDP (Δ 0.36 nm). This is indicative of a better stabilisation. Finally, radius of gyration is an useful estimate of a molecule size and RMS fluctuation that computes the root mean square fluctuation of atomic positions after fitting to a reference frame, are smaller in TGase 2/GTP, showing more compactness in this conformation (Δ 0.05 nm).

Through the dynamic modelling approach we also checked for the occurrence of variation in the protein surface hydrophobicity during ligand induced conformational changes. Unexpectedly results indicated no major variations in exposed hydrophobic regions which could aid

in explaining experimental findings of variation in chromatographic properties of the enzyme. Indeed we noted differences in the retention of human erythrocyte TGase when submitted to hydrophobic chromatography onto phenyl-Sepharose. In the absence of external ligands (i.e. in the GDP bound state) the protein binds completely to the absorbent, while it is completely released when the resin is washed with buffer containing GTP. This behaviour is identical to that we proved previously for the enzyme from calf testes (Bergamini and Signorini 1992). This indicates weakening of hydrophobic interactions between the fixed phase (phenyl groups) and protein hydrophobic regions. These must obviously be located in the protein internal phase (since no changes in surface hydrophobicity was detectable) so that weakening of these interactions must depend on domain movement which restrict accessibility of internal apolar regions for the hydrophobic arms of the matrix.

Conclusions

The roles played by TGase 2 in cell biology are still incompletely understood and the object of intensive investigation because of the relevance in the pathogenesis of human diseases including autoimmune (Koning et al. 2005), neoplastic (Mangala and Mehta 2005) and chronic inflammatory diseases (Sohn et al. 2003), eventually ending into tissue fibrosis, notably in the kidney (Johnson et al. 2003, 2007; Chatziantoniou and Dussaule 2005). These observations have raised great interest in transglutaminase as a potential drug target (Siegel and Khosla 2007; Lai et al. 2008).

Over the years, evidences have been collected that this multifunctional protein (which displays along with the transamidating and the GTPase activity, also protein-disulfide-isomerase activity and, as recently suggested, protein kinase activity) undergoes transfer among different cell compartments (the cytosol, the mitochondrion, the

Table 1 Comparison of the results of molecular dynamics simulations of TGase 2 and TGase 2/GDP, TGase 2/GTP complexes

Results	TGase 2	TGase 2/GDP	TGase 2/GTP
Hydrogen bonds	485 $\pm$ 11	489 $\pm$ 18	503 $\pm$ 10
Distance between oppositely charged groups (nm)	0.348 $\pm$ 0.046	0.308 $\pm$ 0.037	0.298 $\pm$ 0.090
Distance between negative charge (nm)	0.722 $\pm$ 0.062	0.817 $\pm$ 0.067	1.176 $\pm$ 0.108
Distance between positive charge (nm)	0.147 $\pm$ 0.0	0.147 $\pm$ 0.0	0.147 $\pm$ 0.0
Radius of gyration (nm)	2.75 $\pm$ 0.07	2.73 $\pm$ 0.09	2.70 $\pm$ 0.07
Solvent accessible surface apolar $N_{\text{res}}/\text{TOT}_{\text{res}}$	0.51	0.51	0.51
Solvent accessible surface polar $N_{\text{res}}/\text{TOT}_{\text{res}}$	0.49	0.49	0.49
RMSD backbone (nm)	0.25	0.25	0.25
RMS fluctuation	0.098 $\pm$ 0.041	0.089 $\pm$ 0.035	0.076 $\pm$ 0.032

plasma membrane at both the intracellular and the extracellular surface and possibly the nucleus itself). This subcellular redistribution is likely influenced by ligands (Park et al. 2001) and is dependent on conformational changes which influence deeply the activity (Milakovic et al. 2004) and the physico-chemical properties of the protein. As already mentioned, we have obtained confirmation for this proposal by checking the performance of erythrocyte TGase 2 during hydrophobic chromatography, which was identical to that of the enzyme from bovine testes (Bergamini and Signorini 1992).

It is therefore clear that a detailed reinvestigation of the interaction of TGase with ligands is badly required, also because of the discrepancies arising from comparison of results *in vitro* and in the cellular milieu *in situ*. In relation to the calcium-dependent transamidating activity, for which more detailed information is available, it is apparent that TGase 2 behaves *in vitro* as a low-affinity calcium-binding protein, while data *in situ* are indicative of a much tighter interaction with the cation. The present data shed new light on these processes since they demonstrate unequivocally that TGase is indeed a high affinity calcium ligand and that the apparent low affinity displayed by the enzyme *in vitro* is likely dependent on the appearance of additional sites, triggered by conformational changes induced by binding of calcium to the high affinity site with subsequent catalytic self-modification, in the absence of an exogenous protein substrates. Note that indeed formation of the catalytic thioester intermediate is known to lead to rearrangements in the active site region, involving particularly Trp 241 in the stabilization of the intermediate itself (Iismaa et al. 2003). The appearance of additional low-affinity sites is however probably significant during the desensitization of activity to inhibition by GTP, yielding the commonly observed sigmoid saturation plot for the cation. These findings on binding of GTP are on line with the studies by Iismaa et al. (2000) who also explored calorimetrically the interaction of GTP with recombinant rat wild-type and mutant (F174A and R579A) TGase 2. In this case these authors observed major differences between the rat and the human enzyme in relation to nucleotide stripping and preservation of a native-like conformation.

In relation to the reported stabilization of the enzyme to physical challenges and to proteolytic degradation (which could deeply affect the physiologic properties of the intracellular enzyme in relation to cell death or survival) (Mian et al. 1995; Fesus and Szondy 2005), the present data confirm our previous findings of a more compact structure and enhanced stability of the protein complexed with GTP rather than GDP, as already suggested by SAXS experiments (Mariani et al. 2000). An additional comment might relate to the “real” structure in the absence of nucleotides. We tried to get a likely insight into this point

by means of molecular dynamics employing our original model (PDB 1FAU) which was obtained by homology building, tailoring the sequence of human TGase 2 onto the crystal structure of Factor XIII. Obviously this model does not account for any stably bound nucleotide, although sites for GXP binding are clearly present in the sequence. When we submitted this template to docking with either GDP or GTP, we could not obtain any “stable” complex because of displacement of the distal Arg residues (Arg 476, Arg 478). Likewise the crystallographic pictures reported by Pinkas et al. (2007) do not detect stably bound nucleotides in an enzyme thioester intermediate. We wonder that these residues have major structural roles which play on internal protein dynamics-stability and give a reason for the high tendency of TGase 2 to denature upon complete stripping of the nucleotides. Finally we should mention that in their investigations on the mechanism of allosteric regulation of TGase 2 by GTP, Begg et al. (2006a) pointed towards an important role of Arg 579 as a “destabilizer” of the active site region and as a possible regulator of the reactivity of Cys 277. In this perspective the higher localized charge density typical of the protein GTP versus GDP complex is expected to better neutralize the charge and the protein destabilization brought about by Arg 579, thereby influencing directly the reactivity of the active site triad.

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