Opposite Effects of Ca\(^{2+}\) and GTP Binding on Tissue Transglutaminase Tertiary Structure*

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Tissue transglutaminase (tTG) belongs to a class of enzymes that catalyze a cross-linking reaction between proteins or peptides. The protein activity is known to be finely tuned by Ca\(^{2+}\) and GTP binding. In this study we report the effects of these ligands on the enzyme structure, as revealed by circular dichroism, and steady-state and dynamic fluorescence measurements. We have found that calcium and GTP induced opposite conformational changes at the level of the protein tertiary structure. In particular the metal ions were responsible for a small widening of the protein molecule, as indicated by anisotropy decay measurements and by the binding of a hydrophobic probe such as 1-anilino-8-naphthalenesulfonic acid (ANS). Unlike Ca\(^{2+}\), the nucleotide binding increased the protein dynamics, reducing its rotational correlation lifetime from 32 to 25 ns, preventing also the binding of ANS into the protein matrix. Unfolding of tTG by guanidinium hydrochloride yielded a three-state denaturation mechanism, involving an intermediate species with the characteristics of the so-called “molten globule” state. The effect of GTP binding (but not that of Ca\(^{2+}\)) had an important consequence on the stability of tissue transglutaminase, increasing the free energy change from the native to the intermediate species by at least ~0.7 kcal/mol. Also a greater stability of tTG to high hydrostatic pressure was obtained in presence of GTP. These findings suggest that the molecular mechanism by which tTG activity is inhibited by GTP is essentially due to a protein conformational change which, decreasing the accessibility of the protein matrix to the solvent, renders more difficult the exposure of the active site.

Transglutaminases (TGs) are a class of enzymes that can cross-link peptides and proteins by catalyzing the formation of an isopeptidyl bond by transamidation between a side chain of a glutaminyl residue on one substrate and a lysyl residue on the other (1, 2). They can also use polyamines as the second substrate. Their physiological role appears to be increasingly important since they are thought to be at the cross-road between life and death of cells (3). Many different TGs have been described among which the best characterized is the plasma factor XIII, which is crucial for the cross-linking of fibrin fragments, allowing the retraction of the clot (1). Of particular interest is also tissue transglutaminase (tTG), a multifunctional enzyme of 77 kDa that also has GTP (and ATP) hydrolytic activity (4, 5), besides its main role as a protein cross-linking enzyme. The regulation of the transglutaminase activity (TGase) is accomplished through the binding of different cofactors, including calcium ions (6), nucleotides (5), nitric oxide (7), and sphingosylipidophosphocholine (8). Probably the best studied relationship between the function and the structure of tTG is that involving the binding of calcium and GTP. It has been found that the metal ion promotes a transglutaminase activity (6), whereas GTP has an opposite consequence, reducing the affinity of the protein for calcium and thus blocking its cross-linking function (9–11). Experimental evidence has suggested that these effects are due to the occurrence of induced conformational changes of tTG (5, 6, 10). However, despite many experiments that have been done on the enzyme, little is known about the molecular mechanism that characterizes these structural changes. Since tryptophan emission, the major source of protein intrinsic fluorescence, is strongly influenced by changes in tertiary structure, we used fluorescence spectroscopy in order to characterize the interaction between guinea pig liver TG and GTP or calcium ions. In particular we have performed steady-state, dynamic fluorescence, and anisotropy decay measurements in the presence of these ligands. The secondary structure of tTG under different conditions has been studied in parallel by CD spectroscopy in the range 200–250 nm, whereas ANS binding and the CD spectrum in the aromatic region were used to characterize changes in the tertiary structure. The protein stability in the presence and absence of calcium or GTP has been also investigated by studying the effects of guanidinium hydrochloride (GdmHCl) or of pressure.

EXPERIMENTAL PROCEDURES

Samples and Buffers—TGase from guinea pig liver was purchased from Sigma. All spectroscopic measurements were performed dissolving the protein in Tris-HCl 50 mM buffer, pH 7.5, containing 5 mM EDTA. The active site titration was performed according to the procedure described by Kim and co-workers (12). The amount of active TGase was found to be ~97% of the total protein concentration measured by UV absorption spectroscopy.

TGase activity was determined measuring the incorporation of \[^{3}H\]putrescine into \[^{N,N} \]dimethylcasein (13). The reaction mixture contained 150 mM Tris-HCl, pH 9.3, 90 mM NaCl, 30 mM dithiothreitol, 15 mM CaCl\(_2\), 12.5 mg of \[^{N,N} \]dimethylcasein/ml, 0.2 mM putrescine containing 1 μCi of \[^{3}H\]putrescine. 100 μg of TGase were incubated at 37 °C with the reaction mixture in a final volume of 150 μl. After 20 min of incubation, the reaction was stopped by spotting 50-μl triplicate aliquots onto Whatman 3MM filter paper. Unbound \[^{3}H\]putrescine was
removed by washing with 15, 10, and 5% trichloroacetic acid and absolute ethanol. Filters were then air-dried, and the radioactivity was measured by liquid scintillation counting.

GdmHCl and ANS were purchased from U. S. Biochemical Corp. and Molecular Probes Inc., respectively.

Spectroscopic Assays—Steady-state fluorescence and anisotropy were measured with a ISS-K2 fluorometer (ISS, Champaign, IL) thermostating the sample at 20 °C by an external bath circulator. The fluorescence emission decay was extrapolated fitting the phase-shift and demodulation data obtained as described elsewhere (14) using the harmonic content of a frequency-doubled rhodamine 6G-Nd Yag laser (λ = 393 nm) as excitation source. The emission was detected through a WG 305 cut-off filter to suppress scattered light. Either a discrete sum of exponential functions (see Equation 1)

\[ f(t) = \sum_{i=1}^{n} F_i e^{-\frac{t}{\tau_i}} \]  

or a continuously distributed model (see Equation 2)

\[ f(t) = \int_{0}^{t} F(\tau) e^{-\frac{\tau}{\tau}} d\tau \]  

were employed to fit the data using the GLOBAL Unlimited software (15), where for each component \( F_i \) or \( F(\tau) \) and \( \tau_i \) or \( \tau \) represented the fluorescence fractional intensity and lifetime value, respectively. In the case of a continuous function, \( F(\tau) \) had a lorentzian shape according to Equation 3,

\[ F(\tau) = \frac{(w/2)^2}{(\tau - \tau_1)^2 + (w/2)^2} \]  

where \( w_i \) is the full width at half-maximum (FWHM) of each component. Rotational correlation lifetimes were evaluated from the analysis of the anisotropy decay measurements, taking into account the relative fluorescence lifetimes (16).

High pressure experiments were performed on the same instrument, equipped with the ISS high pressure cell. Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter, at 20 °C, using a 0.1- and 1.0-cm quartz cuvette in the peptide and aromatic regions, respectively. The spectra of tTG and tTG in presence of Ca\(^{2+}\) or GdmHCl were corrected by subtracting the blank signal (i.e. Ca\(^{2+}\) or GdmHCl in buffer). In the case of tTG complexed with GTP, the correction was possible only in the aromatic region (260–300 nm), where changes due to the intrinsic absorption of the nucleotide are known to be well distinct from the complex features of the aromatic amino acids transitions (17).

Equilibrium Unfolding Measurements—The unfolding of tTG by GdmHCl was achieved by incubating the protein at 4 °C in the presence of different amounts of denaturant for 12 h. Each measurement was repeated at least three times, and the relative standard deviation has been reported in the figures as an error bar. Refolding of tTG was obtained by diluting a highly concentrated, fully unfolded sample in buffer.

Due to the non-coincidence of the fluorescence and CD data, the analysis of the equilibrium unfolding transition was performed according to a three-state denaturation pathway, following Scheme 1,

\[ K_1 \xrightarrow{\Delta G_1} K_2 \xrightarrow{\Delta G_2} N \]  

where \( K_1 \) and \( K_2 \) are related to the free energy values \( \Delta G_1 \) and \( \Delta G_2 \) which were supposed to vary linearly with the denaturant concentration (18) (see Equation 4).

\[ K = e^{-\frac{\Delta G}{R T}} \]  

The fraction of the native (\( F_N \)), partially (\( F_p \)), and fully unfolded (\( F_u \)) protein species were directly evaluated from the fluorescence and CD data as elsewhere described (19).

RESULTS

Steady-state and Dynamic Fluorescence of tTG, tTG-GTP, and tTG-Ca\(^{2+}\)—The steady-state fluorescence spectrum of tTG has been obtained as a function of the excitation wavelength, and the results are reported in Fig. 1a. The broad emission spectrum (FWHM ~60 nm) and its red-shifted peak, by varying the excitation from 295–305 nm (Fig. 1a, inset), are clear indications of fluorescence heterogeneity as expected for the presence of 12 Trp residues in tTG (20). GTP binding to tTG induced a small decrease (~1%) in the fluorescence quantum yield (Fig. 1b). Even though this change is in the limit of experimental error, the error analysis of the difference spectrum (reported in Fig. 1c) demonstrates that the shape of the tTG fluorescence spectrum is significantly affected by GTP in the wavelength region between 300 and 400 nm. The negative and positive contributions to the difference spectrum almost compensate each other keeping the fluorescence yield practically constant. Ca\(^{2+}\) ions did not affect the overall shape of the emission spectrum but gave a decrease (~8%) of the fluorescence quantum yield (Fig. 1b), in agreement with previous data (21). The fluorescence heterogeneity was confirmed by steady-state anisotropy measurements, which are strongly dependent on the emission wavelength, both in presence and absence of ligands (Fig. 1b, inset).

In order to characterize further the spectroscopic properties of tTG, dynamic fluorescence measurements have been carried out using the phase-shift and demodulation technique (see
under “Experimental Procedures”). The data have been fit according to different decay models, either discrete or continuously distributed lifetimes (Table I). The emission decay was rather complex and, as demonstrated by the reduced \( \chi^2 \) values and weighted residuals (data not shown), the best fit was obtained using two lorentzian-shaped distributions of fluorescence lifetimes (Table I and Fig. 2). This finding is consistent with the fluorescence decay expected in general for heterogeneous systems (22) and in particular for multitransferrin proteins (23–26). Only the longer lifetimes could be detected when the fluorescence of tTG was observed through a cut-off filter at 350 nm (Table I), demonstrating that these lifetimes are associated with tryptophans emitting at the red edge of the spectrum.

The effect of GTP binding on the dynamic fluorescence of tTG is shown in Fig. 2. Although the two components were influenced by GTP to a different extent, the mean lifetime, \( < \tau > \), was not affected by the nucleotide binding (Table I). This finding is in keeping with the results of the steady-state measurements, since the quantum yield of the fluorescence spectrum is directly correlated to the average lifetime of the emission decay (16). The fluorescence lifetime distributions in presence of \( \text{Ca}^{2+} \) are also reported in Fig. 2. Despite much smaller changes in this case, the parameters of both lifetime distributions were significantly different from those of the protein alone (Table I). Also in this case the relative change of \( < \tau > \) (−9%) upon calcium addition correlates fairly well with the corresponding variation of quantum yield.

The different protein fluorescence decays suggested the occurrence of induced conformational changes upon ligand binding, especially in the case of GTP, which could have important consequences on the protein dynamics. In order to test this hypothesis, the rotational correlation lifetimes of tTG and (TGG complexed with GTP and \( \text{Ca}^{2+} \) were also studied measuring the fluorescence anisotropy decay. The data could be fit using at least two rotational correlation lifetimes that are reported in Table II. The slow rotation (\( \phi_2 \approx 32 \text{ ns} \)) is compatible with the motion of a hydrated sphere of \( \approx 70 \text{ kDa} \) (27), and therefore it might be associated with the tumbling of the whole protein molecule. This is the only parameter to be affected by both calcium and GTP, although in an opposite way. In fact, \( \text{Ca}^{2+} \) binding produced a slightly longer rotational correlation lifetime, whereas GTP significantly reduced this value (Table II).

**ANS Binding**—ANS is essentially nonfluorescent in aqueous solution, whereas its emission intensity increases in a hydrophobic environment. This effect is generally accompanied by a blue-shift in the ANS fluorescence spectrum that can be detected by evaluating the change in the spectral center of mass. These properties have been largely exploited to check the presence of hydrophobic pockets in proteins through ANS binding (16, 28). The steady-state fluorescence intensity of ANS increased about 4–5 times in the presence of tTG, whereas the spectrum was shifted toward shorter wavelengths (Fig. 3a), demonstrating that the probe was in fact bound to the protein. This effect was much greater in the presence of \( \text{Ca}^{2+} \), whereas GTP substantially reduced the ANS fluorescence (Fig. 3a).

The binding of ANS was studied as a function of calcium concentration. The results, reported in Fig. 3b, demonstrate that the maximum fluorescence intensity was reached when about 4–6 ions per protein molecule were added. In order to check whether this effect was due to a change of the ionic strength, the same experiment was performed with a ratio [\( \text{Ca}^{2+} \)]:[tTG] \( \approx 1:2 \) and then adding increasing amounts of sodium chloride. No further changes in the fluorescence of ANS were observed upon addition of salt (Fig. 3b).

**Circular Dichroism Measurements**—The effect of \( \text{Ca}^{2+} \) and GTP on the tTG structure has been also studied by circular dichroism, both in the peptide and in the aromatic region (Fig.
The analysis of the protein CD spectrum in the range 200–250 nm yielded a large amount of $\beta$-structure ($\approx 21\%$ $\beta$-strand and $\approx 20\%$ $\beta$-turn) and a significant content of $\alpha$-helix ($\approx 26\%$). As shown in Fig. 4a, calcium binding did not influence the protein secondary structure, whereas a significant change was instead observed with GTP (Fig. 4a). Previous studies have shown that the tTG secondary structure does not vary in the presence of GTP (29). Thus, it must be concluded that this effect is related to the change in the intrinsic CD signal of the nucleotide, which in fact might increase in a hydrophobic environment (17). Assuming that the tTG secondary structure does not change, we have used the difference spectrum in order to follow the GTP binding (Fig. 4a, inset).

When the same experiment was done in the presence of calcium ions, the affinity of GTP for tTG was considerably reduced, as demonstrated in the inset of Fig. 4a. As shown in Fig. 4b, the CD spectrum of tTG in the aromatic region is clearly structured in individual bands, characteristic of phenylalanine (267 nm), tyrosine (271 and 282 nm), and tryptophan (288 and 294 nm) residues (30). In the presence of calcium ions the spectrum was less structured, as demonstrated by the flattening of all the peaks, except at 294 nm. These types of changes are generally associated with an increased mobility of the aromatic side chains, demonstrating a different local conformation of the tTG tertiary structure upon Ca$^{2+}$ binding. An opposite effect was obtained in the presence of GTP (Fig. 4b). In particular a 2-fold increase at 288 and 294 nm and a splitting of the peak at 265 nm into two bands (Fig. 4b) were observed. The profile of the difference spectrum (Fig. 4c) suggests that also in this case important changes in the tTG tertiary structure are occurring. In fact, GTP generally contributes to the intensity of the signal but not to its fine structure, which is instead typical of the aromatic amino acids transitions (17, 31).

Equilibrium Unfolding Measurements—Denaturation of tTG by GdmHCl has been followed by steady-state fluorescence and circular dichroism measurements. The data, reported in Fig. 5a, could not be fit by a simple two-state unfolding process, suggesting the presence of at least one intermediate species. Thus, assuming a three-step transition model, the free energy of unfolding and the population of the native, denatured, and intermediate states were calculated (Table III and Fig. 5b).

Because of the above-mentioned fluorescence properties, ANS binding has been used to study the partial exposition of hydrophobic patches upon loosening of the protein tertiary structure at increasing concentration of GdmHCl (32–33). Actually, the intensity and the position of the ANS emission
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DISCUSSION

Insights on the Conformational Changes Produced by GTP and Ca\textsuperscript{2+}—Since the first evidence obtained by Folk and co-workers (6, 35), several studies on the regulation of tTG activity proposed the occurrence of protein conformational changes upon calcium ions or GTP binding (5, 9). The experimental evidence for these changes was based on the trypsin digestion patterns of tTG as detected by SDS-polyacrylamide gel electrophoresis. We have obtained more direct evidence of tTG conformational changes with spectroscopic techniques, namely CD, steady-state, and dynamic fluorescence, which are particularly suitable to investigate even subtle modifications of protein structure. Of course a detailed analysis of the fluorescence properties of every tryptophan contained in tTG is simply impossible, since proteins containing several tryptophan residues are expected to exhibit a very complex fluorescence dynamics (22–26). Nevertheless, we have shown that the fluorescence decay may be resolved into two main distributions of lifetimes, probably associated with two classes of differently solvated tryptophans. An efficient energy transfer among different tryptophans might be another source of heterogeneous emission decay in transglutaminase. Whatever the origin of this complex fluorescence dynamics, we have shown that whereas Ca\textsuperscript{2+} does not perturb significantly the fluorescence and CD of tTG, the binding of GTP induces major changes that might be summarized as follows: (i) the change of environment of some tryptophan detected through the difference CD spectrum in the aromatic region (Fig. 4c) with a concomitant quenching of the shorter wavelength emission (Fig. 1a) and shorter lifetimes distribution (Fig. 2); (ii) a general stiffening of the molecule as shown by a shorter rotational lifetime (Table II); (iii) a more compact structure of tTG that requires higher GdmHCl concentration to be unfolded and withstands high pressure (Figs. 5a and 7, and see below); (iv) a reduced binding of ANS (Fig. 3a). All these changes do not appear to involve the protein secondary structure (29), although they are reflected in the loss of enzymatic activity (9).

On the contrary Ca\textsuperscript{2+} substantially increased the binding of ANS to tTG (Fig. 3a) and the protein rotational correlation lifetime somewhat (Table II). Thus, the conformational change induced by Ca\textsuperscript{2+} seems to produce a partial “opening” of the protein structure, slowing down the rotational dynamics and allowing ANS molecules to penetrate better into a protein hydrophobic pocket. The binding of ANS may outline the mechanism by which tTG is activated by calcium ions. In fact it is known that TGase activity requires the exposure of a cysteine located in the active site (1) which is highly conserved throughout different tTG molecules (20). The binding of Ca\textsuperscript{2+} may open the access of substrates to this cysteine. In this regard, it is worth mentioning that our estimate of the Ca\textsuperscript{2+} ions linked to tTG at the plateau of Fig. 3b is in agreement with previously published results (10, 36). The opposite effects of Ca\textsuperscript{2+} and GTP on the tTG structure reflect their effect on the activity of the enzyme. It is tempting to speculate that the former ligand opens the active site which must be large enough to allow the entrance of the protein substrates, whereas the latter makes it impossible.

Effects of GTP Binding on the Stability of the tTG Tertiary Structure—Equilibrium unfolding experiments show that the unfolding of tTG by GdmHCl occurs with the formation of at least one discrete intermediate species (Fig. 5b). The most relevant properties of this transition state are as follows: an intact secondary structure with a largely solvated tridimensional folding, as shown by the CD data and by the red-shifted fluorescence spectrum (Fig. 5a); the loss of biological activity (Fig. 6); the enhanced affinity for ANS. These features are

Table III
Thermodynamic parameters characterizing the unfolding of tTG

| Sample    | $\Delta G_1$ (kcal/mol) | $m_1$  | $\Delta G_2$ (kcal/mol) | $m_2$  |
|-----------|------------------------|--------|------------------------|--------|
| tTG       | 2.1 ± 0.2              | 2.3 ± 0.2 | 2.8 ± 0.2              | 1.0 ± 0.1 |
| tTG + Ca\textsuperscript{2+} | 2.0 ± 0.1              | 2.3 ± 0.2 | 2.7 ± 0.2              | 1.1 ± 0.2 |
| tTG + GTP | 2.5 ± 0.1              | 2.2 ± 0.1 |                       |        |

spectrum (reported in terms of the spectral center of mass) indicate that ANS binding mostly occurred at 1.25 M GdmHCl, where the fraction of the intermediate species is maximum (Fig. 5b). This conformational modification is also accompanied by a total loss of enzymatic activity (Fig. 6). A somewhat different behavior was observed when the native protein was previously incubated at 20 °C in the presence of GTP. In particular, the unfolding transition was shifted toward higher denaturant concentration and characterized by a more pronounced blue-shift of the ANS spectrum (Fig. 4c) with a concomitant quenching of the shorter wavelength emission (Fig. 1a) and shorter lifetimes distribution (Fig. 2). This conformational modification is also accompanied by a shorter rotational lifetime (Table II); (iii) a more compact structure of tTG that requires higher GdmHCl concentration to be unfolded and withstands high pressure (Figs. 5a and 7, and see below); (iv) a reduced binding of ANS (Fig. 3a). All these changes do not appear to involve the protein secondary structure (29), although they are reflected in the loss of enzymatic activity (9).

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![Fig. 5. Denaturation of tTG. a, dependence of tTG circular dichroism at 220 nm (squares) and of tTG fluorescence emission maximum (open circles) on GdmHCl concentration. Solid lines represent the best fits obtained using a three-state model (see Table III). The filled symbols represent the fluorescence unfolding curves of tTG + GTP (1:40). b, percentage of native (circles), intermediate (triangles), and unfolded (squares) molecules of tTG upon unfolding. Solid lines represent the best fit obtained using a three-state denaturation model.](image-url)
those typical of the so-called “molten globule” state, an intermediate species with a native-like secondary structure (but an almost unfolded tertiary arrangement) that has been found in the denaturation pathway of several other proteins (37–40). We have shown that GTP binding slightly increased the tTG tertiary structure stability (Fig. 5 and Table III) having a major effect on the fluorescence signal in the range from 1 to 2 M GdmHCl. This finding is consistent with the results obtained by Tanfani and co-workers (29) using infrared spectroscopy. On the other hand, following the denaturation transition through the changes in the ANS fluorescence spectrum (Fig. 6), it has been found that GTP stabilized the intermediate species, suggesting a “protective” action extended also to the protein secondary structure. The nucleotide-complexed enzyme was even more resistant to pressure (Fig. 7). Fluorescence (41) and phosphorescence (42) studies of monomeric proteins under high pressure have recently demonstrated that tryptophan emission is particularly sensitive to conformational changes occurring already in the range 1–3 kbar. In particular, since the mechanism of pressure-induced unfolding is known to be the entrance of water inside the hydrophobic core of proteins (43), a red-shift of the fluorescence spectrum is expected, due to the increasing hydration of buried tryptophans. Thus the effect of GTP might be due to a tTG conformational change that would prevent solvent penetration in the protein core. This hypothesis is in line with a more compact protein structure, as demonstrated by the decrease of the long rotational correlation lifetime (Table II).

Detailed knowledge of the structural effects due to Ca$^{2+}$, GTP, and other ligands might be crucial to understand better the regulation in vivo of the tTG activity and thus its role during cell apoptosis. In particular the higher stabilization by GTP might be crucial. In a viable cell it is in fact expected that cross-linking activity of tTG is suppressed, whereas its role as a signal-transducing GTP-binding protein is maintained (3). It has been found that both in vitro (5) and in situ (44) tTG is protected by proteolysis in the presence of GTP. Thus, the protein conformational change induced by the nucleotide binding responds to a dual function, keeping tTG inactive as transglutaminase but stable and efficient for ATP/GTP hydrolysis. An increase of calcium concentration and the presence of specific cofactors that enhance the affinity of the enzyme for Ca$^{2+}$ reverse the effects of GTP, regenerating the cross-linking activity of tTG (5, 8). Our results suggest that this change is mainly due to the protein structure that is probably required for the exposure of the active site.

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