

GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase

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Calcium binding to erythrocyte transglutaminase was determined by equilibrium dialysis. Results indicate that 6 ions are bound to the enzyme both in the absence and in the presence of GTP and that the nucleotide reduces the affinity of the enzyme for calcium. Furthermore, I^- fluorescence quenching and proteolytic inactivation experiments proved that GTP also alters the conformation of the enzyme. It is thus suggested that multiple mechanisms are involved in the regulation of the enzyme activity by GTP.

Transglutaminase; Enzyme regulation; GTP; Ca^{2+} binding; Proteolysis; Fluorescence quenching; (Human erythrocyte)

1. INTRODUCTION

The activity of cell transglutaminase is regulated by enzyme induction by chemicals, hormones and bacterial toxins [1–5] and by activation by calcium ions [6]. The absolute cation requirement *in vitro* and the apparently selective activation of the enzyme *in situ* under conditions associated with altered calcium fluxes [7,8] suggest that the availability of calcium ions represents the main regulatory mechanism *in vivo*. Quite recently it was recognised that the enzyme is also sensitive to guanine nucleotides, notably GTP, in a concentration range which is indicative of a physiological action of the nucleotide [9,10]. In particular it was observed that GTP inhibits the enzyme assayed at micromolar but not at saturating (millimolar) concentrations of calcium. The present experiments, performed to elucidate the mechanism of regulation by GTP, demonstrate that the nucleotide inhibits calcium binding and the subsequent conformational changes.

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2. MATERIALS AND METHODS

Methods for enzyme purification and assay were previously detailed [10,11]. Calcium binding was determined by equilibrium dialysis allowing equilibration of the enzyme (0.5–1.8 mg/ml) against 100 vols of buffer, 50 mM Tris and 5 mM mercaptoethanol, pH 7.5, supplemented with increasing amounts of $^{45}CaCl_2$ (about 300 cpm/nmol) and 50–200 μM GTP, when required. After 48 h at 4°C, aliquots of the enzyme solution and of the dialysate were withdrawn for liquid scintillation counting. The results were normalised to the protein content of the sample determined by the Lowry procedure [12]. The concentration of free calcium in the presence of GTP was determined assuming a dissociation constant of $31000 M^{-1}$ [13]. The sensitivity of erythrocyte transglutaminase to chymotrypsin was determined by incubating the enzyme (0.5 mg/ml) with variable amounts of chymotrypsin in Tris/mercaptoethanol buffer, pH 7.5, in the presence of 2 mM free calcium and 0.3 mM GTP for 10 min at 30°C, before dilution with a buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and an activity assay. It was verified that at the final concentration employed in the assay, PMSF did not influence the activity of the enzyme. The fluorescence quenching experiments were performed by adding small amounts of a concentrated solution of potassium iodide, containing a few crystals of sodium thiosulfate, to the enzyme solution (0.2 mg/ml) in the fluorescence cell of a Perkin-Elmer MPF 3L fluorimeter operated in the ratio mode. The wavelengths for excitation and emission were 290 and 340 nm, respectively. Corrections were performed for the dilution of the enzyme.

Radioactive calcium was obtained from Amersham, Bucks,

England. Chymotrypsin and GTP were obtained from Sigma (St. Louis, MO) and potassium iodide from Merck (Darmstadt, FRG).

3. RESULTS

The binding of calcium to erythrocyte transglutaminase was investigated by equilibrium dialysis (fig.1): the enzyme binds up to 6 ions per molecule, all with similar affinity. This gives rise to a hyperbolic saturation curve with an apparent affinity constant of $90 \mu\text{M}$. In the presence of $190 \mu\text{M}$ GTP, this pattern is altered since the binding at low calcium is selectively inhibited, with a shift of the curve to a sigmoid one and an increase of the affinity constant for free calcium to $500 \mu\text{M}$. These values are in good agreement with those recalculated from previously published experiments [10] in which the influence of the nucleotide was investigated by measuring enzyme inhibition at variable calcium levels (calcium activation constants of 65 and $600 \mu\text{M}$ were obtained in the absence and in the presence of $250 \mu\text{M}$ GTP, respectively).

To verify whether GTP has additional influences on the enzyme structure, beside altering the binding of calcium, we have measured the sensitivity of transglutaminase to chymotrypsin in the absence

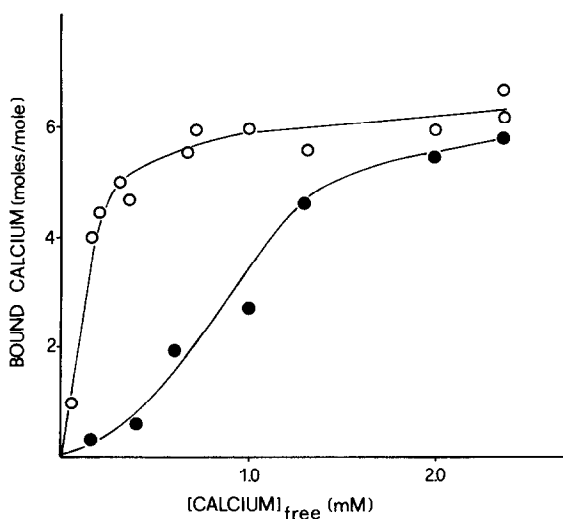


Fig.1. Binding of calcium to erythrocyte transglutaminase in the absence (○) and in the presence (●) of $190 \mu\text{M}$ GTP, measured by equilibrium dialysis at 4°C . Further details are described in section 2.

of ligands and in the presence of calcium and GTP. The cation was added to keep the free concentration constant at 2 mM , in order to saturate the enzyme and to avoid complications from effects of GTP on calcium binding. The results presented in fig.2, demonstrate that the sensitivity of transglutaminase to chymotrypsin is markedly increased by calcium and that the enzyme is almost insensitive to the protease when GTP is added along with the cation. Similar conclusions were obtained in experiments in which the quenching of tryptophanyl fluorescence by iodide was employed to get information on the conformation of the enzyme. The resulting Stern-Volmer plots, presented in fig.3, demonstrate that iodide is a very poor quencher in the absence of calcium, with a collisional quenching constant of 0.73 M^{-1} and that its efficiency is largely increased by the presence of calcium to 2.85 M^{-1} . This effect of calcium is again suppressed by the addition of $250 \mu\text{M}$ GTP, the measured quenching constant being 0.78 M^{-1} , in these conditions.

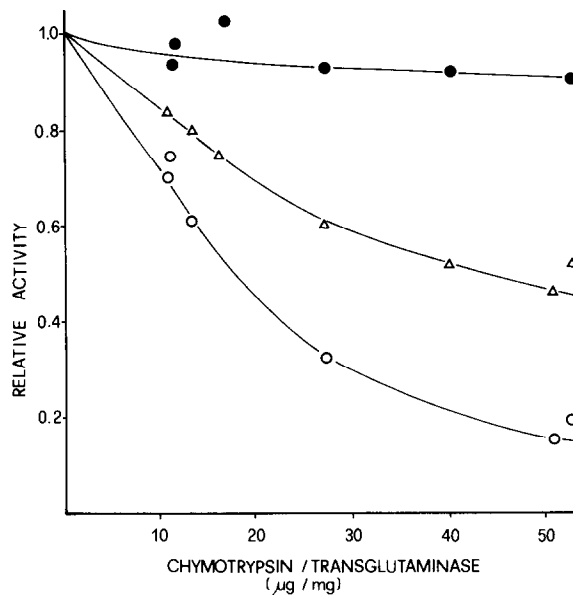


Fig.2. Influence of GTP on the sensitivity of erythrocyte transglutaminase to degradation by chymotrypsin. The enzyme (0.5 mg/ml) was incubated with chymotrypsin in a final volume of $60 \mu\text{l}$ in the absence of calcium (Δ) or in the presence of 2 mM free calcium (\circ) or of 2.27 mM CaCl_2 and 0.3 mM GTP (\bullet). After 10 min at 30°C , the incubations were diluted with 1.0 ml of buffer containing 0.5 mM PMSF and immediately assayed for enzyme activity.

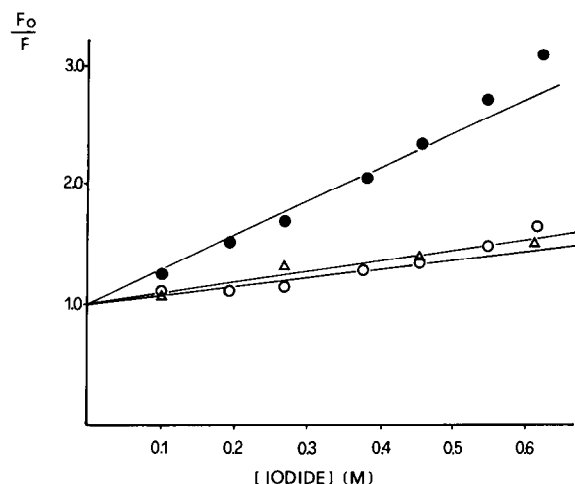


Fig.3. Influence of GTP on the quenching of the protein intrinsic fluorescence by iodide. 5 μ l aliquots of a 5 M iodide stock solution were added to fluorimeter microcells containing 250 μ l of purified transglutaminase (0.2 mg/ml) diluted with buffer alone (○) or with buffer supplemented with 2 mM CaCl₂ (●) or with 2.27 mM CaCl₂ and 0.3 mM GTP (△). Further details are presented in section 2.

4. DISCUSSION

As recalled in section 1, it is presently believed that transglutaminase is maintained in the latent state in the resting cells and that the enzyme is activated transiently by a rise in cytosolic calcium. Our present view of the activation mechanism by calcium is based on large conformational changes of the enzyme after calcium binding, with the exposition of the active site: these effects were proved by differential spectroscopy and by studies of the chemical reactivity of an essential cysteine residue involved in substrate binding [14]. Quite recently we demonstrated that diethylpyrocarbonate [11] and acrylamide [15] can also be employed as probes of the calcium-dependent conformational changes.

The discovery of an additional regulation by GTP, independently of a calcium chelating action [9,10] is particularly interesting in the light of the role calcium and GTP play directly or indirectly in the pathways of intracellular transduction of external messages. Our present data contribute to clarify the mechanism of regulation by GTP demonstrating that two distinct effects take place: at calcium concentrations prevailing inside cells,

i.e. in the micromolar range, the nucleotide reinforces the latency of erythrocyte transglutaminase by decreasing the affinity for calcium, while at saturating calcium levels it still prevents conformational changes of the enzyme. This was proved by the ability of GTP to protect the calcium saturated transglutaminase from inactivation by *N*-ethylmaleimide (unpublished) and by acrylamide [15] as well as by the present data on the protection against inactivation by proteolytic enzymes. Qualitatively similar results were obtained by others [9] in an electrophoretic study of the degradation of enzyme protein by trypsin, but no data were presented on enzyme activity.

Also the data on the intrinsic fluorescence quenching deserve comment: Recently we utilised quenching by acrylamide to study conformational changes of the enzyme exposed to calcium ions. Our data suggested that acrylamide was a relatively efficient quencher, with a quenching constant of 5 M⁻¹, and that the quenching process was not affected by the addition of divalent cations [16]. In contrast, the present data suggest that iodide is a very poor quencher and that the quenching process is influenced by calcium. A possible explanation for these findings is that the tryptophan fluorophores are located rather buried in a negatively charged region which prevents quenching by an anionic but not by a neutral quencher. The binding of calcium thus alters the exposition of the fluorophores to the solvent and therefore the efficiency of quenching by iodide, through either a generalised conformational change of the enzyme or the local neutralisation of the hypothetical anionic sites. The ability of GTP to prevent the calcium-dependent changes in both the iodide quenching and the susceptibility to proteolytic enzymes, but not to alter the binding of the cation at saturating calcium levels, suggest that the former explanation is correct.

In conclusion our data demonstrate that GTP binds to erythrocyte transglutaminase with separate effects on calcium binding and on the overall conformation of the enzyme: the molecular details of these events are still unclear.

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