Studies on tissue transglutaminases: interaction of erythrocyte type-2 transglutaminase with GTP

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Ca^{2+} and GTP are the main modulators of type-2 transglutaminases. To study the interaction of the enzyme with GTP, we have employed periodate-oxidized GTP as an affinity-label probe. Dialdehyde GTP bound irreversibly to type-2 transglutaminase in a time-dependent way with 1:1 stoichiometry at complete modification. The reaction took place in the absence, but was more rapid in the presence, of cyanoborohydride. Native GTP prevented incorporation of dialdehyde GTP, and Ca^{2+} significantly slowed down the reaction rate. The modified enzyme displayed decreased sensitivity to Ca^{2+}, with a sigmoid saturation curve. We conclude that type-2 transglutaminase has a single GTP-binding site, the modification of which by dialdehyde GTP mimics nucleotide binding to the enzyme.

INTRODUCTION

At variance with most protein post-translational modifications, that catalysed by transglutaminase (TGase) is chemically and functionally irreversible, since products are insoluble protein aggregates stabilized by glutamyl-lysine isopeptide bonds. These polymers can be removed from tissues only by extensive proteolytic digestion, resulting in accumulation of isodipeptide derivatives in body fluids [1,2]. Therefore tight regulation of TGase activity is required to keep the enzyme inactive under physiological conditions. Its activation is limited to conditions that mark irreversible cellular changes, with subsequent cell death.

This regulation is achieved by activation by Ca^{2+} and inhibition by GTP. Ca^{2+} is a low-affinity but essential cofactor of TGases [3], with apparent K_d of 0.2 mM, well above the physiological cell concentrations. GTP is a powerful inhibitor of type-2 TGases when activity is measured at suboptimal concentrations of Ca^{2+} [4,5]. The requirement for high concentrations of Ca^{2+} and the inhibition by GTP act in concert to keep type-2 TGase latent inside the cell.

Kinetic and equilibrium-dialysis studies have disclosed details on the effects of GTP on Ca^{2+} binding and enzyme activity [5,6]. In contrast, clear data on the stoichiometry and specificity of GTP binding are not available. This information might be relevant to the understanding of the regulation and structure–function relationships of TGases. We have now explored this issue by affinity-labelling with periodate-oxidized GTP.

MATERIALS AND METHODS

Materials

[1,4-^{14}C]Putrescine and [8-^{14}C]GTP were from NEN and Amersham respectively; the purity of the labelled GTP was stated to be 92% by the producer. $NaBH_4$ was from Sigma, and $NaCNBH_3$ and $NaIO_4$ were from Merck. Other reagents were of analytical grade. 2′,3′-Dialdehyde GTP (dialGTP) was prepared by oxidation of [^{14}C]GTP (specific radioactivity 2500–3500 d.p.m./nmol) for 1 h with periodate, and purified by gel filtration on Sephadex G-10 equilibrated with distilled water.

Enzyme purification and assay

Studies were performed on homogeneous type-2 TGase, purified from human erythrocytes as described [7], but replacing h.p.l.c. by chromatography on Q-Sepharose with NaCl linear-gradient elution. Purified TGase had a specific activity of 3.5 units/mg, in agreement with previous data from our laboratory; it displayed a single band of 92 kDa on SDS/PAGE, but a few peptides (78 kDa, 42 kDa and 38 kDa) were occasionally present, and accounted for less than 12% of the protein; these were most likely produced by oxidative enzyme degradation during purification (S. I. Chung, personal communication). Activity was measured as Ca^{2+}-dependent putrescine incorporation into dimethylcasein [7,8].

Affinity-labelling

TGase (15–20 μM) was incubated for 3 h at 20 °C with a solution containing 200 μM [^{14}C]dialGTP, 1 mM EDTA, 5 mM 2-mercaptoethanol and 50 mM Tris/HCl, pH 7.5, in the presence or absence of 5 mM $NaCNBH_3$. Labelling was determined by spotting samples of incubation mixture on to squares of filter paper, washing with 5% trichloroacetic acid and counting for radioactivity. SDS/PAGE was performed [9] and the gels were subjected to protein staining with Coomassie Blue R-250 and autoradiography after equilibration with 1 M sodium salicylate in 1% glycerol.

RESULTS

Experiments to characterize GTP binding to TGase by equilibrium dialysis gave inconsistent results, possibly because of GTP hydrolysis during the time of equilibration [10]. As an alternative, we employed affinity-labelling techniques, choosing dialGTP rather than azidoGTP [4] as the probe; these probes have different specificities, since azido derivatives are likely to label any amino acid close to the binding site, whereas dialdehyde nucleotides selectively modify lysine residues in nucleotide-binding sites in proteins, to form Schiff base or dihydroxymorpholine derivatives [11,12].

During incubation with dialGTP and $NaCNBH_3$, type-2 TGase was progressively labelled, with formation of covalent

Abbreviations used: dialGTP, 2′,3′-dialdehyde GTP; TGase, transglutaminase (EC 2.3.2.13).

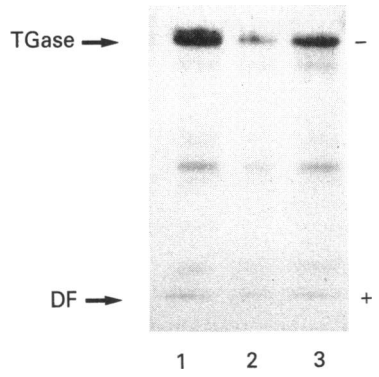

Figure 1 Autoradiographic pattern of dialGTP incorporation into erythrocyte TGase

TGase was subjected to affinity-labelling with [^{14}C]dialGTP in the presence of $NaCNBH_3$ as detailed in the Materials and methods section. After 2 h, the reaction was quenched by addition of denaturating buffer and boiling [9]; electrophoresis was performed on 10% slab gels. Lane 1, standard reaction mixture; lane 2, the same but supplemented with 1 mM GTP; lane 3, the same supplemented with 200 μM Ca^{2+} and 200 μM dialGTP, to keep the initial concentration of the free probe constant. Arrows indicate the migration of TGase and the dye front (DF).

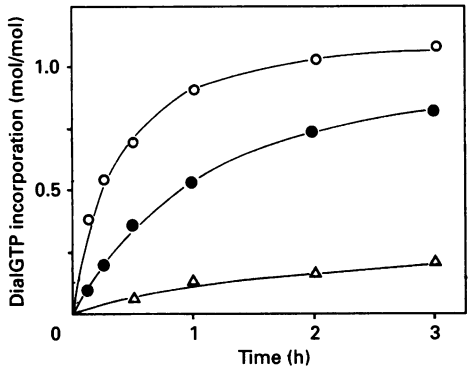

Figure 2 Time course of dialGTP binding to erythrocyte TGase

Incubations were carried out in the presence of 200 μM dialGTP and 5 mM $NaCNBH_3$, as detailed in the Materials and methods section. ○, Standard incubation; ●, +500 μM Ca^{2+}; △, +0.95 mM GTP. Samples of the incubation mixtures were withdrawn at timed intervals, to quantify dialGTP binding.

Table 1 Stoichiometry and reversibility of binding of dialGTP to type-2 TGase

These are representative data from two to four independent determinations, with a range of variations of ±15% of the reported values.

Conditions	Incubation time (h)	Stoichiometry
Standard	2	1.12
Minus $NaCNBH_3$	2	0.96
Minus $NaCNBH_3$	4	1.05
Minus $NaCNBH_3$ plus dialysis	4	0.98
Plus $CaCl_2$ (0.5 mM)	2	0.38
Plus $CaCl_2$ (0.5 mM)	4	0.62
Plus GTP (2 mM)	4	0.12

derivatives easily detectable by SDS/PAGE and autoradiography. The label comigrated with the TGase peptide chain, but labelling was also observed in enzyme-degradation

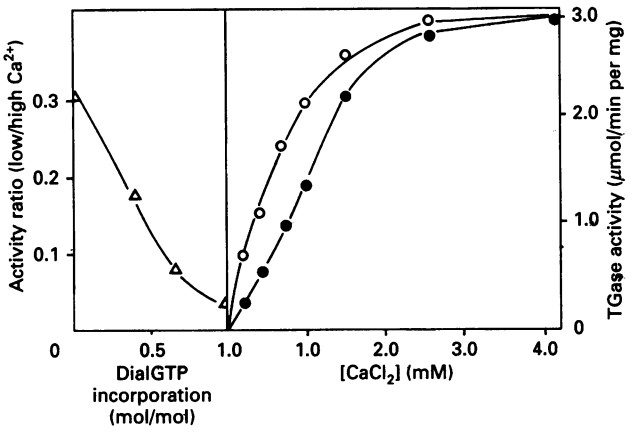

Figure 3 Influence of affinity-labelling by dialGTP on activity of TGase

(**a**) Activity of TGase was determined by standard procedures in substrate mixtures containing saturating and subsaturating concentrations of Ca^{2+}. Enzyme samples were withdrawn during dialGTP incorporation, as in Figure 2, and utilized for determination of label incorporation and of activity. Activity ratios are calculated as activity at 0.32 mM Ca^{2+} divided by that at 5 mM Ca^{2+}. (**b**) After 2 h of incubation, TGase was dialysed to remove unbound nucleotide and utilized to determine Ca^{2+} saturation (●). Data obtained with native TGase are also included (○).

products, when present. Ca^{2+} and GTP influenced the reaction, decreasing the extent of labelling of TGase (Figure 1). Quantitative determination of the reaction by the filter assay demonstrated progressive binding of dialGTP, with maximal incorporation within 2 h of incubation and 1:1 stoichiometry (Figure 2). Again, native GTP almost completely prevented labelling and Ca^{2+} greatly reduced its rate, without significantly affecting the final amount of dialGTP incorporated (Table 1). Interestingly, labelling was stimulated by $NaCNBH_3$, but proceeded also in its absence. The interaction of the probe with tissue TGase survived protein denaturation by alkali and dialysis in glycine buffers, excluding formation of Schiff-base intermediates in the reaction pathway.

The modification has functional consequences on enzyme activity, since ratios of activities measured at suboptimal (0.32 mM) and saturating (5 mM) concentrations of Ca^{2+} decreased from 0.3 to less than 0.1 in the fully modified TGase (Figure 3). This pattern stems from a decrease in Ca^{2+} sensitivity, without changes in maximal activity, because of a shift of the saturation curve from hyperbolic to sigmoid. Apparent affinity constants for activation by Ca^{2+} were 0.19 and 0.75 mM for native and modified TGase respectively.

DISCUSSION

Sensitivity of tissue TGase to GTP is considered a distinctive feature of the enzyme [13]. GTP inhibits activity at low Ca^{2+} concentrations, decreasing Ca^{2+} binding and preventing structural changes essential for catalysis [6]. The enzyme also displays GTPase activity and is possibly modulated by GTP-hydrolytic cycles, similarly to G-proteins [10].

Our results add to the present knowledge by determining the GTP-binding stoichiometry; the nucleotide binds at a single site per subunit, the modification of which induces enzyme conformations functionally equivalent to those induced by native GTP, at least in relation to Ca^{2+} activation. The GTP-binding site is expected to contain lysine residues reactive to dialGTP, forming hypothetical dihydroxymorpholino complexes, rather than a Schiff base, as suggested by the poor reversibility even in the absence of $NaCNBH_3$.

A most important point in understanding regulation of TGases by GTP is represented by locating the GTP- and Ca^{2+}-binding sites. Amino acid sequences of several tissue TGases are now known [14–16], but not that of erythrocyte TGase. TGases form a family of well-conserved proteins; for instance, homology between bovine endothelial TGase and other type-2 TGases is over 80% [16]. The GTP-recognition site should be well conserved, as it is the physiological function of the nucleotide in controlling enzyme activity and sensitivity to Ca^{2+}. Data in this perspective are still lacking, since putative effector sites have not been identified within the primary structure by sequence comparison with other proteins. This, however, is not so surprising when one considers the low affinity of TGases for Ca^{2+} and GTP in comparison with other typical binding proteins. The isolation and sequencing of the labelling peptide will probably help in answering some of these questions.

Note added in proof (received 14 January 1993)

Takeuchi and associates [17] recently reported the binding of radioactive GTP (and ATP) to immobilized guinea-pig liver TGase or to synthetic peptides thereof.

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