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A kinetic study of gamma-glutamyltransferase (GGT)-mediated S-nitrosogluthione catabolism

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ABSTRACT

S-Nitrosogluthione (GSNO) is a nitric oxide (NO) donor compound which has been postulated to be involved in transport of NO *in vivo*. It is known that γ -glutamyl transpeptidase (GGT) is one of the enzymes involved in the enzyme-mediated decomposition of GSNO, but no kinetics studies of the reaction GSNO-GGT are reported in literature.

In this study we directly investigated the kinetics of GGT with respect to GSNO as a substrate and glycyl-glycine (GG) as acceptor co-substrate by spectrophotometry at 334 nm. GGT hydrolyses the γ -glutamyl moiety of GSNO to give S-nitroso-cysteinylglycine (CGNO) and γ -glutamyl-GG. However, as both the substrate GSNO and the first product CGNO absorb at 334 nm, we optimized an ancillary reaction coupled to the enzymatic reaction, based on the copper-mediated decomposition of CGNO yielding oxidized cysteinyl-glycine and NO. The ancillary reaction allowed us to study directly the GSNO/GGT kinetics by following the decrease of the characteristic absorbance of nitrosothiols at 334 nm. A K_m of GGT for GSNO of 0.398 ± 31 mM was thus found, comparable with K_m values reported for other γ -glutamyl substrates of GGT.

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Introduction

Gamma-glutamyl transpeptidase (GGT; E.C. 2.3.2.2)¹ is a well-characterized type II heterodimeric glycoprotein anchored to the outside surface of the plasma membrane of both plant and animal cells. GGT catalyses the transfer of a γ -glutamyl moiety from the *in vivo* donor substrate, glutathione (GSH), to an active site nucleophile to form an acyl-enzyme intermediate during the acylation step of its catalytic cycle. This intermediate can then react, during the deacylation step, with an acceptor substrate containing a free amino group (normally an alpha amino acid) to form a new γ -glutamyl isopeptide bond and regenerate the free enzyme. In the absence of a primary amine acceptor substrate, the acyl-enzyme reacts slowly with water, giving a ping-pong catalytic cycle, modified to include hydrolytic deacylation [1].

GGT is mainly known as a central enzyme for the metabolism of GSH [2,3], although studies from the latest decade have highlighted its wider influence on cellular and extracellular redox equilibria [4].

S-Nitrosogluthione (GSNO) results from nitrosation of GSH, and several reports have proposed its involvement in storage and transport of NO [5,6], as well as a mediator of its endocrine effects of NO [7,8]. Several authors have hypothesized that GGT is involved in *in vivo* GSNO decomposition [9,10]. However, the kinetic parameters (K_m^{GSNO} , V_{max} , K_{cat}) of this reaction have not yet been reliably assessed.

In this study the kinetics of the reaction GSNO/GGT was investigated by a new spectrophotometric method, based on the coupling of two reactions: (i) the enzymatic cleavage of GSNO to CGNO, and (ii) the release of NO from CGNO mediated by copper ions. As both substrate GSNO and product GCNO actually present with the same characteristic absorption at 334 nm, previous studies have employed chromatographic separation of the reaction mixture prior to analysis and quantification [9]. Our method in fact avoids this need, insofar as the Cu(II)-mediated decomposition of CGNO allows to follow GGT enzyme activity by UV/visible spectrophotometry, directly as a decrease of absorbance at 334 nm.

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¹ Abbreviations used: GGT, γ -glutamyltransferase; GSNO, S-nitrosogluthione; NO, nitric oxide; GG, glycyl-glycine; CGNO, S-nitroso-cysteinylglycine; NEM, N-ethylmaleimide; DTPA, diethylenetriamine-pentaacetic acid pentasodium salt; PBS, phosphate buffer solution.

Materials and methods

Chemicals

Analytical reagent-grade chemicals were used without further purification. GGT (G-8040, Type I crude from bovine kidney), glycyl-glycine (GG, G-1002) and glutamic acid 5-(3-carboxy-4-nitro-anilide) ammonium salt (Glu-cp-NA, BioChemika 49525), a synthetic substrate for GGT, were purchased from Sigma-Aldrich (Sigma, Chemical Co., Milan, Italy). Copper sulfate, anhydrous (2791) was purchased from Merck (Laborchimica, Firenze, Italy). *N*-Ethylmaleimide (NEM, 04259 BioChemika Ultra, $\geq 99.0\%$) and diethylenetriamine-pentaacetic acid pentasodium salt (DTPA, 17969) were purchased from Fluka (Fluka GmbH, Buchs, Switzerland).

CuSO₄ and NEM stock solutions were prepared in MilliQ water. NEM solution was prepared daily.

Phosphate buffer solution (PBS) was prepared from monobasic monohydrate sodium phosphate and dibasic anhydrous potassium phosphate (BDH Laboratory Supplies, Poole, England). GGT stock solutions were prepared in 0.1 M PBS pH 8.0. GG stock solution was prepared in 1 M PBS pH 8.0.

Stock solution of GSNO (N4148, CAS No. 57564-91-7, Sigma) was prepared in 0.1 M PBS pH 8.0. In these experimental conditions GSNO standard solution was stable during the working day (9 h time, tested at room temperature) and for 4 weeks if stored at -80°C .

GSNO and CGNO were prepared by reacting 1 M NaNO₂ in H₂O with 1.1 M thiols in 0.5 M HCl, 0.5 mM EDTA at 0°C for 40 min [11], and used after measuring their concentration from the absorbance measured at 334 nm, using the extinction coefficient $977\text{ M}^{-1}\text{ cm}^{-1}$ [12]. RSNOs were diluted in 0.1 M PBS pH 8.0, 0.5 mM DTPA and their stability tested before any other use.

Methanol for liquid chromatographic analysis was purchased from Carlo Erba (Rodano, MI, Italy). Water deionized with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

Measurements of GGT activity

GGT activity was determined by following the hydrolysis of Glu-cp-NA as previously described [2]. Briefly, purified enzyme was incubated with Glu-cp-NA (1 mM) and GG (20 mM) in PBS (0.1 M, pH 8.0) at 37°C and the increase in absorbance at 405 nm was monitored. Enzyme activity was determined using a molar absorption coefficient of $8800\text{ M}^{-1}\text{ cm}^{-1}$ for formation of *p*-nitroaniline.

Spectrophotometric analysis

GSNO and CGNO decomposition measurements were made spectrophotometrically in 0.1 M PBS pH 8.0 at $37 \pm 0.1^\circ\text{C}$ by a Beckman DU-600 spectrophotometer, following the decrease of 334 nm absorbance. Values of rate constants are means of at least three determinations, and the standard error was always better than $\pm 3\%$. Autozero was performed on solutions before adding GSNO. After absorbance stabilization the enzyme was added.

HPLC analysis

The reaction of GGT and GSNO was followed also by HPLC, by sampling the reaction solution at various time points and quenching the reaction by the addition of 1 M HCl followed by centrifugation. The HPLC unit consisted of a pump (P4000, ThermoQuest) equipped with a mechanical degassing system (SC1000, ThermoQuest, Milan, Italy), a Rheodyne 7125 injector (Rheodyne, Cotati,

CA, USA), a 20 μL injection loop, a diode array detector equipped with a 5 cm path length flow cell (DAD, UV6000, ThermoQuest), and a column (Gemini RP C₁₈ 250 \times 4.6 mm, silica particle size 5 μm , equipped with a Guard Cartridge KJ0-4282, Phenomenex). The eluent for analysis was 97% 0.02 M PBS pH 6.0/3% methanol (97:3 v/v), flowing at 1 mL/min. Column effluent was monitored by absorbance at 334 nm. GSNO and CGNO were quantified by reference to authentic standards. All the solutions were filtered by 0.45 μm cellulose acetate filters (Microcon, Millipore Corporation).

Results and discussion

Principles of the spectrophotometric method

In preliminary experiments we observed that GSNO affects the kinetics of the reaction between GGT and γ -Glu-cp-NA. Spectrophotometric kinetics at 405 nm performed in 0.1 M PBS pH 8.0, 4.5 mM GG at 37°C , in the absence and presence of three different concentrations of GSNO, in fact showed that GSNO, being a γ -Glu donor, behaves like a competitive inhibitor in the γ -Glu-cp-NA/GGT reaction. Fig. 1A shows the double reciprocal plots of data corresponding to these experiments. The K_i value for GSNO ($309 \pm 34\ \mu\text{M}$) was calculated from the double reciprocal plot by applying the Eq. (1) (Fig. 1B):

$$\alpha = 1 + \frac{[I]}{K_i} \quad (1)$$

where α corresponds to the ratio of the slopes of the uninhibited and inhibited reactions. Thus, we designed a spectrophotometric study for the determination of the kinetics parameters of the reaction.

Hogg et al. demonstrated that GGT stimulated the release of NO from GSNO in the presence of a transition metal (copper being the most effective) [9]. The enzymatic mechanism did not result however in the release of NO if chelating agents (such as DTPA or EDTA) were present in the reaction medium. This result can be explained by the different stability of GSNO as compared to CGNO (the first product of the enzymatic transnitrosation reaction) toward copper ion-catalysed decomposition. The reaction of Cu(II) with CGNO is very rapid, with half-lives of about 5 s, while reaction of GSNO is 3–4 orders of magnitude slower [13].

On this basis, we optimized suitable operating conditions in order to follow the enzymatic reaction (2) by an ancillary reaction (reaction (3)) based on the copper-catalyzed decomposition of CGNO:



where glycyl-glycine (GG) was chosen as acceptor substrate of GGT. Reaction (3) is fast as compared to reaction (2) [13], thus allowing to follow the enzymatic reaction by the decrease of the absorbance at 334 nm.

Fig. 2 reports UV/visible absorbance spectra of GGT solution and GSNO/GGT/Cu(II) reaction mixture, before and after the addition of transpeptidation acceptor GG. The enzyme is responsible for the absorption band around 280 nm (spectrum a), GSNO for the band at 334 nm (spectrum b). As expected, GGT activity decreased the absorption band at 334 nm (spectrum c, 10 min reaction).

Fig. 3 reports the time-dependent plots of the enzymatic decomposition of GSNO by GGT, in absence or presence of CuSO₄ and GGT. In the absence of GGT (Fig. 3, curve a') Cu(II) itself did not decompose GSNO in the 5 min observation time, in agreement with previous literature data [13]. In the presence of GGT, GSNO was hydrolyzed to CGNO, which decomposed slowly (Fig. 3, curve

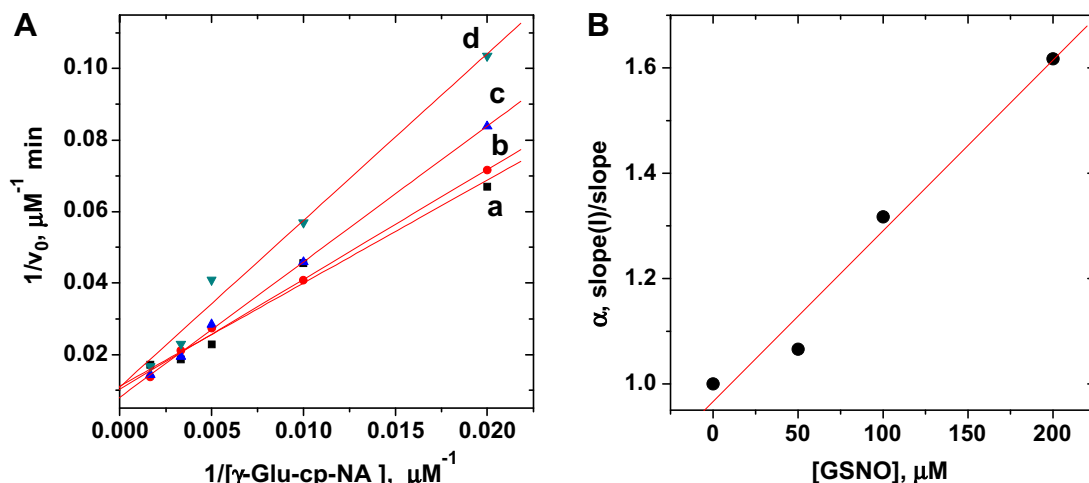


Fig. 1. (A) Double reciprocal plots for $\gamma\text{-Glu-cp-NA}$ at different GSNO concentrations: (a) 0, (b) 50, (c) 100 and (d) 200 μM . (B) Variation of value α as a function of GSNO concentration (intercept = 0.966 ± 0.042 , slope = 0.00324 ± 0.00036 , $R = 0.9877$).

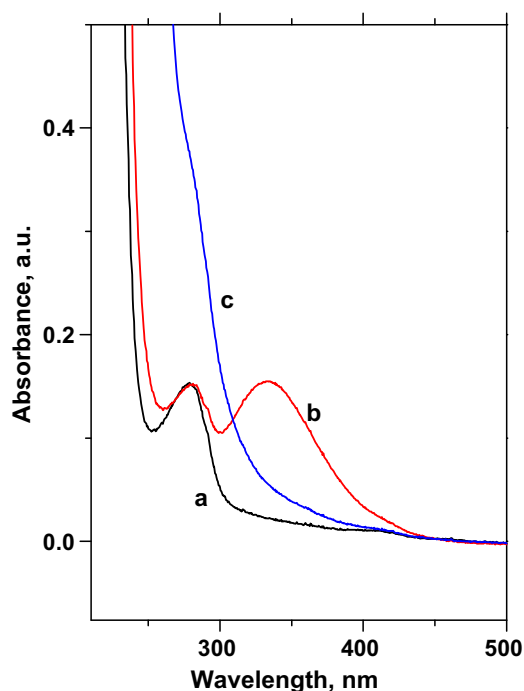


Fig. 2. UV/visible absorbance spectra of 525 mU mL^{-1} GGT in 0.1 M PBS pH 8.0, at 37 °C (a), 525 mU mL^{-1} GGT, 100 μM GSNO, 0.1 mM Cu(II) reaction mixture before (b) and 10 min after the addition of 4.5 mM acceptor substrate GG (c).

a). This was likely due to transition metals accidentally present in the reaction medium, as confirmed by the complete inhibition obtained by the addition of EDTA (0.5 mM, data not shown). On the other hand, in the presence of Cu(II) CGNO was rapidly decomposed (Fig. 3, curve b).

In order to verify if Cu(II) affects the enzymatic reaction, we (i) studied the kinetics $\gamma\text{-Glu-cp-NA}/\text{GG}/\text{GGT}$ in the presence or absence of Cu(II), and (ii) analyzed the reaction products by HPLC, alternatively in the presence of DTPA or Cu(II). The presence of Cu(II) did not significantly affect enzyme activity (Fig. 4). In the HPLC experiments, the enzymatic reaction was stopped at different times by adding HCl (0.5 M final concentration) and analysing the reaction mixture after ultrafiltration. Fig. 5 shows the absorbance chromatograms at 334 nm of the reaction products after 0.5, 1

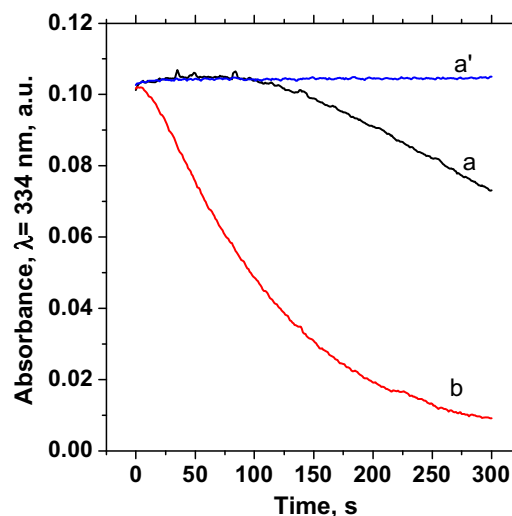


Fig. 3. Time-dependent plots of enzymatic decomposition of 100 $\mu\text{mol L}^{-1}$ GSNO in 0.1 mol L^{-1} PBS pH 8.0, at 37 °C, by 525 mU mL^{-1} GGT, 4.5 mM GG, with and without CuSO_4 : (a) 0 μM , (b) 100 μM . In a' the buffer contained CuSO_4 100 μM , but GGT was omitted.

and 5 min after addition of the enzyme (panel A), and the time-dependent plots obtained by plotting areas of CGNO and GSNO peaks (at 6.69 ± 0.01 and 7.21 ± 0.01 min, respectively; panel B). The assignment of chromatographic peaks to CGNO and GSNO was based on the injection of standard solutions of pure compounds of known concentration measured by UV ($\epsilon_{334} = 977 \text{ M}^{-1} \text{cm}^{-1}$) [12], and quantitation was based on calibration curves. As can be seen in Fig. 5A (in the presence of DTPA), both CGNO and GSNO peaks can be observed, the first increasing, the latter decreasing as the reaction proceeds. In the presence of 0.1 mM Cu(II) only the GSNO peaks were observed, because of the rapid decomposition of CGNO. The rate of GGT-mediated GSNO metabolism was however not significantly affected (Fig. 5B). Thus, on the basis of these results we can conclude that copper does not interfere with the enzyme kinetics.

Kinetic parameters of GSNO/GGT reaction

The ancillary reaction (copper-mediated CGNO decomposition) was used to determine spectrophotometrically the kinetics param-

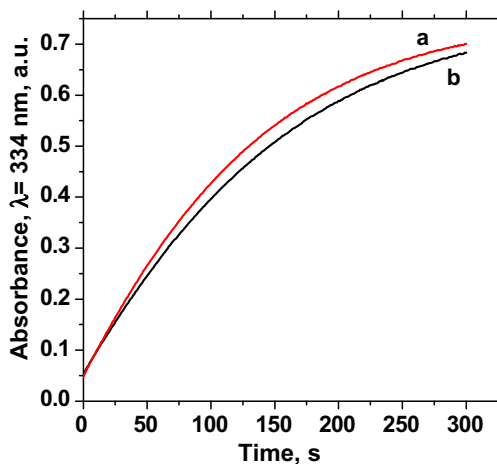


Fig. 4. Time-dependent plots of enzymatic hydrolysis of 100 μM $\gamma\text{-Glu-cp-NA}$ in 0.1 mol L^{-1} PBS pH 8.0, at 37 $^{\circ}\text{C}$, by 525 mU mL^{-1} GGT, 4.5 mM GG without (curve a) and with 100 μM Cu(II).

eters of the GGT/GSNO reaction. K_m was determined at a fixed concentration of the enzyme, by varying the GSNO concentrations between 50 and 300 μM . K_m and V_{max} for GSNO were estimated by linear regression from the double reciprocal plot.

K_m values were derived from kinetics repeated in different operating condition: Cu(II) concentration varying from 50 to 600 μM , GG from 4.5 to 20 mM, and GGT from 200 to 525 and 1000 mU mL^{-1} . Different concentrations of Cu(II) were used in order to ascertain that the presence of the metal did not affect the enzymatic kinetics, while different concentrations of acceptor substrate were used to verify that glycyl-glycine did not affect Cu(II)-mediated CGNO decomposition. It was thus possible to calculate an average K_m value equal to $390 \pm 30 \mu\text{M}$ ($N = 9$ experiments, with Cu(II) concentrations of 50, 100 ($N = 6$), 200 and 600 μM), and a V_{max} value equal to $165 \pm 16 \mu\text{M/min}$ ($N = 6$ experiments with 525 mU mL^{-1} GGT; varying concentrations of Cu(II) and acceptor did not affect the results).

Being aware of the uncertainty deriving from analysis of kinetic parameters using crude enzyme preparations, we also determined K_m for the synthetic substrate $\gamma\text{-Glu-cp-NA}$ using the same lot of commercial GGT. A value of $301 \pm 9 \mu\text{M}$ was found, in agreement with values reported in literature for the same substrate ranging

between 210 and 650 μM , depending on GGT source and reaction conditions [14–17].

K_m for GG was also determined, by keeping constant the concentrations of the enzyme (525 mU mL^{-1}) and GSNO (100 μM), while varying GG concentrations from 0.5 to 8 mM. The fitting of data of the linear part of the double reciprocal plot gave in this case a K_m value of $687 \pm 63 \mu\text{M}$.

Effect of GSNO on $\gamma\text{-Glu-cp-NA}$ /GGT reaction

In order to confirm the reliability of the K_m value found for the GSNO/GGT reaction, we studied the kinetics of the reaction $\gamma\text{-Glu-cp-NA}$ /GGT in the presence of 0, 50, 100 and 200 μM GSNO. K_m was determined at a fixed concentration of the enzyme (525 mU mL^{-1}), by varying the $\gamma\text{-Glu-cp-NA}$ concentrations between 50 and 600 μM . K_m and V_{max} for $\gamma\text{-Glu-cp-NA}$ were estimated by linear regression from the double reciprocal plot.

The results showed that GSNO acts as a competitive inhibitor of the reaction. From the ratio of the slopes of regression lines from the double reciprocal plot obtained with/without GSNO ($\text{slope}_{\text{GSNO}}/\text{slope}_0 = \alpha$) it is possible to calculate K_i , according to the equation:

$$\alpha = 1 + \frac{[I]}{K_i} \quad (1)$$

where K_i is the dissociation constant of the EI complex ($K_i = [E][I]/[EI]$).

Fig. 6 is a plot of the ratio α as a function of GSNO concentration. From linear fitting of data, an intercept = 0.966 ± 0.04 and a slope = 0.00324 ± 0.00036 ($R = 0.9877$) are calculated, corresponding to a $K_i = 309 \pm 34 \mu\text{M}$. This value is in good agreement with the K_m value for GSNO reported above. It is in fact accepted that K_i ($K_i = k_{-1}/k_{+1}$) by definition is $< K_m$:

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad (4)$$

Determination of K_{cat} of GGT for GSNO

A valuable catalytic constant in addition to K_m and V_{max} is the turnover coefficient K_{cat} , defined as:

$$K_{\text{cat}} = V_{\text{max}}/[Et] \quad (5)$$

where $[Et]$ is the total enzyme concentration.

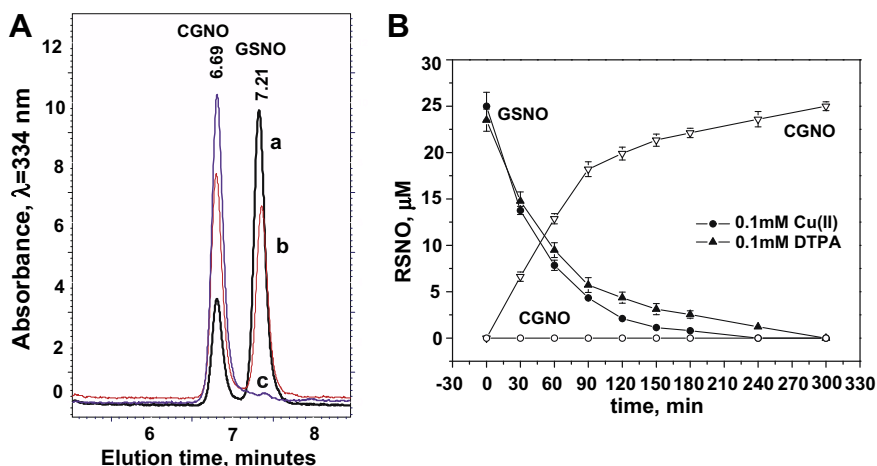


Fig. 5. (A) Absorbance chromatogram at 334 nm of the products of the hydrolysis of 50 μM GSNO (closed symbols) to CGNO (open symbols) by 525 mU mL^{-1} GGT in 0.1 M PBS pH 8.0, 0.1 mM DTPA, at 37 $^{\circ}\text{C}$, 4.5 mM GG [observation times = 0.5 min (a), 1 min (b), 5 min (c)]. (B) GSNO and CGNO time-dependent concentrations in the reaction mixture analysed by HPLC in experiments similar to that represented in (A), in the presence of 0.1 mM CuSO_4 (circles) and 0.1 mM DTPA (triangles).

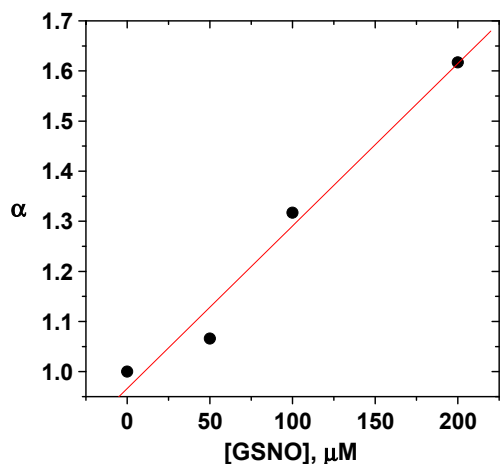


Fig. 6. Inhibitory effect of GSNO on the γ -Glu-cp-NA/GGT reaction. Plot of α ($=1 + [I]/K_i$) as a function of GSNO concentration. $R = 0.9877$, intercept $= 0.9663 \pm 0.042$, slope $= 0.00324 \pm 0.00036$.

K_{cat} was calculated from our data following two different approaches: (i) from the slope of the plot V_{max} as a function of $[Et]$ (Fig. 7A); (ii) by plotting V_0 as a function of GGT concentration at fixed, non-saturating concentrations of GSNO (Fig. 7B), by the equation:

$$V_0 = \frac{K_{cat}[S]}{K_m} [Et] \quad (6)$$

where we can reasonably assume that $[S] = [S_0]$ at $t = 0$.

With the procedure (i), a value of $K_{cat} = (2.88 \pm 0.03) 10^{-4} \mu\text{mol min}^{-1} \text{mU}^{-1}$ was found. With the procedure (ii), by using the K_m value calculated above ($386 \pm 30 \mu\text{M}$), a fully comparable value of $K_{cat} = (3.01 \pm 0.23) 10^{-4} \mu\text{mol min}^{-1} \text{mU}^{-1}$ was found. Thus, the two procedures employed yielded fully comparable results, which represents an independent confirmation of the validity of the K_m value calculated by the spectrophotometric method described above.

Regulation of GSNO/GGT reaction by transpeptidation acceptor: the ratio GSNO/GG

As concentrations of both γ -glutamyl donor and acceptor substrates concur in regulation of GGT activity [14,18], we studied the GGT/GSNO reaction by varying the concentrations of donor GSNO, in the presence of different GG/GSNO ratios (Fig. 8).

In the absence of GG (curve a) the reaction proceeded slowly— γ -glutamyl groups being transferred to water acting as acceptor [14,19]—up to GSNO $> 175 \mu\text{M}$, when V_0 was likely inhibited by donor substrate. For GG/GSNO = 0.5 (curve b), GSNO was little decomposed and V_0 was independent of GSNO concentration; this might be due to enzyme inactivation by Cu(II)–GG complexes: GG is in fact known to form complexes with copper [20]. Only for GG/GSNO ratio = 15 (curve d) we observed a $K_m = 380 \mu\text{M}$ (double reciprocal plot fitting parameters: slope $= 3.538 \pm 0.266$, intercept $= 0.0093 \pm 0.003$, $V_{max} = 107 \mu\text{M/min}$; $R = 0.9916$, $N = 5$), i.e., a value in agreement with data reported above.

Concluding remarks

Despite the envisaged role of GSNO as the physiological substrate for GGT activity, to date the kinetics of the reaction of GGT with GSNO as substrate had not been investigated in full detail, probably due to the difficulty of detecting the reaction product CGNO. The latter compound in fact presents with the same absorption at 334 nm as the substrate GSNO itself, which required the introduction of a HPLC separation step in order to perform quantification [9]. On the other hand, the method described in this study overcomes product detection problems and permits straightforward determination of GGT-mediated GSNO metabolism, giving therefore more reliable kinetics parameters for the GSNO/GGT reaction. In our assay conditions an apparent K_m of $386 \pm 30 \mu\text{M}$ was calculated for the GGT/GSNO reaction, using commercial bovine kidney GGT and GG as transpeptidation acceptor. This value is closely comparable with GGT K_m values reported in literature for synthetic substrates such as γ -glutamyl-*p*-nitroanilide (610 – $680 \mu\text{M}$, [21]), γ -glutamyl-3-carboxy-4-nitroanilide ($370 \mu\text{M}$ [15]), as well as its physiological substrate GSH ($400 \mu\text{M}$ [22]), thus suggesting that GGT may have a role in GSNO metabolism. A signif-

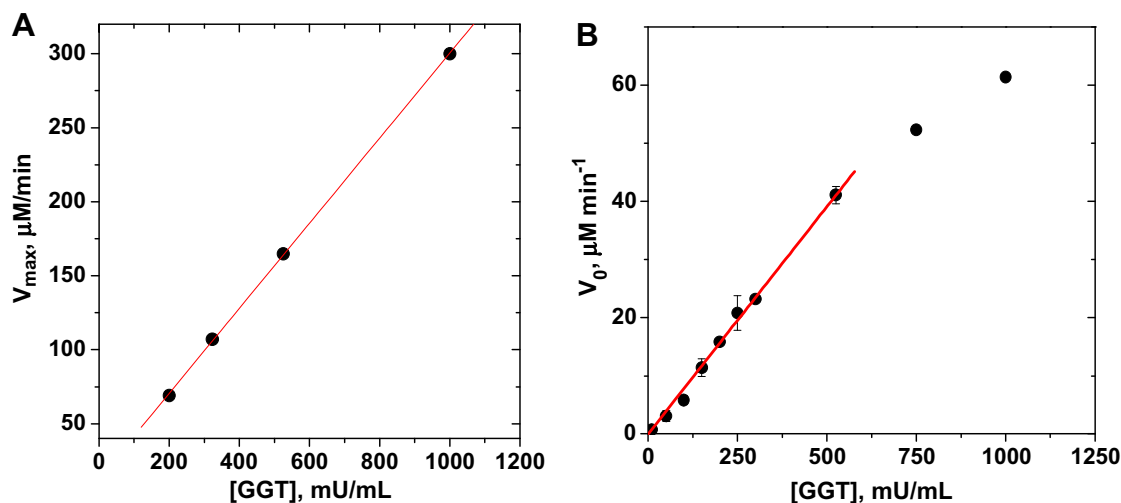


Fig. 7. (A) Plot of V_{max} as a function of GGT concentration. Reaction conditions: $100 \mu\text{M}$ GSNO in 0.1 M PBS pH 8.0, at 37°C , 0.1 mM CuSO_4 . Fitting parameters: intercept $= 13.0 \pm 1.5$, slope $= 0.288 \pm 0.003$; $R = 0.9990$, $N = 4$). (B) Plot of V_0 vs. GGT concentration derived from time-dependent plots of enzymatic decomposition of $100 \mu\text{M}$ GSNO in 0.1 M PBS pH 8.0, at 37°C , 4.5 mM GG, 0.1 mM CuSO_4 , at various concentrations of GGT (10, 50, 100, 150, 200, 250, 300, 525 mU mL^{-1}). Fitting parameters of linear part: slope $= 0.078 \pm 0.0014$; $R = 0.9979$, $N = 8$).

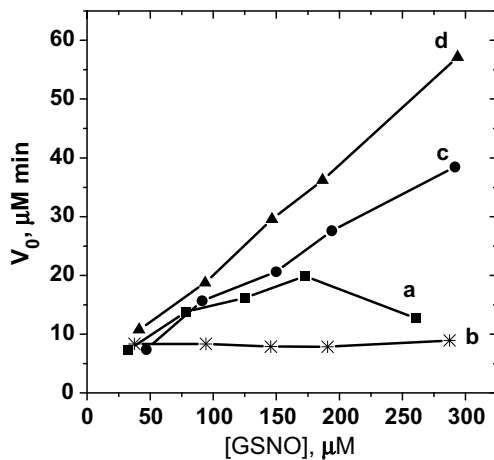


Fig. 8. Plot of V_0 as a function of GSNO concentration at different constant GG/GSNO ratios: (a) GG = 0, (b) GG/GSNO = 0.5, (c) GG/GSNO = 5, (d) GG/GSNO = 15. Reaction conditions: 323 mU/mL GGT, 0.1 mM Cu(II) in 0.1 M PBS pH 8.0, 37 °C.

icant association of serum GGT values with evolution of cardiovascular diseases has been repeatedly reported [23]. The possibility thus exists that an altered GSNO handling may occur in these patients; further studies are clearly needed to investigate this hypothesis.

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