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Mechanism for the inhibition of transglutaminase 2 by cystamine

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Abstract

Cystamine is neuroprotective in a number of models of neurodegeneration. The therapeutic benefit of cystamine has been attributed, in part, to its inhibition of transglutaminase activity. Cystamine [β-mercaptoethanolamine (MEA) disulfide] is reduced within cells to MEA which is largely responsible for the in vivo effects of its disulfide precursor. In the current study, the amine group of MEA was shown to act as a transglutaminase (TG) substrate resulting in the formation of N^{β}-(γ-L-glutamyl)-MEA bonds. The formation of such bonds would compete with the generation of other TG-catalyzed reactions that may contribute to neurodegeneration such as polyamination, protein cross-linking, deamination and the covalent attachment of ceramide to proteins. The demonstration that cystamine-derived MEA can form N^{β}-(γ-L-glutamyl)-MEA bonds offers a unique tool for identifying the TG substrates that occur in diseased brains in vivo. Structure-function studies also indicated that the mercapto group of MEA significantly influences the substrate behavior of this compound. These structure-function studies also identified the following hierarchy of physico-chemical characteristics: hydrophobicity > S as the group VIII atom > distance separating the N and group VIII atom, as the major determinants contributing to the substrate behavior for low-molecular weight amine substrates of TG 2.

1. Introduction

Cystamine is neuroprotective in a number of disease models [1–5]. Consequently, there is a great interest in both the therapeutic potential of this agent and its mechanism of action. The therapeutic benefit of cystamine has been attributed to its inhibition of transglutaminases (TGs) [1,2,6] and caspase 3 [7], as well as, to its stimulation of glutathione (GSH) synthesis [7,8]. Nevertheless, some actions of cystamine may be more important than others. For example, the inhibition of caspase 3 by cystamine has been only demonstrated in cultured cells and not in animals [7]. Cystamine does stimulate GSH synthesis in vivo but the increase is very modest in organs that have a high rate of GSH production and, therefore, is unlikely to be significant in the brain which has a low rate of GSH turnover. Cystamine stimulates the catalysis of GSH by supplying one of the substrates, namely, cysteine. Thus, the ability of cystamine to stimulate GSH production is determined primarily by the cellular levels of GSH and γ-glutamylcysteine synthetases. The liver has significant amounts of these enzymes. Even so, cystamine causes only a modest stimulation of hepatic GSH synthesis [9]. Compared to the liver, the brain has a very low capacity to produce GSH [10,11] and therefore, cystamine would be expected to have little effect on cerebral GSH levels. This conclusion has been borne out by the experimental studies (A.J.L. Cooper et al., in preparation). In contrast, the inhibition of TG activity has been demonstrated in brain lysates of mice treated with cystamine and is consistent with many

Abbreviations: DMC, *N,N*-dimethylcasein; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione disulfide; MEA, β-mercaptoethanolamine; TG, transglutaminase

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studies indicating a role for TGs in Huntington disease [1,2,12–15].

Rodents and humans have eight separate TGs (TG 1–7 and Factor XIII) that catalyze the formation of substituted amide linkages between glutaminyl (Q) residues and either lysyl residues or polyamines [16]. In vitro, these enzymes can also utilize amines and diamines as acyl acceptors. The amide bond is formed by a Ping-Pong mechanism involving two successive nucleophilic attacks. In the first reaction, the thiolate anion in the active site of TG attacks and substitutes for the amine group of the carboxamide moiety at the γ position of a Q residue to form a thioester. This thioester is then attacked by either the primary amine of a lysyl residues or a polyamine to form an N^{ε}-(γ-L-glutamyl)-L-lysyl isopeptide or *N*-(γ-L-glutamyl)-polyamine linkage, respectively. The unmodified terminal amine of the *N*-(γ-L-glutamyl)-linked polyamine can then attack another acyl TG intermediate to form a bis-glutamyl-polyamine bridge. The ability of TGs to cross-link proteins through the formation of either N^{ε}-(γ-L-glutamyl)-L-lysyl or bis-γ-glutamylpolyamine linkages prompted the hypothesis that these enzymes contribute to the formation of the insoluble proteinaceous aggregates that characterize neurodegenerative diseases [17,18]. This hypothesis is supported by the observation that TG activity and the frequency of the N^{ε}-(γ-L-glutamyl)-L-lysyl linkage are increased in the brains of patients with Progressive Supranuclear Palsy, Alzheimer, Huntington or Parkinson diseases [2,12–14,19–22]. Additionally, the level of free N^{ε}-(γ-L-glutamyl)-L-lysyl dipeptide, which is proteolytically-derived from N^{ε}-(γ-L-glutamyl)-L-lysyl cross-linked proteins, is elevated in affected areas of Huntington disease brains and cerebrospinal fluid [2,23]. The administration of cystamine reduces TG activity and normalizes the levels of free N^{ε}-(γ-L-glutamyl)-L-lysyl dipeptide in the brains of huntingtonsonian mice and suggests that the inhibition of TGs is one of the major in vivo actions of this drug [1,2].

Cystamine [NH_2–$(CH_2)_2$–S–S–$(CH_2)_2$–NH_2] is the disulfide of cysteamine [β-mercaptoethanolamine (MEA), NH_2–$(CH_2)_2$–SH]. The disulfide linking the two MEA moieties of cystamine is readily reduced by thiols such as dithiothreitol (DTT) and intracellular GSH. Thus, the actions normally attributed to cystamine are most likely, under reducing conditions, to be due to MEA. Indeed, we have shown that twice the amount of MEA is required to inhibit in vitro TG 2 activity to the same extent as any given amount of cystamine (in the presence of DTT), consistent with the yield of two moles of MEA per mole of cystamine [24]. It has been suggested that cystamine inhibits TGs by forming a mixed disulfide with the active site thiol through a thiol-disulfide interchange mechanism [7,25] analogous to the inhibition of this enzyme by 5,5′-dithiobis-(2-nitrobenzoic acid) [26]. However, if cystamine is reduced to MEA intracellularly, as indicated by numerous studies [27–29], it is unlikely that the mechanism of thiol-disulfide exchange is responsible for the inhibition of TG by cystamine. In the following study, we examine the possibility that MEA inhibits TG 2 by acting as an alternate amine-bearing substrate for this enzyme and thereby generating the N^{β}-(γ-L-glutamyl)-MEA bond.

2. Materials

Butanolamine (4-amino-1-butanol), propanolamine (3-amino-1-propanol), 2-aminothiophenol, 3-aminothiophenol, 4-aminothiophenol, 2-aminophenol, 3-aminophenol and 4-aminophenol were purchased from Aldrich whereas DTT, *N*-α-carbobenzyyglutaminylglycine, diethylenetriaminepentaacetic acid, *N,N*-dimethylcasein (DMC), hydroxylamine-HCl, MEA, dansylcadaverine, β-selenocystamine, TG 2 (guinea pig liver), Substance P were purchased from Sigma Chemical Company (St Louis, MO). [1,4-^{14}C] Putrescine dihydrochloride (111 pCi/pmol) was bought from Amersham Life Science (Piscataway, NJ) and counted with aquasol scintillation fluid (New England Nuclear).

3. Methods

3.1. Continuous fluorescence assay for TG activity

TG 2 activity was measured by monitoring the covalent attachment of dansylcadaverine to DMC. The reaction mixture for optimally measuring TG activity contained 0.5 mg/mL DMC, 32 μM dansylcadaverine, 100 mM Tris–HCl (pH 8.0), 10 mM DTT and 10 mM $CaCl_2$ in a final volume of 0.2 mL. After warming the reaction mixture for 10 min at 37 °C, the reaction was initiated by the addition of 10 mU TG 2. The covalent attachment of dansylcadaverine was monitored fluorometrically (excitation 280 nm, emission 538 nm) for at least 20 min at 37 °C using a 96-well plate SpectraMax Gemini fluorometer (Molecular Devices, Sunnyvale, CA). Note that because the fluorescence procedure yields relative values, the amount of enzyme added in the fluorescence experiments is given in terms of units predetermined by the standard hydroxaminolysis reaction as described by Folk and Cole [30] and modified by Cooper et al. for well plate analyses [18]. The hydroxaminolysis reaction was monitored in a SpectraMax 25096-well analyzer (Molecular Devices, Sunnyvale, CA).

3.2. Radiometric TG assay

TG-catalyzed incorporation of radiolabeled putrescine into DMC was measured essentially as described by Cooper et al. [18] using a reaction mixture of 1 mg/mL DMC, 0.2 μCi [1,4-^{14}C] putrescine dihydrochloride, 0.5 mM putrescine, 200 mM Tris–HCl (pH 8.0), 10 mM DTT and 10 mM $CaCl_2$ in a final volume of 0.1 mL. This mixture was pre-warmed for 10 min at 37 °C prior to the

addition of TG 2 to start the reaction. The enzyme reaction was terminated after 10 min incubation by the addition of 20 μL ice-cold 50% TCA after which the tubes were placed in ice for at least 30 min. Centrifugation at 10,000 × *g* for 15 min at 4 °C was used to collect the precipitated proteins, which were washed twice more with 0.1 mL 10% trichloroacetic acid using the same centrifugation conditions. The final pellet was dissolved with 0.1 mL 1 M NaOH and combined with 4 mL of aquasol for liquid scintillation counting.

3.3. Preparation of TG 2 and reagents

Guinea pig liver tissue TG 2 is supplied as a lyophilized powder and this preparation loses activity within hours of dissolution in various buffers at 0 °C. The enzyme can be stabilized, however, by dissolving the lyophilized extract at a concentration of 70 mU/min 10 mM DTT and 1 mM diethylenetriaminepentaacetic acid, pH 7.0. Under these conditions, the enzyme is stable for at least 10 days at 0 °C. Dansylcadaverine was dissolved in glacial acetic acid at a concentration of 50 mg/mL (149 mM) and buffered as described previously [31].

3.4. Molecular modeling

Molecular modeling was performed on 11 structural congeners of MEA to ascertain whether the differences in activity could be correlated with the physico-chemical determinants of the compounds. The structures of the compounds were, if available, derived from X-ray crystal data. These matrices were then entered into Chem-X (Chemical Design Ltd., 1995) and optimized using functions within Chem-X. The optimized structures were then submitted to a Molecular Orbital Package, version 6.0 [32] and optimized using reduced Hartree–Fock, Austin model 1 theory. The resultant optimized data were then submitted to the Molecular Orbital Package [32] to obtain single point charges for each atom. This was achieved using minimal neglect of differential overlap, electrostatic potential theory with a net charge on each compound equal to zero.

3.5. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on an Applied Biosystems Voyager-DE™ STR Biospectrometer™ Workstation. Saturated solutions of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/water (v/v) containing 0.1% trifluoroacetic acid were used as a matrix.

3.6. Statistics

All statistical analyses (reported as mean ± S.E.M., *n*), linear and non-linear curve fitting were performed using SigmaPlot 8.0 (Systat Software Inc., Richmond, CA).

4. Results

4.1. Development of the TG assay

Michaelis-Menten analysis can distinguish among the several models of enzyme inhibition. In order to apply this analysis to the study of the attenuation of TG 2 activity by MEA, we developed an assay to satisfy the necessary conditions for this analysis. This was done by modifying an earlier TG assay that utilized dansylcadaverine as amine substrate and *N,N*-dimethylcasein as the Q substrate (methylation renders an otherwise lysyl residue substrate unreactive in the TG reaction)[33]. The method of Lorand et al. [33] requires extraction of the fluorescent reaction product into *n*-heptane, prior to its detection. To eliminate this extraction step and to obtain a continuous fluorescent assay, the product of the TG 2-catalyzed amide linkage between dansylcadaverine and DMC was scanned to identify its optimal excitation and emission wavelengths which were found to be 260 and 538 nm, respectively. Using these spectral parameters, we found that dansylcadaverine and DMC at concentrations of 16 μM and 0.5 mg/mL, respectively, maximally stimulated TG 2 activity (not shown). The $K_{m,app}$ for dansylcadaverine under the conditions (derived from Lineweaver–Burke plots, not shown) was 2.35 ± 0.02 ($n = 46$) μM and is comparable to the value obtained by Case and Stein (1.9 μM) using a comparable assay [34]. The covalent attachment of dansylcadaverine to DMC at saturating substrate concentrations was linear for the first 15 min.

4.2. MEA acts like a competitive inhibitor of TG 2

MEA attenuated the initial rate of TG-catalyzed incorporation of dansylcadaverine (Figs. 1A and 2A) and ^{14}C putrescine into DMC (Fig. 1B). The attenuation of dansylcadaverine incorporation was concentration-dependent and persisted for at least an hour (Figs. 1A and 2A). Greater amounts of MEA than dansylcadaverine were required to attenuate the incorporation of ^{14}C putrescine into DMC consistent with the lower K_m of the dansylated polyamine for TG 2 [31,33]. The Lineweaver–Burke plots of the attenuation of TG 2 by MEA are consistent with a model of competitive inhibition with respect to dansylcadaverine (Fig. 2B). A K_i concentration of 147 μM was derived by plotting the slopes from the double reciprocal plot (K_m/V_{max}) as a function of MEA concentrations (Fig. 2C, Table 1). The conclusion that MEA acts so as to compete with dansylcadaverine for catalysis by TG 2 is supported by the observation that diluting TG 2, which had previously been treated with 0.4 mM MEA, also decreased the attenuation of this enzyme by MEA (Fig. 2D). In other words, the effect of MEA on TG 2 was reversible and not due to a direct modification of the enzyme.

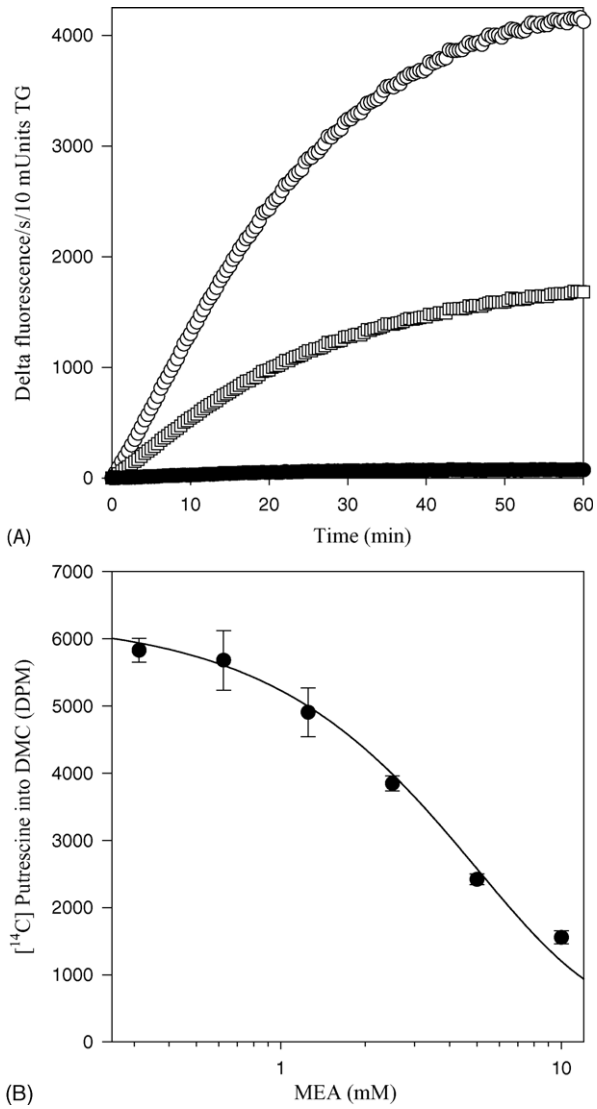

Fig. 1. Measurement of TG activity and its inhibition by MEA.The inhibition of TG 2 by 0 (○), 0.98 (□) and 25 mM (●) MEA over 60 min is depicted in panel A and represents the mean and S.E.M. of eight experiments (the S.E.M. values are less than the width of the symbols). Panel B shows the inhibition of TG 2-catalyzed [^{14}C] putrescine incorporation into DMC by indicated final concentrations of MEA. The asymptote of the fitted curve tends to $y = 0$ and was confirmed with experiments using 20 and 40 mM MEA concentrations (not shown). The mean and S.E.M. of three experiments is presented in B.

4.3. MEA attenuates TG activity by acting as an alternate substrate

Two mechanisms may account for the apparent competitive inhibition of TG 2 by MEA. These mechanisms differ based on whether or not MEA undergoes catalytic transformation subsequent to competing with dansylcadaverine for binding to TG 2. Since MEA resembles a number of amine substrates of TG, we hypothesized that this compound may attenuate TG 2 activity by acting as an alternate amine substrate for the enzyme. This hypothesis is supported by the demonstration that β-mercaptoethanol [**HO–**CH_2–CH_2–SH], which has a hydroxyl in place of the amine group of MEA, did not attenuate TG 2 activity even at a concentration of 50 mM (data not shown). The possibility that MEA was acting as an alternate TG substrate was tested in the following experiment. MEA was used to attenuate the covalent attachment of dansylcadaverine to Substance P, a neuropeptide substrate of TG [35]. This compound possesses two adjacent Q residues, only one of which can act as a TG substrate, and contains no amine substrates in the form of lysine residues [35]. These factors simplify the interpretation of the results of TG 2-catalyzed cross-linking between Substance P (Q substrate) and MEA (amine substrate). Separation of the products of this reaction on SDS-PAGE allowed further mass spectrometric identification of the N^{β}-(γ-L-glutamyl)-MEA residue on Substance P (Fig. 3). The M_r of Substance P is 1347.7. The sulfoxide and sulfone of Substance P, therefore, have M_r values of ∼1364 and 1380, respectively. The upper trace (panel A) clearly shows that all three are present in the mass spectrum of Substance P eluted from the gel, with the sulfoxide predominating. Traces of other impurities are also present at higher M_r values but the nature of these impurities is not known. When Substance P was treated with cystamine and activated TG 2, the peaks at M_r (=m/z) values of 1348, 1364 and 1389 almost totally disappeared to be replaced by six major peaks each separated by ∼16 amu, beginning with a M_r value of about 1408 (panel B). This peak corresponds to Substance P covalently modified by the attachment of MEA. Peaks at M_r values of ∼1424, 1440, 1456, 1472 and 1488 correspond to successive additions of O atoms to the two sulfurs in the covalent adduct. A maximum of five O atoms can be added (methionine residue fully oxidized to a sulfone plus the –SH group fully oxidized to a sulfonic acid). The additional peak possibly corresponds to a MEA adduct to one of the impurities with a higher M_r than that of Substance P. Notice that an M_r value of ∼1408 cannot distinguish between Substance P containing a substituted amide [$RC(O)NHCH_2CH_2SH$] and Substance P containing a thioester [$RC(O)SCH_2CH_2NH_2$]. However, only a maximum of four O atoms (at the methionine residue) can be accommodated for the oxidation of the latter structure whereas the former structure can accommodate the addition of up to five extra O atoms. Thus, the multiple peaks separated by ∼16 amu are in accord with TG 2-catalyzed covalent attachment of MEA to Substance P through its nitrogen rather than through its sulfur.

In summary, these studies show that MEA acts as an alternate substrate for TG 2 and the mechanism accounts for the apparent competitive inhibition observed in the Michaelis-Menten studies above. Thus, MEA does not inhibit TG activity per se but rather diverts its activity to catalyzing the covalent attachment of MEA to proteins.

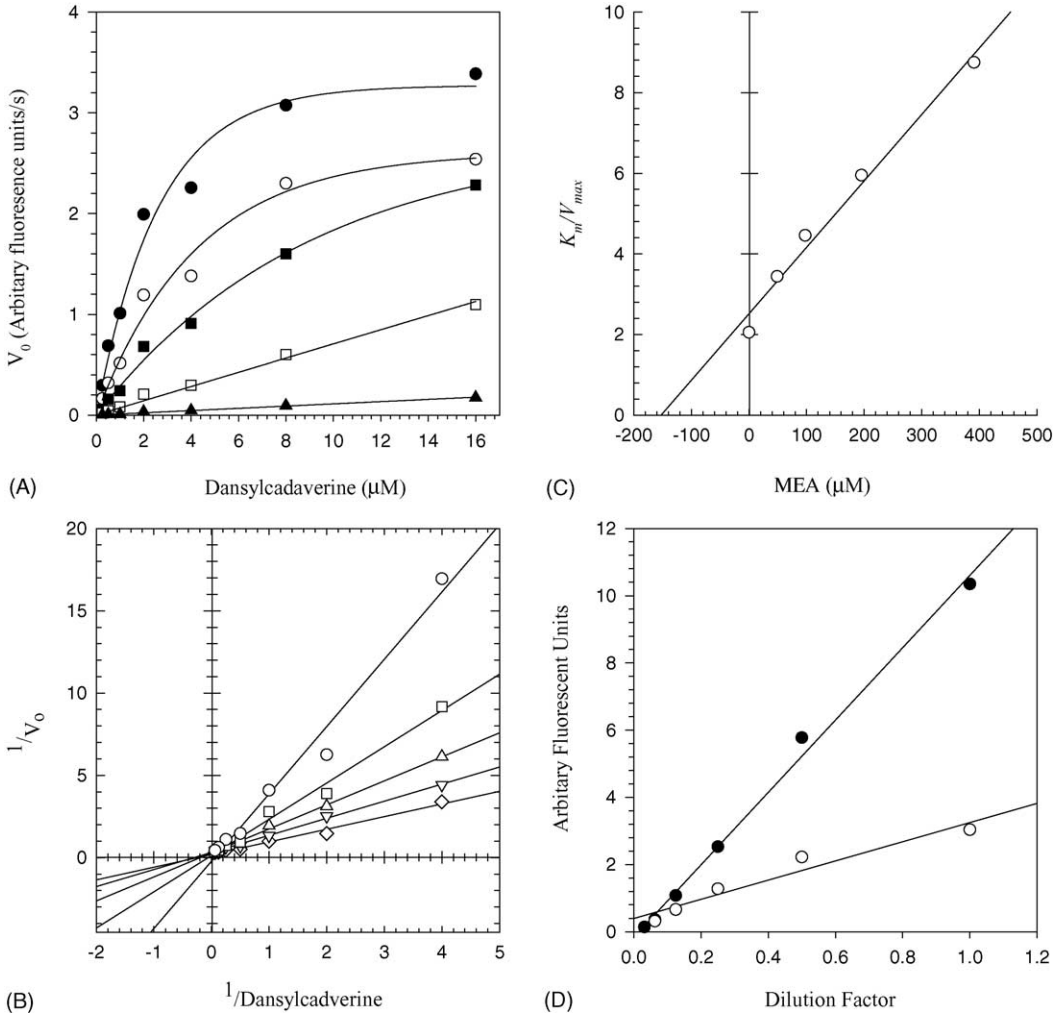

Fig. 2. MEA acts as a competitive inhibitor of TG 2. The effect of 0 (●), 0.1 (○), 0.4 (■), 1.6 (□) and 6.3 mM (▲) MEA on the initial rates of TG 2 activity and the indicated concentrations of dansylcadaverine are presented in panel A. The mean of four experiments is depicted. Panel B represents the data in (A) as Lineweaver–Burke plots. Panel C shows the $K_{m,\,app}/V_{max}$ values derived from the data in (B) as a function of MEA concentration. Panel D shows the activity of TG 2 that had previously been treated with (●) and without (○) 0.39 mM MEA and then serially diluted with reaction mixture. Shown are the results of a single experiment of which two were performed. Note that the V_o were obtained under initial velocity conditions.

Table 1
K_i, pK_i and other physico-chemical properties of MEA and it homologs

Compound	Formula	K_i (mM)	N charge (eV)	Group VIII atom charge (eV)	Distance (Å)[f]	$-NH_3^+$ pK_a	–SH or –SeH pK_a
Ethanolamine	$NH_2(CH_2)_2$**OH**	2.29 ± 0.39 (4)	4.75	5.63	3.66	9.5[a]	
MEA	$NH_2(CH_2)_2$**SH**	0.15 ± 0.02 (4)	4.53	6.20	3.98	10.7[b]	8.4 [b]
β-Seleoethanolamine	$NH_2(CH_2)_2$**SeH**	5.14 ± 0.19 (3)	4.86	6.52	3.98	11.0[c]	5.0 [c]
Propanolamine	$NH_2(CH_2)_3$**OH**	2.05 ± 0.32 (3)	4.94	6.01	4.84	10.2[d]	
Butanolamine	$NH_2(CH_2)_4$**OH**	0.28 ± 0.39 (4)	4.70	6.01	6.13	10.4[e]	
2-Aminothiophenol	$NH_2(C_6H_4)$**SH**	0.16 ± 0.03 (4)	5.11	5.81	3.03		
3-Aminothiophenol	$NH_2(C_6H_4)$**SH**	0.16 ± 0.06 (5)	5.30	5.95	5.16		
4-Aminothiophenol	$NH_2(C_6H_4)$**SH**	0.63 ± 0.09 (4)	5.05	5.33	5.92		
2-Aminophenol	$NH_2(C_6H_4)$**OH**	1.04 ± 0.06 (4)	4.78	6.09	2.85		
3-Aminophenol	$NH_2(C_6H_4)$**OH**	1.91 ± 0.13 (4)	4.98	5.90	4.87		
4-Aminophenol	$NH_2(C_6H_4)$**OH**	1.09 (2)	5.74	6.08	5.57		

[a] [72–74].
[b] [62,75,76].
[c] [62].
[d] [74,77,78].
[e] [78].
[f] Distance between N and group VIII atom.

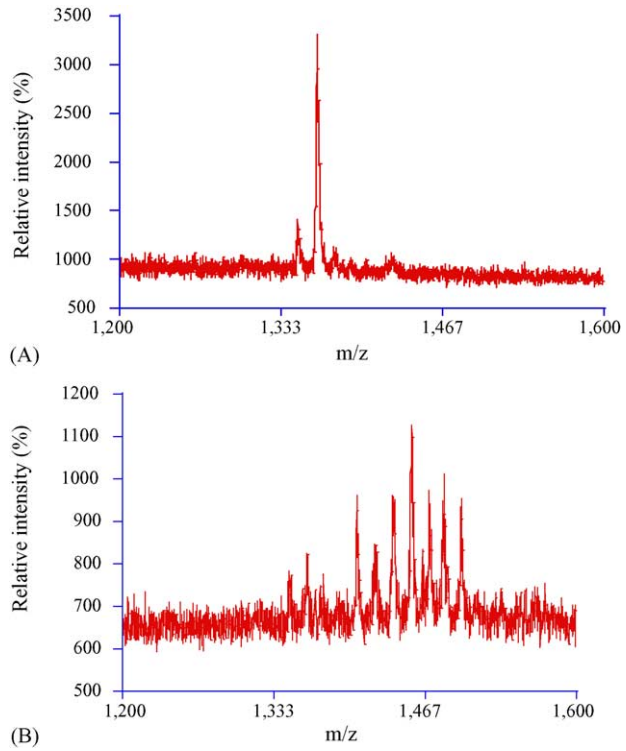

Fig. 3. N^{β}-(γ-L-Glutamyl)-MEA bond formation by TG 2. This experiment was essentially the same as Fig. 2A except that 5 mg/mL Substance P was used in place of DMC and that 25 mM MEA alone was used to inhibit the activity of TG 2. The products of the samples treated with and without MEA were then separated on 12.5% SDS-PAGE gels and the bands corresponding to Substance P (detected with Coomassie Blue staining) were excised for mass spectrometry as described in Section 3. Panel A shows the mass spectrometry of Substance P and panel B the mass spectrometry of the same peptide treated with TG 2 and MEA. The reaction inferred from these experiments is given in the text.

4.4. Structural requirements for the attenuation of TG 2 activity by MEA

The structural requirements for the alternate substrate activity of MEA were investigated by comparing the attenuation of TG 2 activity by MEA (as measured by the covalent attachment of dansylcadaverine) with that produced by its homologs (Table 1). Molecular modeling studies were also carried out to ascertain which physico-chemical determinants of the MEA congeners significantly contributed to the attenuation of TG 2 activity. The results of these studies are given in Table 1 as well as the known pK_a values of the appropriate functional groups. The first major determinant of the ability to attenuate TG activity relates to the hydrophobicity of the investigated compounds. The aromatic compounds (e.g., the aminophenols and aminothiophenols) were, in general, more effective attenuators of TG 2 activity than the aliphatic compounds (e.g., MEA, β-selenoethanolamine and the aminoalcohols: ethanolamine, propanolamine and butanolamine). Moreover, the aromatic compounds did not conform to the relationships observed between the physico-chemical characteristic and K_i of the aliphatic compounds, suggesting that the effects of hydrophobicity, for the most part, outweighed the effects of charge on the N or group VIII atom, pK_a or distance between the amine and group VIII atom-containing functional group (Table 1).

The pK_a of the amine moieties of the aminoalcohols and MEA correlated well with the pK_i values for the attenuation of TG 2 activity ($r^2 = 0.71$, Fig. 4A). Interestingly, the correlation between the charge on the N of these amine moieties and the K_i values for attenuation of TG 2 activity by the aminoalcohols and MEA was not as good ($r^2 = 0.59$, Fig. 4A) as the aforementioned correlation of the pK_a and pK_i values (Fig. 4A), despite an excellent correlation between N charge and amine pK_a of these compounds ($r^2 = 0.98$, not shown). One factor that clearly influenced the K_i, pK_a and the charge on the N was the distance separating N and O atoms in the aminoalcohol series (Fig. 4B). Thus, both the pK_a and charge on the N increased with increasing separation between the N and O atoms in the aminoalcohols ($r^2 = 0.91$ and 0.73, respectively, Fig. 4B). The K_i values, however, decreased with increasing distance between the N and O for the same compounds ($r^2 = 0.86$, Fig. 4C) and correlated with the pK_a values for the aminoalcohols ($r^2 = 0.59$, not shown). These observations further support the hypothesis that the pK_a of the amine is a critical determinant of the ability to attenuate TG 2 activity.

The charge on the O of the amino alcohols (Table 1) did not correlate with K_i, pK_a or charge on the N at the α position (not shown). However, the charge for the group VIII atoms (e.g., O, S and Se) was highly correlated with the K_i and pK_i of the β-substituted ethylamines: ethanolamine, MEA and β-selenoethanolamine ($r^2 = 0.93$ and 0.98, respectively, Fig. 4D). Moreover, S at the β position of β-substituted ethylamines conferred the greatest ability to attenuate TG 2 activity (Fig. 4D). There were no other significant correlations for the K_i values of ethanolamine, MEA and β-selenoethanolamine and the charges on the N atom or distances separating the α and group VIII atoms. This illustrates that an important physico-chemical determinant for one series of compounds (e.g., distance for the aminoalcohols) may be overshadowed by another determinant in a different series of structurally related compounds (e.g., group VIII atom charge for the β-substituted ethylamines). Thus, there was no correlation between the K_i values for the aromatic compounds and distance between the N and group VIII atoms or the charge of these atoms (not shown). This suggests the following hierarchy of physico-chemical determinants for the attenuation of TG 2 activity: hydrophobicity > S as the group VIII atom > distance separating the N and group VIII atom. There is some overlap in this order as the presence of an S atom is influential even in the aromatic compounds as evidenced by significant differences ($p < 0.05$, unpaired t-test) between the K_i values of the aminothiophenol (0.16–0.63 mM) and those of the aminophenols (1.04–1.91 mM) (Table 1).

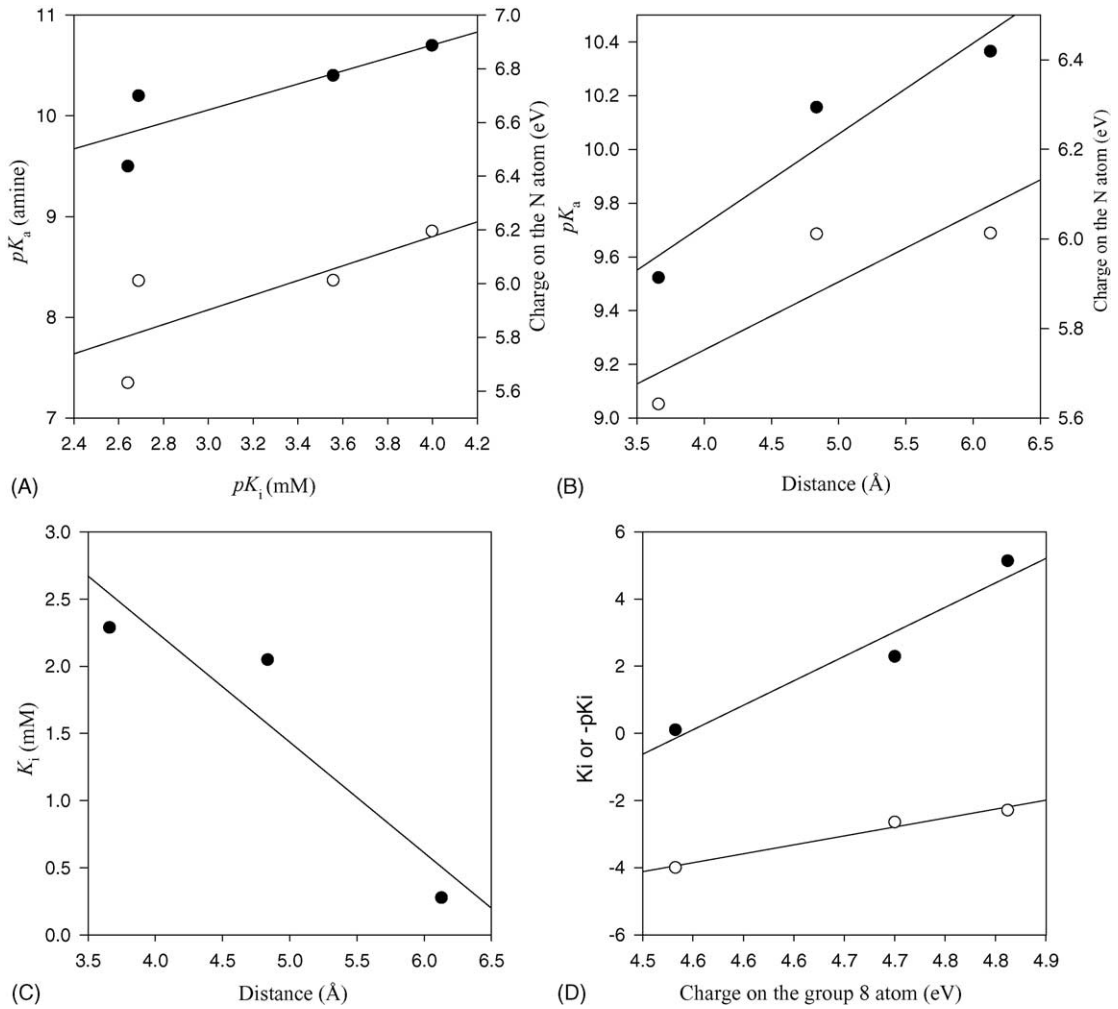

Fig. 4. Correlation of physico-chemical characteristics and pK_a of the functional groups with the K_i of the tested compounds. The correlations of pK_a (●, left axis, $r^2 = 0.71$) and charge on the N (○, right axis, $r^2 = 0.59$) for the amine moiety of ethanolamine, propanolamine, butanolamine and β-mercaptoethanolamine with the pK_i values of these compounds is shown in the Hammett plot (A). The correlations of pK_a (●, left axis, $r^2 = 0.91$) and charge on the N (○, right axis, $r^2 = 0.73$) with the distances separating the N and O in ethanolamine, propanolamine and butanolamine is shown in (B). The correlation of the K_i of ethanolamine, propanolamine and butanolamine with the distances separating the N and O of these compounds ($r^2 = 0.86$) is given in (C). Panel D depicts the correlations of the charge of the group VIII atoms of ethanolamine, β-mercaptoethanolamine and β-selenoethanolamine with the K_i (●, left axis, $r^2 = 0.93$) or pK_i (○, right axis, $r^2 = 0.98$) of these compounds.

5. Discussion

This study has demonstrated that MEA is an alternate amine-bearing substrate for TG 2 and acts as an apparent competitive inhibitor of this enzyme with a K_i of $\sim 10^{-4}$ M. Many of the cellular effects produced by 10^{-4} M concentrations of MEA can only be replicated in test tube assays by 10^{-2} M concentrations of MEA [36–38; J.R. Oliver, personal communication], a 100-fold difference, that suggests that this agent is actively concentrated within the cells. Thus, MEA may attain intracellular concentrations that approach the K_i value for the attenuation of TG 2 activity reported here (Table 1). Another possibility is that cystamine-derived MEA may attenuate TG activity in specific subcellular compartments. It is well established that MEA accumulates within lysosomes and these organelles contain TG activity [39–41]. Thus, MEA may preferentially affect lysosomal rather than cytosolic TGs.

Cystamine has been shown to decrease in situ TG activity in cultured cells and also the activity present in cellular extracts [1,2,4,5,42–49]. Central to our hypothesis is the notion that cystamine is reduced intracellularly to MEA, which has been experimentally validated [27–29]. Since all TGs share the same catalytic mechanism and accept polyamines or monoamines as amine-bearing substrates [50,51], it is also likely that the activity of all cerebral TGs, namely TG 1–3 [20], will also be attenuated by cystamine-derived MEA.

MEA is a remarkable compound that has a number of biological and chemical actions that clearly distinguish it from other closely related mercaptoalkylamines [7,36,37,52–61]. In an attempt to understand the unique

biochemical pharmacology of MEA, we and other investigators have compared the reactivity of this compound with its homologs in various settings to identify the physico-chemical properties that best account for its behavior [53,56,58–62]. For the most part, these studies have focused on the reactivity of the mercapto group as modulated by the α position amine. The studies presented here indicate the importance of both the amine and mercapto groups in the TG substrate activity of MEA. MEA could potentially react with TG via its mercapto or amino moieties. Under the reducing conditions of our assay, however, it is unlikely that the mercapto group of MEA undergoes a thiol-disulfide interchange reaction with the TG active site cysteinyl residue. It is well established that primary amines are TG substrates [30,31,33,63] and the studies presented here, indicate that the amine of MEA is used by TG to form the N^{β}-(γ-L-glutamyl)-MEA bond. Since all the TG assays require 10^{-3}–10^{-2} M concentrations of thiols, the previous reports of the competitive inhibition or alternate substrate behavior of cystamine, are in fact due to MEA and support the conclusions of the present study and our earlier report [24]. In addition to demonstrating that the amine of MEA can form a N^{β}-(γ-L-glutamyl)-MEA bond, these studies have indicated that the mercapto moiety has a significant influence on the substrate behavior of the amine. This influence is manifested via the electron-withdrawing capacity of the mercapto group and its effect on the pK_a of the amine which is a major determinant of its nucleophilicity and TG substrate behavior [64]. The electron-withdrawing capacity of the mercapto group of MEA is reflected both by the pK_a of this group and the calculated charge on the sulfur (Table 1). The charge on the sulfur of MEA correlates very well with the ability of this molecule to attenuate TG 2 activity. We speculate that the thiolate of MEA may associate with a cationic functional group in the TG active site to position the amine close to the thioester intermediate of the enzyme and favor the use of MEA as a substrate. The association of the thiolate with a cationic group would also have the advantage of neutralizing the charge on the sulfur, thereby increasing the nucleophilicity of the amine.

Although attenuating TG activity is therapeutic in murine models of neurodegeneration [1,2,15], the role of TGs in the etiology of this disease has not been fully elucidated. MEA is the drug of choice for the treatment of cystinosis [52]. Since mice tolerate greater amounts of cystamine than MEA [1,2], it may be possible to give humans beneficial doses of cystamine. The covalent attachment of MEA to Q-bearing TG substrates may account for the decreased levels of protein cross-linking observed following the treatment of animals and cells with cystamine [2,5] because the formation of N^{β}-(γ-L-glutamyl)-MEA bond would preclude the generation of the N^{ϵ}-(γ-L-glutamyl)-L-lysyl linkages. TGs also catalyze the formation of bis-γ-glutamylpolyamine linkages [65], the generation of ester linkages with ceramide [66], the deamination of Q residues [67,68] and the covalent attachment of polyamines to proteins [69–71]. The role of these TG reactions in neuronal death has, for the most part, not been investigated. Nevertheless, the formation of N^{β}-(γ-L-glutamyl)-MEA bonds after cystamine treatment may provide a unique and specific tool for identifying the Q-bearing TG substrates that contribute to neurodegeneration. The relevant proteins would be those that bind MEA following the treatment of cells or animals with cystamine. Identifying the proteins that bind MEA in a TG-dependent manner has the potential to significantly advance our understanding the role of TGs in neurodegeneration and the therapeutic actions of cystamine.

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