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Cystamine and cysteamine as inhibitors of transglutaminase activity in vivo

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Cystamine is commonly used as a transglutaminase inhibitor. This disulphide undergoes reduction in vivo to the aminothiol compound, cysteamine. Thus, the mechanism by which cystamine inhibits transglutaminase activity in vivo could be due to either cystamine or cysteamine, which depends on the local redox environment. Cystamine inactivates transglutaminases by promoting the oxidation of two vicinal cysteine residues on the enzyme to an allostERIC disulPhide, whereas cysteamine acts as a competitive inhibitor for transamination reactions catalyzed by this enzyme. The latter mechanism is likely to result in the formation of a unique biomarker, N-(γ-glutamyl)cysteamine that could serve to indicate how cyst(e)amine acts to inhibit transglutaminases inside cells and the body.

Introduction

Cystamine is a symmetric organodisulphide commonly used as an inhibitor of transglutaminases. This disulphide is also reduced to cysteamine within the body. Cystamine and cysteamine both inhibit transglutaminases but by different mechanisms. Therefore, the purpose of this discussion is to highlight the redox behavior of cystamine and cysteamine in vivo and the mechanisms by which cystamine and cysteamine inhibit the activity of transglutaminases inside the body.

Transglutaminases and the formation of cross-linked proteins in disease

Transglutaminases catalyze nucleophilic substitutions of the carboxamide group of glutaminyl residues [1,2]. The attacking nucleophiles are typically the amines of various compounds, but can include hydroxyl moieties and H2O depending on the transglutaminase isozyme or conditions. Thus, subject to the nucleophile, transglutaminases catalyze transamination, esterification, or deamidation of glutaminyl residues. Transamination involving the ε amine of lysyl residues is the reaction most often catalyzed by transglutaminases and results in the formation of Nε-(γ-glutamyl)lysine isopeptide linkages between polypeptide chains (Figure 1A). A number of important pathologies exhibit both aberrant transglutaminase activity and increased production of Nε-(γ-glutamyl)lysine cross-linked proteins (e.g., neurodegenerative disorders [3-12] and cardiovascular disease [13-20]). The involvement of increased transglutaminase activity in neurodegenerative or cardiovascular diseases is supported by the observation that genetic inactivation of various transglutaminases in animal models slows progression of these diseases [21-26]. The preceding observation and others prompted a search for medicinal transglutaminase inhibitors [27-29], as well as testing of cystamine in disease models and patients [30-45]. These tests indicate that cystamine might be of benefit in the treatment of selected diseases.

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Figure 1. Reactions of cyst(e)amine with transglutaminases and cystine

(A) Transglutaminase-catalyzed \(N^\epsilon(\gamma\text{-glutamyl})\)lysine isodipeptide formation: transglutaminases catalyze an acyl transfer reaction that proceeds by a Bi-Molecular or Ping-Pong mechanism. Activated transglutaminases first act to form a thioester bond between the active site Cys\(277\) and the carboxamide moiety of glutaminyl residues. Formation of this intermediate involves the release of the amide nitrogen as ammonia, which powers the subsequent catalysis. The thioester bond then undergoes a nucleophilic attack by the \(\epsilon\) amine of lysine to complete the acyl transfer and produce \(N^\epsilon(\gamma\text{-glutamyl})\)lysine isodipeptide. These dipeptides can then be released from the protein by hydrolysis of the peptide linkages. (B) Oxidative inactivation of transglutaminase 2 by cystamine by the mechanism of Lorand and Conrad [46]: in this model, the thiol moiety of Cys\(277\) participates in thiol-disulphide interchange with cystamine to produce cysteamine–Cys\(277\) mixed disulphide. (C) Oxidative inactivation of transglutaminase 2 by cysteamine by our interpretation of the mechanism of Palanski and Khosla [48]: in this model, cystamine first forms mixed disulphides with Cys\(370\) and Cys\(371\). Cys\(370\) then undergoes thiol-disulphide interchange with cysteamine–Cys\(370\) mixed disulphide. The newly reduced Cys\(371\) then reduces the mixed disulphide of cysteamine–Cys\(370\) while being oxidized to the Cys\(370\)–Cys\(371\) disulphide. It is also possible that the Cys\(370\) undergoes thiol-disulphide interchange with the cysteamine–Cys\(370\) mixed disulphide rather than the cysteamine–Cys\(371\) disulphide. In either case, the Cys\(370\)–Cys\(371\) disulphide would form and allosterically regulate the enzyme. (D) Thiol-disulphide interchange of cysteamine and cystine: cysteamine interacts with cystine by thiol-disulphide interchange. Note that the latter resembles the lysyl residue depicted in (A). (E) Transglutaminase-catalyzed \(N^\epsilon(\gamma\text{-glutamyl})\)cysteamine formation: a mechanism for the competitive inhibition of transglutaminase by cysteamine. This mechanism is analogous to that shown in (A) and for the sake of brevity begins with thioester bound intermediate. The thio–ester bond is attacked by the amine nitrogen of cysteamine to complete the acyl transfer and produce \(N^\epsilon(\gamma\text{-glutamyl})\)cysteamine. We propose that \(N^\epsilon(\gamma\text{-glutamyl})\)cysteamine is released from the protein by proteolysis, as is the case for other \(N^\epsilon(\gamma\text{-glutamyl})\)amines.
Oxidative mechanisms for the inhibition of transglutaminases by cystamine

Cystamine was first reported to be an inhibitor of transglutaminase 2 by Lorand and Conrad in 1984 [46]. They hypothesized that cystamine and the active site Cys<sup>277</sup> undergo a thiol-disulphide interchange to produce a mixed disulphide that prevents catalysis (Figure 1B). Cystamine is the disulphide form of cysteamine. Thus, the proposed thiol-disulphide interchange (thiolation) produces free cysteamine and a mixed disulphide of cysteamine and Cys<sup>277</sup>. An ‘oxidative mechanism’ for the inhibition of transglutaminase 2 is supported by subsequent investigations by Jeon <i>et al.</i> [47], and also by Palanski and Khosla [48]. The latter researchers, however, proposed a modified hypothesis, which states that cystamine forms mixed disulphides with a triad of cysteinyl residues on the surface of transglutaminase 2 that regulates the activation of the extracellular pool of this enzyme. According to Palanski and Khosla [48], cystamine reacts with Cys<sup>336</sup>, Cys<sup>370</sup>, or Cys<sup>371</sup> to promote the eventual formation of an allosteric disulphide bond between Cys<sup>370</sup> and Cys<sup>371</sup> as shown in Figure 1C. These mechanisms, however, presume that cystamine is not metabolized <i>en route</i> to the targetted transglutaminases; a presumption that is not supported by pharmacokinetic studies.

Conversion of cystamine into cysteamine within the body

Cystamine is rapidly reduced to cysteamine by serum, as well as by the liver and kidneys [49]. By contrast, cystamine is relatively stable in plasma and rapidly absorbed from blood into tissues [49-53]. Prior to cellular uptake, cysteamine undergoes thiol-disulphide interchange with extracellular cysteine to form cysteamine–cysteine mixed disulphide (Figure 1D), which resembles lysine [54,55]. Consequently, the cysteamine–cysteine mixed disulphide enters cells through amino acid transporters and is then reduced to cysteamine and cysteine. Thus, the major form in which cysteamine inhibits intracellular transglutaminases is cysteamine and not cystamine.

Cysteamine as an inhibitor of intracellular transglutaminases

In earlier studies, we demonstrated that cysteamine acts as a substrate for transglutaminase 2 to link this compound to glutamyl residues by way of an isopeptide linkage forming N-(γ-glutamyl)cysteamine (Figure 1E) [56]. In other words, cysteamine by virtue of being a transglutaminase 2 substrate, acts as a competitive inhibitor of the other amine substrates of this enzyme. Cysteamine has not been shown to be an amine substrate of transglutaminase 2, an assertion erroneously attributed to us elsewhere [48]. Formation of N-(γ-glutamyl)cysteamine by transglutaminases could account for two puzzling observations pertaining to the metabolism of exogenously supplied cysteamine. The first of these observations is that a significant portion of the administered cysteamine is unaccounted for following analysis of established routes of metabolism. Cysteamine generated endogenously by the catabolism of pantetheine is oxidized to hypotaurine and then taurine [57]. The administration of cysteamine to rodents, however, does not result in significant accumulation of hypotaurine or taurine in brain or plasma [49], and indicates that the metabolism of exogenous cysteamine bypasses oxidation to taurine. A small portion of cysteamine administered <i>per os</i> is metabolized to thialysine and then S-(2-aminooethyl)-cysteine ketimine decarboxylated dimer [50]. Based on the levels of cysteine that accompany cysteamine into cells as a mixed disulphide, significant quantities of cysteamine must enter cells [38,49-51,54,55] but it is then rapidly metabolized. The cellular fate of the majority of exogenous cysteamine remains unaccounted for.

A role for transglutaminases in the metabolism of cysteamine

A novel hypothesis for the metabolism of cysteamine is that it is covalently attached to proteins by intracellular transglutaminases. This hypothesis is supported by the observation that a significant portion of radiolabeled cysteamine administered to animals or cells is covalently bound to proteins, but not by disulphide bonds [58,59]. This hypothesis requires that the intracellular transglutaminases be activated while cysteamine is being absorbed by cells. Transglutaminases are activated by calcium. Exogenous cysteamine may stimulate calcium release from intracellular stores and thereby promote transglutaminase activity. This mechanism depends on the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), by micromolar amounts of cysteamine. At these concentrations, thiols (RSH) such as cysteamine reduce transition metals (M<sup>n</sup> → M<sup>n-1</sup>, where n is the oxidation number), while being oxidized to the corresponding disulphide (RSSR):

\[
2RSH + 2M^n \rightleftharpoons RSSR + 2M^{n-1} + 2H^+ \]
The reduced metals, in turn, reduce oxygen ($O_2$) to superoxide ($O_2^-$):

$$M^{n-1} + O_2 \rightleftharpoons M^n + O_2^-$$

Dismutation of superoxide yields hydrogen peroxide ($H_2O_2$), which is a mild oxidant at physiological pH values (7.2–7.4):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Thiols, such as cysteamine, react slowly with hydrogen peroxide under these conditions [60]. Effective scavenging of the peroxide by thiols does not occur until the thiols are present at millimolar concentrations [54]. Thus, at the micromolar concentrations that it attains outside of cells, cysteamine promotes hydrogen peroxide production by the reactions shown above [54]. Hydrogen peroxide readily enters cells and causes a peroxidative stress that is exacerbated by the inhibition of cellular glutathione peroxidases by cysteamine [54].

Hydrogen peroxide promotes the release of calcium from intracellular stores [61,62] and should therefore stimulate transglutaminase activity. In support of this notion, the addition of hydrogen peroxide to cells in culture stimulates their in situ transglutaminase activity [63,64].

The above conjecture could be readily tested by investigating the plasma of cysteamine-treated animals or medium of cells in culture treated with cysteamine for the presence of free N-(γ-glutamyl)cysteamine. Isopeptide linkages are resistant to proteolysis and consequently transglutaminase-made N-(γ-glutamyl)amines are excised as free N-(γ-glutamyl)amines during proteolysis of proteins bearing these species [65]. Free N-(γ-glutamyl)amines are present in various body fluids and reflect the levels of active transglutaminases in tissues [3,66,67]. If our hypothesis is correct, then the simultaneous measurements of taurine, S-(2-aminoethyl)-cysteine ketimine decarboxylated dimer, as well as protein-bound and free N-(γ-glutamyl)cysteamine should provide a comprehensive accounting of the metabolism of exogenous cyst(e)amine, in addition to indicating the mechanism by which cysteamine inhibits intracellular transglutaminases.

### Sites for the oxidative inactivation of transglutaminases by cystamine

Transglutaminases are fully activated by the binding of three calcium ions per enzyme and reducing conditions that maintain the active site cysteine in a fully reduced state [1,2,47]. The cytosol is highly reducing and therefore the activation of intracellular transglutaminases is regulated by the availability of cytosolic calcium. The extracellular environment is different; calcium is readily available whereas reductants are not. Khosla et al. discovered that the activity of extracellular transglutaminase 2 is regulated by the redox status of two vicinal cysteinyl residues on the surface of this enzyme [68]. Under the oxidizing conditions of interstitial fluids [69], these residues: Cys$^{370}$ or Cys$^{371}$ form a disulphide in a manner that involves a third cysteine residue, Cys$^{236}$, and ERp57 [70]. Reduction in the Cys$^{370}$–Cys$^{371}$ disulfide linkage by thioredoxin activates extracellular transglutaminase 2 [71,72]. The activation of extracellular transglutaminase by this mechanism is blocked by cystamine forming mixed disulfides with Cys$^{370}$ and Cys$^{371}$ (Figure 1C). As noted earlier, cystamine is converted into cysteamine in the body [49]. It is possible that a portion of the plasma-derived cysteamine is oxidized to cystamine within the interstitial spaces and in this form inactivates extracellular transglutaminases. The amount of cystamine available to inhibit the extracellular transglutaminases by this mechanism will depend on the amounts of cysteamine and cysteamine–cysteine mixed disulphide; the amounts of the latter are expected to be significant after the administration of cyst(e)amine. It should be noted that cysteamine–cysteine mixed disulphide could also inhibit transglutaminase in an oxidative manner, as shown in Figure 1C with cysteamine–cysteine mixed disulphide replacing cystamine. The lumens of the gut is also likely to be an oxidizing environment because the administration of cysteamine by gavage results in the appearance of cysteine in the plasma [50]; the most likely site for oxidation of cysteamine to cystamine, in this case, is the gut. Thus, cystamine is most likely to inhibit transglutaminases by an oxidative mechanism in the gut. This observation is important since aberrant transglutaminase activities contribute to etiology of several intestinal diseases, in particular, celiac disease. In this disease, transglutaminases act to deamidate glutaminyl residues in the wheat protein gliadin increasing the autoantigenicity of the modified protein in the context of HLA-DQ2 or HLA-DQ8 [73]. Cystamine inhibits the generation of the relevant epitopes in vitro, but only at millimolar concentrations [74]. Given the relatively safe use of cysteamine in humans [75-77] and the potential to assess the mechanism by which this compound inhibits transglutaminases (i.e., by measurement of N-(γ-glutamyl)cysteamine), cysteamine may be of use in the treatment of celiac disease and other diseases involving transglutaminases.
Conclusion
The activities of intracellular and extracellular transglutaminases contribute to a number of important pathologies. Agents that safely inhibit the in situ activities of these transglutaminase pools are therefore of interest as possible therapeutics. The evidence presented here indicates that cystamine inhibits extracellular transglutaminases, while its reduced congener – cysteamine – inhibits intracellular transglutaminases. This distinction is important for the design of other transglutaminase inhibitors based on the mechanisms by which cysteamine or cystamine inhibit these enzymes (e.g., disulphiram [48]). It may also guide the form in which cystamine is administered: as either cystamine or cysteamine. Finally, the measurement of N-(γ-glutamyl)cysteamine) may provide a means of determining the mechanism by which intracellular transglutaminases are inhibited following the administration of cysteamine or cystamine.

Competing interests
The authors declare that there are no competing interests associated with the manuscript.

References


