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Research Article

HOMOCYSTEINE THIOLACTONE FORMS COVALENT ADDUCT WITH ARGININE AND HISTIDINE

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A B S T R A C T

Available evidences suggest that homocysteine thiolactone is solely responsible for the protein N-homocysteinylation (a process in which HTL covalently modifies the targeted protein) which is responsible for the inactivation, aggregation and precipitation of proteins on target. Previous data of MS and MS/MS has already suggested the fact that HTL reacts with the side-chain ε-amino group of lysine residues. Spectroscopic and chromatographic analyses revealed that side-chain amino group of arginine and histidine could also prove to be potential targets for Hcy-thiolactone.

INTRODUCTION

Homocysteine, biosynthesized from methionine, is a non-protein sulphur containing amino acid that is involved in methionine metabolism pathway [1]. Methionine is considered to be the sole source of homocysteine in the human body. Inside the cells, methionine is converted to S-adenosylmethionine (SAM), a methyl group donor. Donating a methyl group to an acceptor, S-adenosylmethionine is converted to S-adenosylhomocysteine (AdoHcy), which is later on hydrolyzed to adenosine and Hcy. Under circumstances when there is an excess of methionine concentration, trans-sulfuration comes into play where cystathione β-synthase (CBS) converts Hcy to cystathione, which further gets converted to cysteine via cystathione γ-lyase. Normal levels of Hcy in the human are 5-10 μM in healthy individuals. However, the elevated cellular and plasma Hcy levels may range from 15-20 μM (mild forms) up to 500 μM (severe forms, a case of hyperhomocysteinemia) [2, 3]. A high level of Hcy level is related to many clinical manifestations including dislocation of eye lens, neurodegenerative diseases, cardiovascular diseases, autoimmune diseases, neural tube defects and even cancer [3-10].

The exact mechanism of cellular toxicity due to excess Hcy is still unclear; however, pioneering work by H. Jakubowski has attempted to explain its toxicity by proposing the 'Hcy-thiolactone hypothesis'[11]. This hypothesis states that Hcy inside the cells is metabolized to its cyclic thioester Hcy-thiolactone (HTL) [12-15]. This reaction is catalyzed by methionyl-tRNA synthetase in an error editing process during protein translation when Hcy, instead of methionine, gets incorporated mistakenly. HTL, which is considered to be the real damaging agent, is a toxic ester in a way that it forms amide bonds with the ε-amino groups of protein lysine residues, a process known as N-homocysteinylation. Various studies confirm that Hcy and its thiolactone can modify nearly all plasma proteins depending on the concentration and lysine content of the protein. Earlier Cornelis E. C. A. Hop *et al.* with the help of MS and MS/MS data has already suggested that HTL essentially reacts with the side-chain amino acid group of lysine residues as well as amino group at the N-terminal and carboxy group at the C-terminal of peptide [16]. In the present study, we were interested to investigate whether homocysteine thiolactone have the potential to form covalent adducts with side chains of arginine as well as histidine. Carrying out Ellman's assay and HPLC experiments, it was fascinating to find that HTL could form adduct with the side chains of arginine and histidine amino acids.

MATERIALS AND METHODS

Commercially lyophilized powder of arginine and histidine was purchased from Sigma Chemical Co. DL-Homocysteine thiolactone hydrochloride, was also obtained from Sigma

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Chemical Co. Potassium chloride and potassium phosphate were purchased from Merck. Double distilled water was used as the aqueous phase. All experiments were carried out in 0.05M potassium phosphate buffer (pH 7.4) containing 0.1M KCl at 37°C.

A solution containing 1 mM Hcy-thiolactone and amino acid (Arg, His) at a concentration of 10 mg/ml in 0.05 M phosphate buffer of pH 7.4 was incubated at 37°C.

Sulfydryl estimation using Ellman reagent

Amino acid (His and Arg) treated with HTL were first prepared in phosphate buffer, pH 7.4. Then using 5, 5′-Dithiobis (2-nitrobenzoic acid), the Ellman's reagent, the amount of thiol groups in control and homocysteinylated protein samples were assayed. Absorbance of the samples was measured at 412 nm, using 1cm path-length cuvette. Measurements were made using a Perkin Elmer Lambda 25 UV/Vis spectrometer. The amount of 5′-nitrothiobenzoate released was estimated from ε, the molar extinction coefficient of 13,700 M^{-1} cm^{-1} [17-20].

High Performance Liquid Chromatography (HPLC) experiment

Measurements were made using LC-6AD Shimadzu Liquid Chromatograph. Arg and His of each 10 mM concentration were incubated with 10 mM HTL (1:1 ratio) in 0.05 M phosphate buffer. Mixture of analytes was then passed through silica column. Column oven temperature was maintained at 37°C in all experiments. Phosphate buffer of 0.05 M (pH 7.4) was used as mobile phase keeping flow rate at 1 ml/min and pressure maintained at 104 ± 5 kgf/cm^2.

RESULTS AND DISCUSSION

To investigate whether homocysteine thiolactone has the potential to form amide bond with arginine and histidine, the two amino acids were first modified by HTL (1 mM). The concentration of Hcy-thiolactone was chosen keeping in mind the pathological conditions of hyperhomocysteinemia in the human body. Each amino acid was treated with HTL and incubated overnight at pH 7.4 and constant temperature of 37°C. Then, both amino acid sets were analyzed for the free –SH groups using Ellman's reagent. **Fig 1** and **2** depicts gradual increase in thiol contents with increasing incubation time. Hcy-thiolactone forms an amide linkage with the side-chain amino group therefore adding up an –SH group, which is finally assessed by the Ellman's reagent. Each covalent adduct formation between HTL and side-chain amino acid corresponds to addition of a single thiol group, therefore, rise in free –SH content upon treatment with HTL confirms covalent adduct formation.

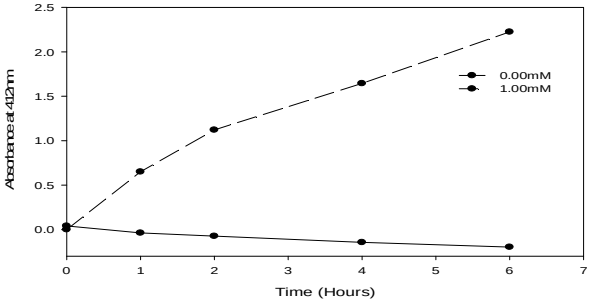

Figure 1 Sulfydryl content measurement of HTL-treated Arginine using Ellman reagent.

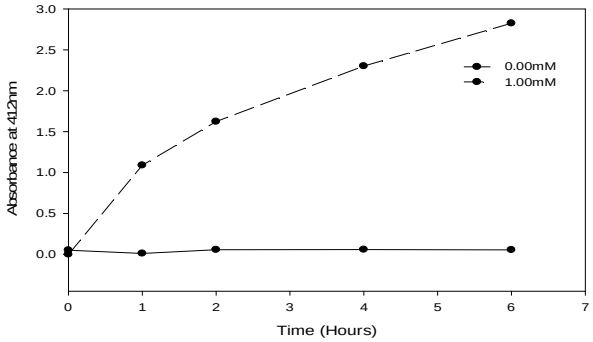

Figure 2 Sulfydryl content measurement of HTL-treated Histidine using Ellman reagent

We further confirmed our results using reverse-phase HPLC. In reverse-phase HPLC technique separation occurs on the basis of hydrophobicity [21]. The elution of the solute molecules depends on their interaction with hydrophobic stationary phase and hydrophilic mobile phase [22]. Therefore, molecule with the highest hydrophobicity takes the maximum time for elution. Reverse-phase HPLC proves to be a very reliable technique for analysis of amino acids, peptides/proteins since very closely related molecules can be assessed with very fine resolution [23, 24]. **Fig 3** and **Fig 4** shows the chromatogram of HTL-modified Arginine and histidine respectively.

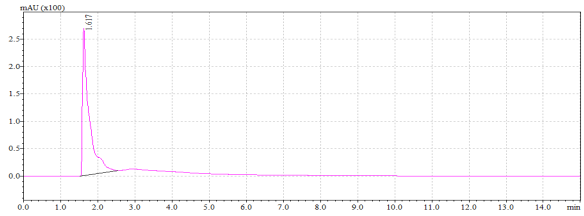

Figure 3 a RP-HPLC of unmodified Arginine

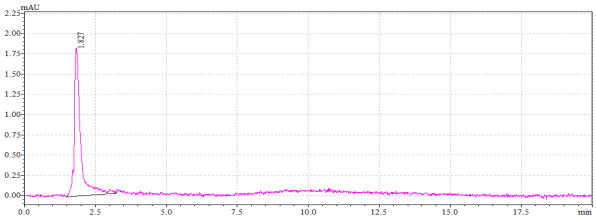

Figure 3 b RP-HPLC of Hcy-thiolactone

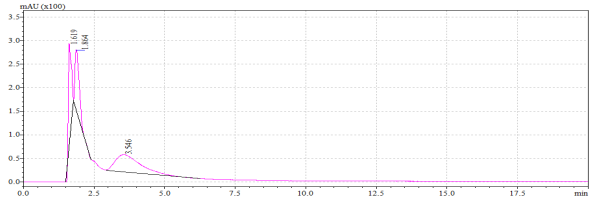

Figure 3c Separation of HTL-modified Arginine using RP-HPLC

Figure 3

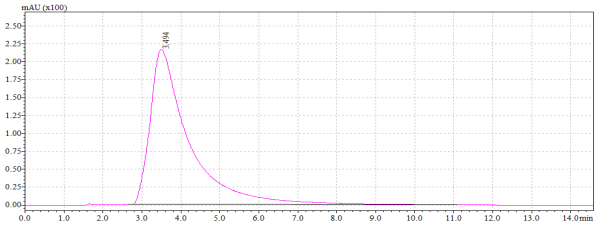

Figure 4a RP-HPLC of unmodified Histidine

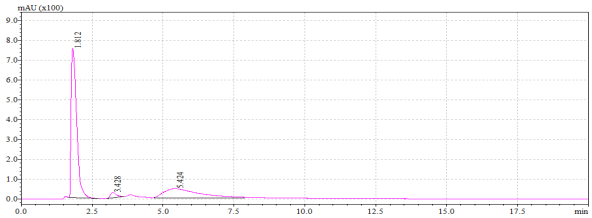

Figure 4 b Separation of HTL-modified Histidine using RP-HPLC

Figure 4

Arginine is a conditionally essential polar and strongly basic amino acid with hydropathy index of -4.5. Similarly, histidine is also a polar, weakly basic amino acid with hydropathy index of -3.2. Therefore, comparing the HPLC peaks of native amino acids with their respective HTL-modified forms, it can be concluded that HTL could form efficient amide bonds even with side-chain amino group of Arginine and histidine, in addition to that with lysine.

Taken together, our results led us to believe that in addition to lysine residues, arginine and histidine residues may also form adduct with HTL, indicating that other than lysine, arginine and histidine residues may also take part in bringing about conformational changes in native protein due to adduct formation by HTL. Results further implies that proteins that have exposed histidine and arginine residues on their surfaces, in addition to lysine residues, have clear chances of undergoing modification by Hcy-thiolactone.

Moreover, arginine and histidine are known to be present in free form in cellular environment. Therefore, increasing free histidine and arginine in cellular environment will be a strategy to lower Hcy-induced cytotoxicity by making covalent complex with HTL and preventing protein modification.

CONCLUSION

Previous works reported that Hcy-thiolactone reacts with side-chain amino group of lysine residues and generates covalent adducts. Now, our spectroscopic analysis and HPLC experiments clearly shows that HTL also has the potential to form covalent adducts with the side-chain amino group of arginine and histidine as well. This study could be helpful in reducing the detrimental effect of elevated Hcy and its thiolactone levels. Further, increasing free arginine and histidine amino acids could scavenge the excess of Hcy/HTL in cellular environment which could be a beneficial strategy to buffer the undesirable consequences of elevated Hcy/HTL by preventing them to react with the cellular proteins. Therefore, this could prove to be a promising therapeutic approach in reducing the deadly hyperhomocysteinemic/homocystinuric conditions.

Conflict of interest

The authors have declared that no competing interests exist.

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