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HTL affects the chaperone activity of IDPs: A study based on α-casein and β-casein

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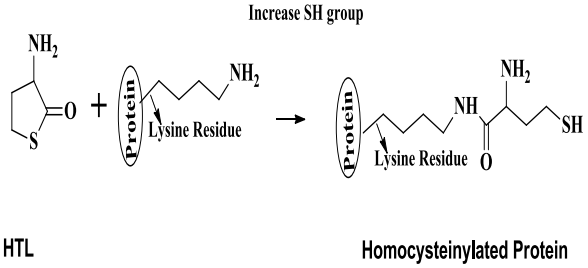
Abstract- *Several factors responsible for the Hcy-associated toxicity have been proposed and well documented, including oxidative stress, lipid peroxidation, neuronal degeneration and apoptosis. Post-translational modification of proteins by the Hcy metabolite, homocysteine-thiolactone (HTL), is one among the several proposed mechanisms of Hcy-induced toxicity. Incubation of proteins with HTL has been shown to form covalent adducts with protein lysine residues which ultimately results in structural and functional alterations. There has been an innumerable work done in understanding the role of N-homocysteinylation in globular proteins. However, in order to understand the effect of homocysteinylation in intrinsically disordered/unstructured proteins, alpha- and beta-casein proteins were used as model. Spectroscopic investigation revealed that HTL induces loss of chaperone activity. The study provides insights for the possible role of disorderness of IDPs for maintaining their biological activity.*

Keywords: - homocysteine, homocysteine-thiolactone (HTL), N-homocysteinylation, chaperone activity, IDPs (Intrinsically disordered proteins).

1. INTRODUCTION

Homocysteine (Hcy) is a sulphur containing toxic non- protein amino acid that is involved in methionine metabolism pathway. This compound is biosynthesized from methionine by removal of its terminal methyl group [1]. A high level of homocysteine in the blood is associated with many clinical manifestations such as mental retardation, dislocation of eye lens, etc. and is known to result in homocystinuria (or hyperhomocysteinemia) [1-3]. 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 hyperhomo-

cysteinemia) [4].



Scheme-1

The cellular toxicity of Hcy is not yet well understood. However, one of the proposed mechanisms of homocysteine toxicity in humans is the modification of proteins by the metabolite of Hcy, homocysteine thiolactone (HTL), a reactive thioester which is formed by methionyl-tRNA synthetase in an error editing reaction[1,5]. HTL, when incubated with proteins, preferentially forms amide bonds with the ε-amino group of protein lysine residues in a non-enzymatic reaction; a process referred to as "protein N-homocysteinylation" [1, 6] (see Scheme-1). Various studies have now shown that modification of protein via N-homocysteinylation results in inactivation of enzymes, aggregation of proteins, amyloid transformation and even protein precipitation [5-9]. Till date, there have been numerous efforts in understanding the reactivities of HTL towards various globular proteins from different sources; however, not much work has been done in understanding the effect of HTL on intrinsically disordered proteins (IDPs). Intrinsically disordered proteins are the ones that lack fixed stable tertiary and/or secondary structures under physiological conditions *in vitro* [10-12]. In the present study, we have investigated the effect of HTL on the chaperone activity of α and β-casein, a milk protein that falls under the category of intrinsically disordered proteins. Interestingly, it was found that initial incubation with HTL did not have any significant alterations in the protein's chaperone activity and α and β-casein was able to provide specific protection against aggregation of catalase, whereas, with increased incubation, HTL tends to induce loss of protein's chaperone function.

2. Materials and Methods

Commercially lyophilized powder of alpha (α-CN) and beta (β-CN) casein (from bovine milk) were purchased from Sigma Chemical Co. DL-Homocysteine thiolactone hydrochloride, 5, 5′ - Dithiobis (2-nitrobenzoic acid) was also obtained from Sigma Chemical Co. Potassium chloride and potassium phosphate were purchased from Merck. Double distilled water was used as the aqueous phase.

α-CN and β-CN solutions were dialyzed against 0.1M KCL at pH 7.0 in cold (~ 4°C). Protein stock was filtered using 0.22µm Millipore syringe filters. Concentration of the protein samples were determined experimentally using ε, the molar extinction coefficient values of 15000 M^{-1} cm^{-1} for α-CN and 11000 M^{-1} cm^{-1} for β-CN at 280 nm. All solutions for optical measurements were prepared in the appropriate degassed buffer. All experiments were carried out in 0.05 M potassium phosphate buffer (pH 7.4) containing 0.1M KCl at 37°C.

2.1 Preparation of N-Hcy α-CN and β-CN

Proteins (0.2mg/ml) were incubated with HTL of different concentrations (0-1000µM) in 0.05 M potassium phosphate buffer, pH 7.4 overnight at 37°C.

2.2 Sulfydryl estimation using Ellman's reagent

Proteins treated with HTL were first precipitated down with 10% TCA. This removes the unbounded HTL. Then the proteins pellets were collected and resolubilized in phosphate buffer, pH 7.0. 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 a 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}.

2.3 Chaperone Activity

For analyzing the effect of N-homocysteinylation on chaperone activity of α-CN and β-CN (0.5mg/ml), activity was measured against heat stressed amorphously aggregating catalase (0.3mg/ml) at 55°C for 20min. The fluorescence emission and excitation was kept at 360 nm keeping the slit width at 5 nm. Measurements were made using Cary Eclipse fluorescence spectrophotometer.

2.4 Light Scattering Measurements/Time-Dependent Aggregation

The effect of N-homocysteinylation (1.00mM) on α-CN and β-CN at different time intervals was measured using light scattering as a probe. We observed that homocysteinylation induced time dependent aggregation in both α-CN and β-CN.

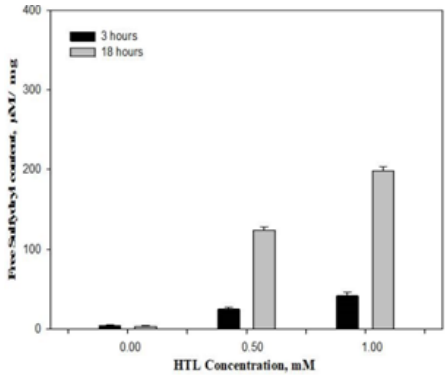


Fig 1: Ellman's assay of α-Casein at 3 hours and 18 hours.

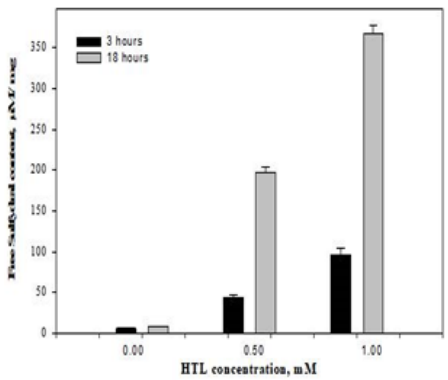


Fig 1: Ellman's assay of β-Casein at 3 hours and 18 hours.

Homocysteinylated α-CN and β-CN in the presence of catalase (approximately 0.3mg/ml) was placed into 10mm path-length quartz cuvette and the aggregation process was followed by monitoring the change in light scattering at 400 nm at 55°C using a V-660 UV/Vis Spectrophotometer equipped with a Peltier-type temperature controller.

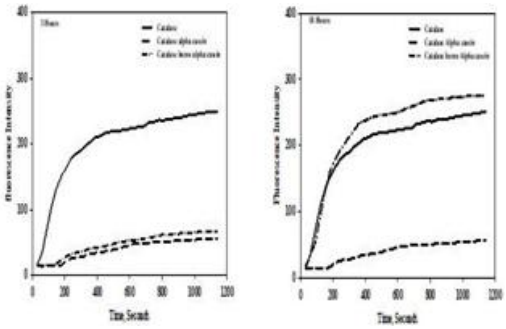


Fig 3: Effect of N-homocysteinylation on activity of α-Casein

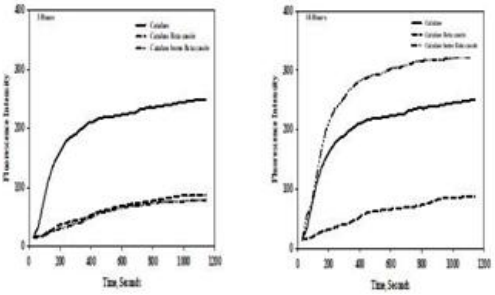


Fig 4: Effect of N-homocysteinylation on activity of β-Casein

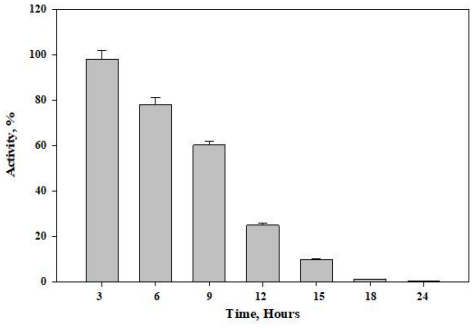


Fig 5: Time dependent Chaperone activity of α-Casein. Percent activity of protein modified with 1.00mM HTL as a function of incubation period.

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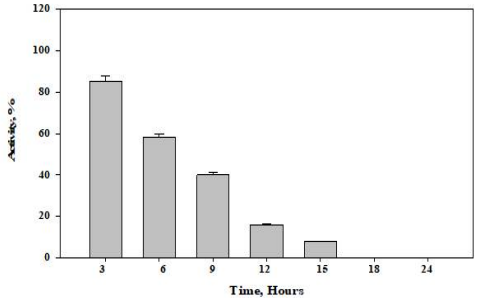

Fig 6: Time dependent Chaperone activity of β-Casein. Percent activity of protein modified with 1.00mM HTL as a function of incubation period.

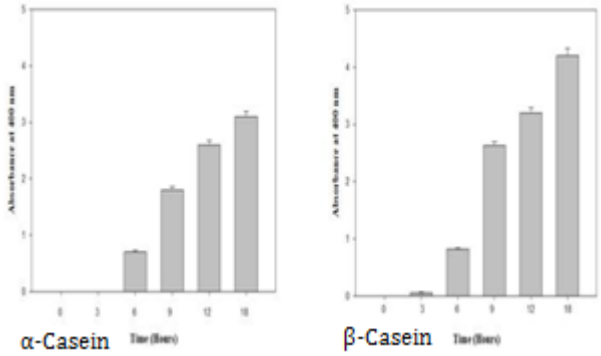

Fig 7: Light scattering of α-Casein and β-Casein proteins was measured by observing the progress change in optical density at 400nm at pH 7.4.

4. Results

To explore the effects of N-homocysteinylation on the chaperone function of the milk protein (α- and β-casein), the two proteins were first modified by HTL. Each protein was treated with HTL and incubated for 3 hours as well as 18 hours at pH 7.4 and constant temperature of 37°C. Then, both α and β casein were analyzed for the free –SH groups using Ellman's reagent. **Fig 1 and Fig 2** depicts gradual increase in thiol contents with increasing incubation time of α- and β-casein, respectively with HTL. **Fig 3** and **Fig 4,** respectively, show the effect of N-homocysteinylation on the molecular chaperone function of α- and β-casein (5mg/ml). It was observed that after 3 hours of initial HTL treatment chaperone activity of both the proteins did not alter. However, after 18 hours of HTL treatment chaperone function of both α- and β-casein was lost.

Fig 5 and Fig 6 show a time-dependent study of the chaperone activity of HTL-treated α-CN and β-CN, respectively. It was observed that till 3 hours α-CN has an activity of nearly 100% and β-CN has an activity of approximately 85% which gradually decreased with increase in the incubation time. To ascertain the true theory behind this loss of activity, we checked the aggregation propensity of HTL-adducted alpha and beta proteins by measuring light scattering intensities with time at 400 nm **Fig 7**. Interestingly, it was found that beyond 3 hours the proteins seem to form aggregate. Taken together, our results on functional analyses of α-CN and β-CN has led us to believe that HTL does not abruptly alter the protein, instead takes a longer period of incubation for complete loss of chaperone activity.

5. Discussion

Functional alteration upon N-homocysteinylation was assessed using different spectroscopic techniques. The concentrations of HTL used in our experiments nearly reflect the pathological conditions of hyperhomocysteinemia. Increase in the free –SH in the modified protein is a result of the covalent adducts formation between HTL and protein lysine residues. Since, **Fig 1 and Fig 2** shows increase in the free –SH content, confirming that the proteins have been well incorporated with HTL. Initial incubation of α-CN and β-CN with HTL for 3 hours did not interfere with the chaperone activity of the protein and both α- and β-casein successfully protects catalase enzyme from forming aggregate. However, on incubating for an extended period of time (18 hours), both α- and β-casein lose chaperone activity, suggesting formation of covalent adducts between protein lysine residues and HTL.

Caseins (α, β and κ) are the major components of milk protein and are secreted as large colloidal aggregates called micelles [13, 14]. They have less ordered secondary and tertiary structures and disorderness is necessary for their biological activity [15]. α-CN and β-CN, ATP-independent molecular chaperones are able to suppress the thermal and chemical aggregation of substrate proteins such as insulin, lysozyme, alcohol dehydrogenase, and catalase by forming stable complexes with the denaturing substrate proteins [16]. In addition, β-casein is known to exhibit a significantly higher chaperone activity than α-casein [17]. In a study carried out by Stroylova et al. [18], it was shown that N-homocysteinylation of casein leads to aggregation in all cases.

Our study is an extension of this previous study, focusing on molecular chaperone activity upon homocysteinylation. During initial incubation time of 3 hours, although there seems to be a little formation of covalent adduct, chaperone activity of both proteins remains unaltered, suggesting the fact that 3 hours of time is not enough for significant interaction of HTL with protein, and also those covalent adducts that are formed do not disturb the disorderness of proteins. This proves that disorderness of IDPs plays a crucial role for retaining biological function of the protein. On the other hand, after 18 hours of incubation with HTL, both α- and β-CN proteins have undergone certain alterations in the native fold, therefore, inducing the functional loss by additional disulfide bonds generated by the introduced HTL residues. Current study shows that N-homocysteinylation of casein (or any IDPs for that matter) induces altered biological function. This may be due to significant changes in physical and chemical properties connected with aggregate or amyloid-like formation which somehow direct the protein from losing its chaperone activity. Thus, our study clearly indicates that covalent modification of alpha- and beta-casein proteins by homocysteine-thiolactone has led to covalent adducts in a time-dependent manner, ultimately resulting in complete loss of chaperone activity.

6. Conclusion

Intrinsically disordered proteins are involved in regulatory functions because disordered segments permit interaction with several proteins. Casein (α and β), a class of IDP family protein has a stronger ability to reduce aggregation of substrate proteins (e.g. catalase) hence showing a strong molecular chaperone activity. Current study on casein as the model protein concludes that even when the covalent adducts is formed during initial incubation with HTL; they do not disturb disorderness of the IDPs. Further, unlike the globular proteins, the lysine residues in alpha- and beta-casein (or any other IDPs) might not be crucial for the biological chaperone activity. Also, the process of loss of chaperone activity is not abrupt but gradual and needs a longer period of incubation. Nevertheless, the study indicates that HTL might affect the function of IDPs.

7. Acknowledgement

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