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Review

Role of heat-shock proteins and cobalamine in maintaining methionine synthase activity

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Atheromatous plaque is one of the most common cardiovascular-related diseases. Reports show a connection between its development and the levels of homocysteine. In pathological states high levels of homocysteine in the organism can be caused by the malfunction of the methionine synthase pathway. Bacterial methionine synthase (MetH) is a homologue of the human methionine syntase (MS). In this study we aimed to investigate the functional relations between MetH and its cofactor – cobalamine — under stress conditions. We have demonstrated that heat shock proteins (Hsp 70/100 system or HtpG) can protect MetH activity under stress conditions. Moreover, in the presence of cobalamine they can restore the activity of partially denatured methionine synthase.

Key words: methionine syntase (MetH), cobalamine, HSP, HtpG, Hsp 70/100, vitamin B12

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INTRODUCTION

Atherosclerotic plaque is the main cause of cardiovascular related diseases. Endothelial dysfunction leads to the development of the atheromatous plaque. Reports have shown a connection between endothelial dysfunction and an elevated level of homocysteine (Lentz *et al.*, 1996; Quere *et al.*, 1997; Bellamy *et al.*, 1998; Chambers *et al.*, 1998; Kanani *et al.*, 1999; Ungvari *et al.*,1999; Eberhardt *et al.*, 2000; Lentz *et al.*, 2000; Dayal *et al.*, 2001; Hofmann *et al.* 2001; Austin *et al.* 2004; McCully 2005; Lentz 2005).

Homocysteine is an endogenous amino acid, not occurring in proteins, that takes part in the methylation process (Finkelstein, Martin, 1986). Even a small increase in the homocysteine level can be a significant risk factor for cardiovascular diseases (Sawuła *et al.*2008). During the metabolic cycle methionine is transformed to S-adenosine methionine and used as a methyl group donor. After the transfer of the SAM methyl group S-adenosine homocysteine is formed from which homocysteine is released. In the next step homocysteine is remethylated to methionine by methionine synthase (Lentz 2005). Methionine synthase is one of the main enzymes of the folate and homocysteine metabolism. Human methionine synthase (MS) shows high homology to the bacterial methionine synthase (MetH) (Guenther *et al.*, 1999). Activity of this protein is cobalamine-dependent (Amaratunga *et al.*, 1995; Muld *et al.*, 2001). MS catalyses the transfer of the CH_3-H_4 folate methyl onto homocysteine. In this reaction methionine and a biologically active form of folic acid — H_4 folate — is formed (Finkelstein & Martin, 1986; Rosenblatt & Fenton, 2001). Vitamin deficiency, mutations and stress conditions are the main cause of dysfunction of the mentioned enzymes (Chanarin *et al.*, 1992). Disorders in the homocysteine metabolic cycle lead to homocysteine accumulation which is a significant risk factor for cardiovascular-related diseases (Wilcken & Wilcken 1976; Sawuła *et al.*, 2009). Additionally, methionine synthase disorders lead to a decrease in folate availability that disrupts cell homeostasis. Stress conditions occurring in atheromatous plaque regions lead to the surplus of heat shock proteins (Xu, 2002).

Heat shock proteins are a class of proteins encoded by highly conserved DNA regions. This class is divided into families based on the molecular weight of proteins. Heat shock proteins are produced either constitutively or are induced by environmental factors (Georgopoulos & Welch 1993; Xu, 2002). They protect cells from adverse environmental factors, aid protein refolding and synthesis (Hartl, 1996). Studies have shown an increase in HSPs synthesis in atheromatous plaque rich regions (Berberian, 1990; Xu, 2002). High concentration of HSPs is supposed to reduce the stress i.e. oxidation stress (Madrigal-Matute *et al.,* 2010) in the plaque region and to inhibit plaque expansion.

The aim of this study was to determine the role of heat shock proteins and cobalamine in maintaining the activity of *Escherichia coli* methionine synthase (MetH). It was demonstrated that the bacterial enzyme may be a proper model for studies on the functions of its human homologue, while possessing all advantages of the prokaryotic experimental system (Jakóbkiewicz-Banecka *et al.*, 2005).

MATERIALS AND METHODS

Overexpression and purification of MetH. *E. coli* K — 12 XL1-Blue strain containing the pCWG-02 plasmid carrying the *metH* gene was inoculated into

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Abbreviations: HSP, heat shock protein; MetH, methionine syntase; SAM, S-adenosyl methionine.

LB medium supplemented with 5 μM cobalamine and ampicillin (100 μg/mL). The culture was carried at 37°C with mechanical shaking. After reaching $OD_{595} = 0.7$ overproduction was induced by addition of 0.5 M IPTG. Four hours after induction the culture was centrifuged (4600 × *g*, 4°C, 15 min). The sediment was suspended in 50 mM phosphate buffer (pH = 7.2). The bacteria were lysed in a French Press. The obtained lysate was centrifuged (78 000 × *g*, 4°C, 40 min) and the resulting supernatant was applied onto a chromatographic column containing a DEAE bed (20 mL). The column was pre-equilibrated with 50 mM phosphate buffer (pH = 7.2). A linear gradient from 50 to 500 mM phosphate buffer was used for the elution of the protein. The obtained fractions were examined using SDS/PAGE gel electrophoresis. Fractions containing the MetH protein were combined and dialyzed against 50 mM phosphate buffer (pH = 7.2). Next the sample was transferred onto a chromatographic column containing a Sorce-Q bed (16 mL) equilibrated with 50 mM phosphate buffer (pH = 7.2). The column was washed with 50 mM phosphate buffer (pH = 7.2) and 118 mM phosphate buffer (pH = 7.2). A linear gradient from 118 to 320 mM phosphate buffer (pH=7.2) was used for the elution of the protein. The collected fractions were examined using SDS/PAGE. Fractions containing pure enzyme were dialyzed against 50 mM phosphate buffer (pH = 7.2) after which they were frozen in liquid nitrogen and stored at –70°C.

Overexpression and purification of heat shock proteins. DnaK, GrpE, DnaJ, ClpB and HtpG were overproduced and purified in accordance with the previously published methods (Banecki & Żylicz, 1996; Żylicz *et al*., 1987; Spence *et al*., 1989; Woo *et al*., 1992; Wawrzynów *et al*., 1995).

MetH activity test. The MetH activity test was performed in accordance with the methionine synthase test developed by Drummond (1995). This test measures the amount of H4 foliate in the $CH^+ = H_4$ foliate form that is formed in the last step of the test. Before conducting the test all solutions were degassed. The test solution contained 100 mM phosphate buffer (pH = 7.2), 19 μM SAM, 500 μM homocysteine, 25 mM DTT, cobalamine (different concentrations — 10, 20, 50, 100 μM) and 0.375 μM MetH. The solution was incubated for exactly 5 minutes at 37°C. After the incubation 250 μM (6-*R,S*)-CH3-H4 foliate was added. The volume of the reaction mixture was equal to 800 μL. The reaction was carried out for 15 minutes at 37°C and then, after the addition of 200 μL of the mixture of hydrochloric acid in acetic acid, the temperature was raised to 80°C and the reaction was continued for another 10 minutes. The aim of this was to convert H4 foliate into $CH^+ = H_4$ foliate. MetH activity was determined based on the absorbance of $CH^+ = H_4$ foliate at 350 nm (ε_{350} 26 000 M^{-1} cm^{-1}).

Thermal denaturation of MetH. 0.375 μM MetH was pre-incubated in the presence of 100mM phosphate buffer (pH = 7.2), 50 mM NaCl, 20 mM KCl and 20 mM MgCl2 for 15 minutes in temperatures varying from 37 to 59°C. After the incubation the sample was cooled down to room temperature and subjected to the MetH activity test.

MetH size exclusion analysis. The separation was performed using a Perkin–Elmer series 200 HPLC system equipped with a GE Healthcare Life Sciences "*Superose 6*, 10/300 GL column (mobile phase: 50 mM Tris/HCl (pH = 8.0), 100 mM NaCl; flow 0.3 mL/min; sample injection: 25 μL) The detection was carried out on a Perkin-Elmer Series 200 UV-VIS detector at 210 nm. The analyzed samples were pre-incubated for 15 minutes at 50°C either with or without the addition of 50 μM cobalamine.

MetH protection by HSP. 0.375 μM MetH was pre-incubated in 100 mM phosphate buffer (pH = 7.2), 50 mM NaCl, 20 mM KCl and 20 mM $MgCl_2$ in the presence of DnaK, DnaJ, GrpE, ClpB(Hsp 70/100 system) and 5 mM ATP or HtpG and 5 mM ATP. The concentration of heat shock protein was 3.7 μM, 1.4 μM, 0.36 μM, 1.5 μM and 3 μM, respectively. Additionally, a variation of this test was performed with the addition of 50 μM cobalamine. The incubation was carried out for 15 minutes at 50°C. Finally the samples were subjected to the MetH activity test.

Renaturation of thermally inactivated MetH by the Hsp 70/100 system. 0.375 μM MetH underwent thermal inactivation through a 15 minute incubation at 50°C in the presence of 100 mM phosphate buffer (pH = 7.2), 50 mM NaCl, 20 mM KCl and 20 mM $MgCl_2$. After incubation the enzyme was renatured in the presence of Hsp 70/100 or HtpG. Additionally, a variation of this test was performed with the addition of 50 μM cobalamine to the denatured protein. Renaturation was carried out for 45 minutes at 20°C. Finally, the sample was subjected to the MetH activity test.

RESULTS

Effect of cobalamine concentration on MetH activity.

The effect of HSPs on the enzyme stability was studied at low and saturated cobalamine concentration. We have found that MetH activity strongly depends on the presence of its cofactor — cobalamine. Additionally, binding of the cofactor can stabilize the structure and stability of the enzyme. Cobalamine, at concentrations up to 40 μM, increases Meth activity. However, at higher concentrations the enzyme becomes saturated with the cofactor. The results of the experiment are presented in Fig. 1.

MetH thermal denaturation

The test was performed to find an optimum inactivation temperature for the analysis with the use of heat shock proteins (Fig. 2). The obtained thermal inactivation curve has shown a temperature-dependent gradual loss of activity by MetH. The temperature

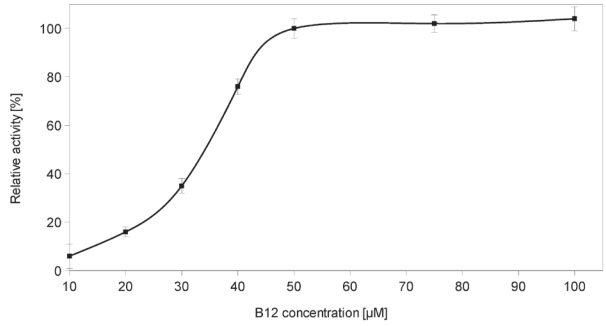

Figure 1. Kinetics of MetH activity in increasing concentration of cobalamine.
The result obtained with 50 μM cobalamine was assumed as 100% of MetH activity. Data points are averages of three independent experiments. The error bars show standard deviation (S.D.).

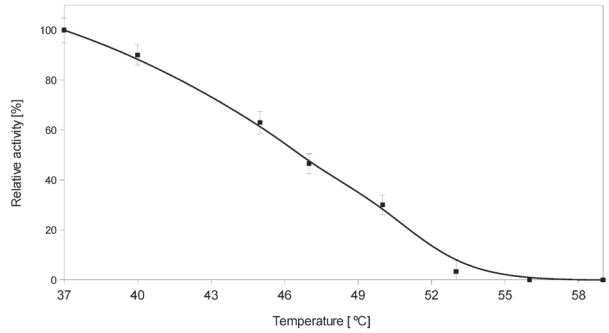

Figure 2. Kinetics of MetH thermal inactivation.
Data points are averages of three independent experiments. The error bars show standard deviation (S.D.).

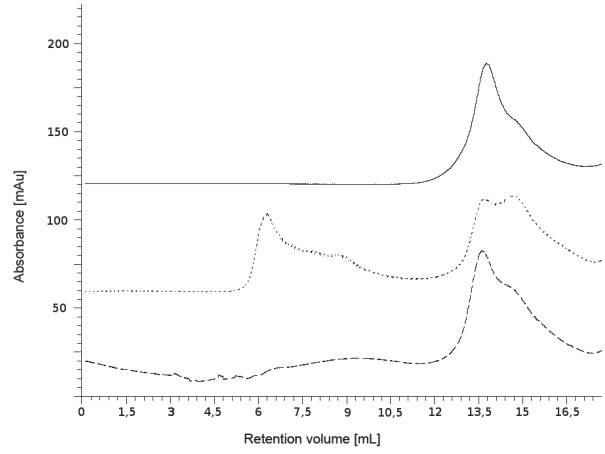

Figure 3. Size exclusion chromatography of MetH.
Experiment was performed on a Superosa 6 column with UV detection at 210 nm. MetH was incubated at 50°C with or without cobalamine. Solid line, native MetH; dotted line, denatured MetH; dashed line, MetH denatured in the presence of cobalamine.

of 50°C was chosen as optimal for further analyses. At this temperature the enzyme is partially denatured, however can still be renatured in the presence of HSPs. Size exclusion chromatography shows that at this temperature MetH starts to form aggregates (Fig. 3). Aggregation can be inhibited in the presence of cobalamine.

Protection against thermal deactivation of MetH

The reported local overproduction of HSPs in the atheromatous plaque regions suggests their important role in maintaining the functionality of the metabolic pathways including the homocysteine transformation pathway. To determine the role of heat shock proteins in MetH protection two tests were performed — the influence of HSPs on the denaturation process and the effect of HSPs on restoring the activity of denatured MetH. The results of the thermal deactivation tests are presented in Fig. 4. HSPs efficiently protect MetH against thermal inactivation. In the presence of Hsp70/100 and HtpG 70% and 59% of the activity was preserved, respectively. When cobalamine was present, up to 92% of the enzyme activity was preserved. These results prove that the Hsp 70/100 system and HtpG protect MetH from inactivation under stress conditions.

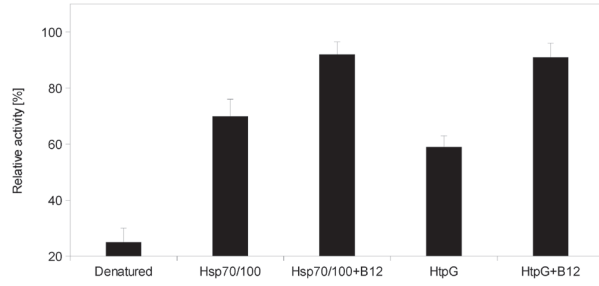

Figure 4. Protection of MetH from thermal inactivation by HSPs.
Test was performed at 50°C. The concentrations of Hsp70/100 and HtpG were as follows: Dnak 3.7 μM, DnaJ 1.4 μM, GrpE 0.36 μM, ClpB 1.5 μM, HtpG 3 μM. Cobalamine concentration was 50 μM. Data points are averages of three independent experiments. The error bars show standard deviation (S.D.).

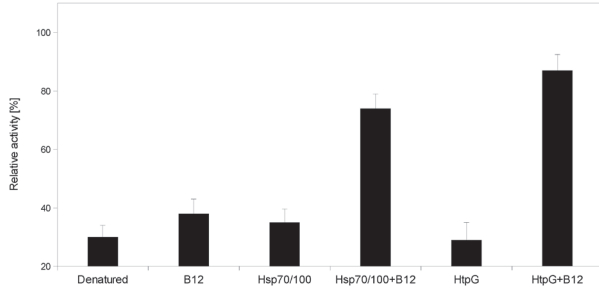

Figure 5. Renaturation of thermally inactivated MetH by Hsps.
Test was performed after thermal inactivation of MetH and renaturation in the presence of selected proteins. The concentrations of Hsp70/100 and HtpG were as follows: Dnak 3.71 μM, DnaJ 1.41 μM, GrpE 0.361 μM, ClpB 1.51 μM, HtpG 3 μM. Cobalamine concentration was 50 μM.

Renaturation of thermally deactivated MetH

The aim of this experiment was to determine the mechanisms involved in MetH protection, activity restoration and cofactor binding (Fig. 5). These mechanisms could play a key role in maintaining homeostasis in the atheromatous plaque region. Neither cobalamine nor HSPs alone could reactivate partially denatured enzyme. However renaturation in the presence of the Hsp 70/100 system and an excess of cobalamine has been efficient and 74% of the enzyme activity was restored. Similar results were obtained in the presence of HtpG. In this case even 87% of the initial activity was restored. Because the Hsp 70/100 system has higher disaggregation capacity, these results suggest that HtpG assists also in cobalamine binding to the active site.

DISCUSSION

Homocysteine is one of the main risk factors of ischemic stroke and cardiovascular diseases. Disorders in the metabolism of homocysteine and its overall high concentration are connected with the formation of the atheromatous plaque and with stress condition conditions in the blood vessels. Increased levels of homocysteine are usually caused by its metabolic pathway dysfunction or diet. Activity of MetH, one of the main enzymes of the metabolic pathway of homocysteine, depends on its

cofactor — cobalamine bound to the active site. Upon stress conditions MetH aggregates, the cofactor dissociates and the activity of the enzyme decreases. The presented study may help to broaden the understanding of the etiology of cardiovascular diseases. We have shown that elevated concentration of cobalamine, can maintain enzyme activity. This result may explain why B — group vitamin diet supplementation is one of the elements in the therapy to prevent stroke (Toole *et al*., 2004).

However, in the atheromatous plaque region the Hsp family proteins are overexpressed and this results in slowing down the process of atherosclerosis development. Under stress conditions the heat shock proteins preserve the native structure of the enzyme and prevent the dissociation of the cobalamine cofactor. This may play a key role in maintaining the activity of methionine synthase ensuring the functionality of the homocysteine and folic acid metabolic pathways. HSPs not only protect MetH from inactivation but also restore its activity after inactivation. The role of the Hsp 70/100 system in enzymatic activity recovery following stress conditions is limited to structure refolding. We speculate that Hsp 70/100 restores MetH structure but does not restore enzyme activity. Only in the presence of cobalamine a significant increase in activity is observed. This proves that the MetH molecule has a natural ability to bind the cofactor to the active site and this reaction is limited by the concentration of cobalamine.

HtpG is a bacterial Hsp 90 protein with established activity in maintaining homeostasis. As the representative of the Hsp 90 family, HtpG interacts with many substrates and is involved in protein protection and synthesis. The results of our experiments show that HtpG not only protects MetH from thermal inactivation but also restores its activity. It is even more efficient than the Hsp70/100 system. Probably, when cobalamine is present in excess, HtpG inflicts conformational changes in the MetH molecule that lead to rebinding of the cofactor. In the case of minor structural changes of MetH, the activity of HtpG may play an important role in maintaining MetH activity. However under high stress conditions the activity of HtpG alone may not by sufficient.

Probably in case of severe structural damage, the Hsp 70/100 system and HtpG cooperate as a complete system maintaining and restoring MetH activity (Genest *et al*., 2011). Taking into account the number of substrates that can interact with Hsp70/100 and HtpG its activity is not specific towards MetH. Probably under stress conditions both systems are responsible for the maintenance of MetH activity. This activity leads to a local decrease in homocysteine level. Increased homocysteine concentration induces oxidative stress in the cell. Thus decreasing the level of homocysteine can contribute to alleviating stress conditions and reducing the effects of development of the plaque.

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