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The Effect of Homocysteine on MetR Regulation of *met*E, *met*R and *met*H Expression *In Vitro*

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An *Escherichia coli* S-30 DNA directed protein synthesis system was used to study the effect of homocysteine on the *in vitro* expression of the *met*E, *met*H and *met*R genes. In the presence of purified MetR protein, which is known to regulate the expression of these genes, homocysteine activates *met*E expression and inhibits both *met*R and *met*H expression. These findings support the recent *in vivo* results of Urbanowski, M.L. and Stauffer, G.V. (1989), *J. Bacteriol.* **171**, 3277-3281.

The terminal reaction in methionine synthesis involves a methyl transfer from N^5-methyltetrahydrofolic acid to homocysteine (reviewed in 1). In *Escherichia coli* there are two different enzymes that catalyze this reaction. One, the product of the *met*H gene, requires vitamin B_{12} (B_{12} dependent methyltransferase), whereas the second enzyme, is the product of the *met*E gene, (non-B_{12} methyltransferase). This latter enzyme is a major protein in *E. coli* representing up to 5% of the soluble protein under derepressed conditions (2) and the expression of the *met*E gene is regulated in a complex fashion. Thus, it has been known for many years that the expression of this gene is repressed by both methionine and vitamin B_{12} (3-8). The mechanism of methionine repression is now known to involve a repressor protein (MetJ protein) and S-adenosylmethionine (AdoMet) (9-11). Studies *in vivo* suggest that the repression by vitamin B_{12} involves the *met*H gene product (3.4), and perhaps the *metF* gene product (8,12). In addition, a new regulatory locus, called *met*R, that is involved in *met*E and *met*H regulation, has recently been described (13) in both *E. coli* and *Salmonella typhimurium*. In genetic studies, Urbanowski, *et al* (13) showed that *met*R mutants do not make the MetE protein and have markedly reduced levels of MetH protein. It was concluded that the *met*R gene, which is located close to the *met*E gene, both in *S. typhimurium* (14) and *E. coli* (15), codes for a transactivator of both *met*E and *met*H expression. We have recently extended these studies, using an S-30 protein synthesis system, and have demonstrated that the purified MetR protein from *E. coli* can stimulate the *in vitro* expression of the *met*E and *met*H genes and also autoregulate its own synthesis (16). In addition, Urbanowski and Stauffer (17,18) have presented *in vivo* data indicating that homocysteine may modulate the ability of the MetR protein to regulate the

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expression of the *met*E, *met*H and *met*R genes. In the present study, we show a direct effect of homocysteine on the MetR regulation of these genes *in vitro*.

MATERIALS AND METHODS

E. coli strain, RK4536, (*met*E, *met*H) was obtained from the *E. coli* Genetic Stock Center (Yale University) and was used as the source of the S-30 extract. The construction of plasmid pRSE562 (*met*E and *met*R genes) and plasmid pQN1011 (*met*H) was reported earlier (15,19). The latter plasmid was obtained from Dr. R. Glass (Queen's Medical Center, Nottingham, England). Homocysteine thiolactone was purchased from Sigma Chemical Co., hydrolyzed by boiling for 5 min. in 20mM NaOH and neutralized with 1M Tris-HCl, pH5.6. The purification procedures for the MetR and MetJ proteins have been described previously (15,20), as well as the preparation of the S-30 extract for the *in vitro* system for gene expression (21). The incubation conditions for gene expression, using plasmid DNA as template and the assays used to detect MetE synthesis either enzymatically or by autoradiography have been described (2, 16).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of homocysteine on the *in vitro* expression of the *met*E and *met*R genes, using plasmid pRSE562 as template (Panel A), and the *met*H gene using plasmid pQN1011 as template (Panel B). For these experiments, the ^{35}S labelled proteins were separated by gel electrophoresis and detected by autoradiography. As shown previously (16), using an S-30 extract, there is very low expression of the *met*E gene under these conditions, but good expression of the MetR protein and β–lactamase from pRSE562 (Lane 1). In the presence of purified MetR protein, there is a significant increase in MetE synthesis and a small decrease (autoregulation) in the synthesis of MetR (Lane 2). As seen in Lane 3, the addition of homocysteine to an incubation containing the MetR protein markedly stimulates *met*E expression,

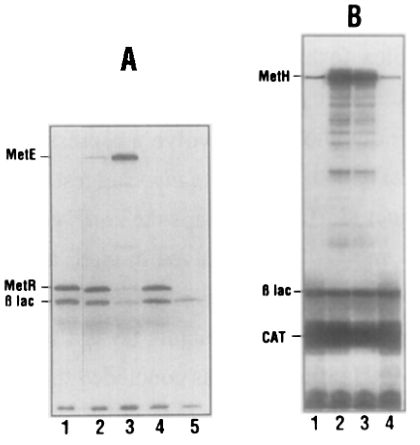

Fig. 1. DNA-directed *in vitro* synthesis of MetE and MetH proteins. Plasmids containing the *met*E, *met*R and *met*H genes were incubated in an S-30 extract containing [^{35}S] methionine. MetR protein (1μg), MetJ protein (0.4 μg), AdoMet (50 μM) and L-homocysteine (0.3 mM) were added as indicated. After 60 min. at 37° aliquots were removed, subjected to $NaDodSO_4$ polyacrylamide gel electrophoresis and autoradiographed. A, plasmid pRSE562 (*met*E, *met*R); lane 1, no addition; lane 2, MetR protein; lane 3, MetR protein and homocysteine; lane 4, homocysteine; lane 5, MetR protein, homocysteine, MetJ protein and AdoMet ; B, plasmid pQN1011 (*met*H); lane 1, no addition; lane 2, MetR protein; lane 3, MetR protein and homocysteine; lane 4, homocysteine.

but almost completely inhibits *met*R expression. On the other hand, homocysteine alone shows no effect on either *met*E or *met*R expression (Lane 4 vs Lane 1). Lane 5 shows that the addition of MetJ protein (repressor) and AdoMet inhibit *met*E expression in the presence of MetR and homocysteine, as shown previously when MetR alone was added (16). It should be noted that the MetR protein and homocysteine also caused a decrease in the expression of ß-lactamase from plasmid pRSE562 (Lane 3). This effect is still unexplained since it was not observed with other plasmid templates that carry the amp^r gene (see below). The results of *met*H expression are seen in Fig. 1B using pQN1011 as template. Lanes 1 and 2 show the stimulatory effect of the MetR protein on *met*H expression in this *in vitro* system as previously reported (16). When homocysteine is added to incubations containing MetR, there is a modest decrease of about 30-50% in the synthesis of the MetH protein (Lane 3 vs Lane 2), whereas homocysteine by itself has no effect (Lane 4 vs Lane 1). The decrease by MetR and homocysteine, although small, has been routinely observed and is consistent with recent *in vivo* results (17) that showed homocysteine decreased the expression of a *met*H-lac gene fusion protein in *E. coli* . There is no effect of the MetR protein and homocysteine on either ß-lactamase or CAT synthesis from this template (Lane 3).

Fig. 2A shows the effect of homocysteine on the expression of the *met*E gene in incubations containing varying amounts of the MetR protein. In these experiments the amount of MetE synthesized was assayed enzymatically (2, 16). A maximal stimulatory effect of homocysteine is obtained with about 1.0 µg of MetR protein. Fig. 2B shows that homocysteine, at a concentration of $3x10^{-4}M$, is saturating for the expression of the *met*E gene in the presence of 1 µg of MetR protein. With regard to specificity, S-adenosylhomocysteine and cystathionine were both found to give partial activity, but this may be due to their conversion to homocysteine

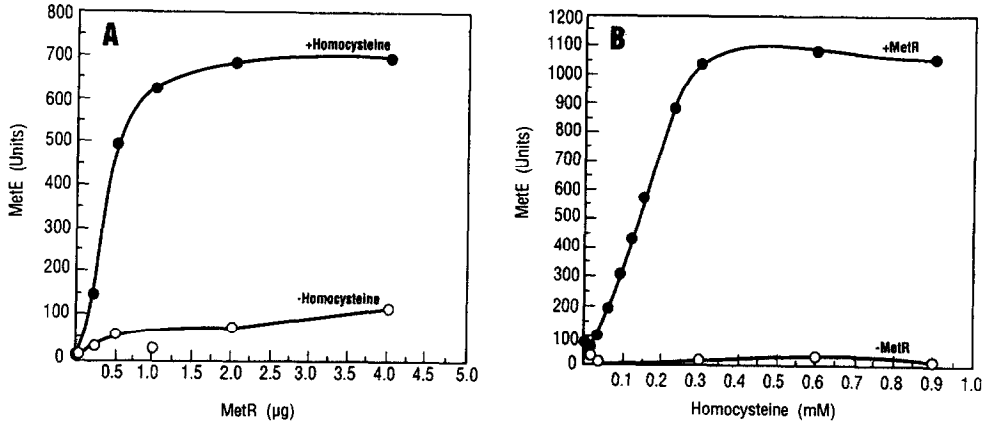

Fig. 2. Effect of MetR and homocysteine concentrations on the expression of *met*E from plasmid pRSE562 in an S-30 protein synthesis system. After 60 min. at 37° aliquots were removed and assayed for MetE enzymatic activity (pmol of methionine formed in 60 min. per 35 µl incubation). A, incubations were carried out in the presence of varying concentrations of MetR protein in the absence (o) or presence (•) of 0.3 mM homocysteine; B, incubations were carried out in the presence of varying concentrations of homocysteine in the absence (o) or presence (•) of MetR (1 µg).

in this crude system. Cysteine and methionine could not be tested since they are essential components of the *in vitro* protein synthesis system.

The present *in vitro* results provide direct evidence to support recent *in vivo* experiments that indicated that homocysteine, in the presence of the MetR protein, is a positive effector of *met*E expression (17), but a negative regulator for both *met*R (18) and *met*H expression (17). The mechanism by which homocysteine acts as a coinducer or corepressor with MetR is not known. However, previous DNA footprint experiments have demonstrated that the MetR protein binds to only one region in the *met*E and *met*R intergenic region (16) and recent experiments have shown that homocysteine has no effect on this binding even at sub-saturating levels of MetR protein (data not shown). These results suggest that the effect of homocysteine is at a step subsequent to the binding of MetR to the DNA. Thus, it is possible that the MetR dependent regulation of the *met*E, *met*R and *met*H genes is a two-step process. The first involves the binding of the MetR protein to a specific DNA sequence, followed by a homocysteine dependent reaction that results in the activation of transcription initiation by MetR. This hypothesis is consistent with recent genetic studies that showed a MetR mutant protein from *E. coli* could bind to its target DNA , but could no longer use homocysteine as a modulator (22).

It is of interest to note that the MetR protein is a member of the LysR family of bacterial activator proteins (23). Each member of the LysR family of activator proteins is transcribed divergently from one of its target genes. Additionally, these bacterial activators all range between 30 and 35kD and are highly homologous at their N-termini in a region predicted to have a helix-turn-helix structure. The ability of homocysteine to modulate MetR activity may be similar to that seen in the lysine biosynthetic pathway, where diaminopimelate functions with LysR as a coinducer for *lys*A expression (24,25).

Physiologically, the stimulatory effect of excess homocysteine on *met*E expression is consistent with the reasoning that under conditions in which homocysteine accumulates, the cell could lower the level of homocysteine by increasing the amount of MetE protein. However, the reason for the increased autoregulation of *met*R expression by MetR and homocysteine is not clear, especially since the MetR protein is required for *met*E expression. In addition, it is important to note that all of these experiments have been performed with S-30 extracts. Although we have developed partially defined *in vitro* protein synthesis systems to study gene expression (26, 27), we have found that many of the effects seen in the S-30 extracts are lost in the more defined protein synthesis systems. For example, the regulation by MetR of *met*E,H and R expression and the effect of homocysteine were much less evident in the more defined *in vitro* systems. The situation is further complicated by the presence of an inhibitor in cell extracts that appears to act specifically to antagonize the effect of MetR on *met*E and possibly *met*H expression (data not shown). These observations suggest that the regulation of these *met* genes involves other factors in addition to the MetR protein, homocysteine, MetJ protein and AdoMet. Further studies are in progress to identify these components.

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