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Structure–function studies on *Escherichia coli* MetR protein, a putative prokaryotic leucine zipper protein

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ABSTRACT **The *Escherichia coli metR* gene has been sequenced. The sequence predicts a protein of 317 amino acids and a calculated molecular weight of 35,628. This is about 15% larger than the protein from *Salmonella typhimurium* reported previously [Plamann, L. S. & Stauffer, G. V. (1987) *J. Bacteriol.* 169, 3932–3937]. The protein is a homodimer and contains a leucine zipper motif characteristic of many eukaryotic DNA-binding proteins. Replacement of two of the leucines in the leucine zipper region of the MetR protein, or substitution of proline for one of the leucines, results in loss of biological activity of the protein. In addition, truncation studies have identified a region on MetR that may be involved in the homocysteine activation of *metE* expression.**

The terminal reaction in methionine synthesis in *Escherichia coli* involves a methyl transfer from N^5-methyltetrahydrofolate to homocysteine. In *E. coli* there are two enzymes that catalyze this reaction (reviewed in ref. 1). One, the product of the *metH* gene, referred to as the B_{12}-dependent methyltransferase, has a cobamide prosthetic group. The other is the product of the *metE* gene, termed the non-B_{12} methyltransferase, and is a major protein in *E. coli* under derepressed conditions (2). It is now apparent that the regulation of the expression of the *metE* gene is under both negative and positive control. As with other *met* genes in *E. coli* (except for *metH*), the *metE* gene is repressed by high levels of methionine in the growth medium (3–9). A number of studies have shown that this regulation involves the MetJ protein and *S*-adenosylmethionine (10–14). The expression of the *metE* gene is also repressed when *E. coli* is grown in the presence of vitamin B_{12} (4, 5). Although the mechanism of this regulation has not been elucidated, the MetH holoenzyme (4, 5) and possibly the MetF protein (8, 15) are involved in this repression.

A *met* regulatory locus referred to as *metR* has been identified in both *E. coli* and *Salmonella typhimurium* (9). The MetR protein has been purified and shown to be a positive effector of both *metE* and *metH* expression *in vitro* (16, 17). The MetR protein is a DNA-binding protein that protects ≈24 base pairs (bp) in the intergenic region between the *metE* and *metR* genes (17). We noted from a partial nucleotide sequence of the *E. coli metR* gene that the predicted amino acid sequence of the MetR protein has a putative leucine zipper region as has been identified in several eukaryotic transcription factors (18). In the present study, we have sequenced the entire *E. coli metR* gene and have examined the effect of site-directed mutagenesis of the leucine residues in the leucine zipper region on the ability of the MetR protein to transactivate the *metE* and *metH* genes *in vivo*. We have also studied the effect of truncation of the MetR protein on its functional activity.*

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MATERIALS AND METHODS

E. coli strain GS244 (*metR, pheA905, thi*), described previously (9), was obtained from G. Stauffer. A *recA* derivative of the strain was constructed by P1 transduction (19) with PRC102 (*srl*::Tn*10 recA*). The MetR protein was purified as described (16). Cells were grown routinely in the presence of methionine at 10 μg/ml in M9 salt medium supplemented with phenylalanine (20 μg/ml), thiamin (0.2 μg/ml), and glucose (0.4%) and were harvested at an OD_{600} of 0.6. The cells were disrupted by sonication and the MetE activity in extracts was determined (2). For the determination of MetH activity (20) the growth medium contained vitamin B_{12} at 0.5 μg/ml. Protein concentrations were determined by the method of Lowry *et al.* (21).

DNA sequencing was performed using the dideoxy sequencing technique for double-stranded templates (22, 23) and plasmid pRSE562 (16) as template. Oligodeoxynucleotides were chemically synthesized (Applied Biosystems model 350B) and used as primers for the sequence determination of both complementary strands. The wild-type *metR* gene was obtained by cleaving plasmid pRSE562 with restriction endonucleases *Sma* I and *Bam*HI. An ≈3000-bp fragment was isolated by agarose gel electrophoresis and ligated into the Bluescript M13$^-$ vector (Stratagene), which had also been cut with the above restriction enzymes. This plasmid (pBLR) carries the wild-type *metR* gene under control of its own promoter. Site-directed mutagenesis of the *metR* gene was carried out by published procedures (24, 25). The mutations are described in the legend of Fig. 1 and in Table 1.

In order to truncate the *metR* gene, plasmid pRSE562 containing the wild-type *metR* gene was sequentially digested with *Sma* I and *Bgl* II. *Sma* I cleaves the DNA in the *metE–metR* intergenic region, 162 bases upstream of the site of initiation of translation of *metR* (17), while *Bgl* II cuts within the *metR* gene, 546 bases downstream of the site of initiation of translation (see Fig. 1). The 708-bp fragment liberated was isolated after agarose gel electrophoresis. This fragment was ligated into pBR322 that had been digested with *Eco*RV and *Bam*HI. This recombinant plasmid contains 182 codons of *metR* that are in frame with 299 codons of pBR322. In order to truncate the protein further, plasmid pMRIII (17) was digested with *Dra* I and *Mlu* I, which cut the *metR* gene 104 nucleotides upstream (17) and 265 nucleotides downstream, respectively, of the initiation of translation (see Fig. 1). The 369-bp fragment was isolated and ligated into the Bluescript vector that had been digested with *Eco*RV. This plasmid contains 88 codons of *metR* that are in frame with 20 codons of the Bluescript vector. The two plasmids were isolated from cleared lysates by CsCl centrifugation and used to transform GS244. Growth of these transformants and assay of their MetE and MetH enzyme activities were as described above.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37630).

RESULTS

Sequence of *E. coli metR* Gene Encodes a Putative Leucine Zipper Motif. Fig. 1 shows the nucleotide sequence as well as the deduced amino acid sequence of the *E. coli metR* gene. The protein contains 317 amino acids and has a calculated molecular weight of 35,628. This latter value agrees well with a molecular weight of about 34,000 as determined by SDS/polyacrylamide gel electrophoresis. The MetR protein from *S. typhimurium* has been reported to contain only 276 amino acids, with a calculated molecular weight of 30,991. However, similar to the *E. coli* MetR, the *S. typhimurium* MetR also migrates with a molecular weight of about 34,000 as estimated by SDS/polyacrylamide gel electrophoresis (27). The amino acid sequence of the *S. typhimurium* MetR protein is about 90% identical to the first 276 amino acids of the *E. coli* protein. In this context, it is of interest that an insertion of a guanine residue at nucleotide 853 of the *S. typhimurium* sequence (26) would cause a frameshift that would lead to the synthesis of a protein similar in size to the *E. coli* protein. Assuming such an insertion, the deduced amino acid sequence of the additional carboxyl-terminal region of the *S. typhimurium* MetR protein would be about 80% identical to the *E. coli* MetR protein.

Of particular interest in the MetR amino acid sequence is the presence of a typical leucine zipper motif. Thus, between amino acids 19 and 40 (Fig. 1) there is a helical region containing a leucine residue every seventh amino acid. In order to investigate the role of the leucines in this region on the activity of the MetR protein, site-directed mutagenesis of each of the four leucine residues was performed (see legend to Fig. 1; Table 1). The resultant plasmids were transformed into GS244 ($metR^-$) and the transformants were assayed for their ability to grow in the absence of methionine as well as for MetE (non-B_{12} methyltransferase) and MetH (B_{12} methyltransferase) activity. The results are summarized in Table 1. GS244 did not grow in the absence of methionine and had only a trace level of MetE activity when grown in the presence of low levels of methionine (10 μg/ml). Transformation with the plasmid carrying the wild-type gene (pBLR) relieved the auxotrophy and showed high levels of MetE activity. When this strain was transformed with plasmid pBL_{26} or pBL_{40}, the mutant transformants also grew in the absence of methionine and the cell extracts had MetE activity similar to that of transformants containing the wild-type *metR* gene. It is of interest that although the pBL_{19} transformant grew in the absence of methionine at the same rate as the wild-type transformant, the MetE activity was only about 15% of wild type. These results suggest that although the mutation of the first leucine in the putative zipper region decreased the ability of the mutant MetR to fully transactivate *metE* expression, this level of MetE was still sufficient for normal growth. More dramatic results were obtained when the $metR^-$ mutant was transformed with plasmid pBL_{33} or $pBL_{26,40}$. In both cases, the transformants did not grow in the absence of methionine and had levels of MetE activity that were similar to that of the $metR^-$ mutant. A similar pattern was also observed in the case of MetH activity. As previously reported (9) and shown in Table 1, GS244 contains ≈20% of the wild-type MetH activity. The level of this activity was increased to wild-type levels

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                                        1                           30                           60
CTCAAGCGCAAATTATTCATGCCGAAGTGAAGGACTTTC ATG ATC GAA GTA AAA CAC CTG AAA ACG CTA CAA GCG TTG CGG AAC TGC GGC TCG CTC GCA
                                         M   I   E   V   K   H   L   K   T   L   Q   A   L   R   N   C   G   S  [L]  A
                       90                           120                          150
GCC GCT GCG GCG ACG TTG CAT CAG ACG CAA TCC GCC CTG TCT CAC CAG TTT AGC GAT CTG GAA CAA CGC CTT GGC TTC CGG CTA TTT GTG
 A   A   A   A   T  [L]  H   Q   T   Q   S   A  [L]  S   H   Q   F   S   D  [L]  E   Q   R   L   G   F   R   L   F   V
                       180                          210                          240
CGT AAG AGC CAG CCG CTA CGC TTT ACA CCG CAG GGA GAA ATC CTG TTG CAA CTG GCA AAC CAG GTA CTG CCG CAA ATT AGC CAG GCC CTG
 R   K   S   Q   P   L   R   F   T   P   Q   G   E   I   L   L   Q   L   A   N   Q   V   L   P   Q   I   S   Q   A   L
                    Mlu I  270                          300                          330
CAA GCC TGC AAT GAA CCG CAG CAG ACG CGT CTG CGC ATT GCC ATT GAG TGC CAT AGC TGT ATT CAG TGG CTG ACA CCC GCG TTA GAA AAT
 Q   A   C   N   E   P   Q   Q   T   R   L   R   I   A   I   E   C   H   S   C   I   Q   W   L   T   P   A   L   E   N
                       360                          390                          420
TTC CAT AAG AAC TGG CCG CAG GTA GAG ATG GAT TTT AAA TCG GGC GTG ACA TTT GAC CCG CAG CCC GCC TTG CAA CAG GGA GAG CTG GAT
 F   H   K   N   W   P   Q   V   E   M   D   F   K   S   G   V   T   F   D   P   Q   P   A   L   Q   Q   G   E   L   D
                       450                          480                          510
CTG GTA ATG ACG TCC GAT ATT CTG CCG CGC AGT GGC CTG CAT TAT TCG CCG ATG TTC GAC TAT GAA GTG CGT CTG GTG TTA GCA CCT GAC
 L   V   M   T   S   D   I   L   P   R   S   G   L   H   Y   S   P   M   F   D   Y   E   V   R   L   V   L   A   P   D
                       540      Bgl II              570                          600
CAT CCA CTG GCG GCG AAA ACG CGA ATT ACA CCG GAA GAT CTC GCC AGC GAG ACG CTA TTA ATT TAT CCG GTG CAG CGT AGT CGA CTG GAT
 H   P   L   A   A   K   T   R   I   T   P   E   D   L   A   S   E   T   L   L   I   Y   P   V   Q   R   S   R   L   D
                       630                          660                          690
GTC TGG CGG CAT TTT CTT CAG CCG GCA GGC GTC AGC CCG TCA CTG AAA AGC GTC GAT AAC ACC TTA TTG TTG ATT CAG ATG GTT GCC GCG
 V   W   R   H   F   L   Q   P   A   G   V   S   P   S   L   K   S   V   D   N   T   L   L   L   I   Q   M   V   A   A
                       720                          750                          780
CGG ATG GGT ATT GCC GCG CTA CCG CAT TGG GTA GTA GAG AGT TTT GAG CGC CAG GGT CTG GTG GTG ACC AAA ACC CTG GGC GAA GGC TTG
 R   M   G   I   A   A   L   P   H   W   V   V   E   S   F   E   R   Q   G   L   V   V   T   K   T   L   G   E   G   L
                       810                          840                          870
TGG AGC CGA CTG TAC GCG GCC GTG CGC GAT GGC GAG CAG CGT CAG CCA GTT ACG GAA GCG TTT ATT CGC TCA GCG CGC AAT CAC GCC TGC
 W   S   R   L   Y   A   A   V   R   D   G   E   Q   R   Q   P   V   T   E   A   F   I   R   S   A   R   N   H   A   C
                       900                          930                          960
GAT CAT CTG CCG TTT GTG AAG AGC GCG GAG CGA CCC ACT TAC GAT GCA CCC ACA GTG AGG CCA GGA TCA CCA GCG CGC CTG TAATAAACGTT
 D   H   L   P   F   V   K   S   A   E   R   P   T   Y   D   A   P   T   V   R   P   G   S   P   A   R   L  END

GGCCAGTGCGGC
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FIG. 1. DNA sequence of the *metR* gene of *E. coli*. The sequence shown starts with the nucleotide previously shown to be the start of transcription (17). The nucleotides are numbered starting with the first in-frame ATG codon. The deduced amino acid sequence is shown with single-letter abbreviations and is 317 amino acids in length. Leucines that comprise the putative leucine zipper region are boxed and are located at amino acid positions 19, 26, 33, and 40. Site-directed mutagenesis was performed as described (24, 25) to change the first of these leucines to arginine (L_{19}), the second to serine (L_{26}), the third to proline (L_{33}), and the fourth to glutamine (L_{40}). A double mutant was also constructed that converted the second and fourth leucines to serine and glutamine, respectively ($L_{26,40}$). The positions of the restriction sites within the *metR* gene that were used to generate the truncated MetR proteins are denoted by arrows. *Mlu* I cleaves at nucleotide 265 (after amino acid 88) and *Bgl* II at 546 (amino acid 182).

Table 1. Enzymatic activities of MetE and MetH in GS244 transformed with *metR* mutant plasmids

Plasmid	Amino acid substitution(s)	Methionine auxotrophy	Activity, nmol of methionine per mg of protein	
			MetE	MetH
None	—	+	0–0.4	15
pBLR	—	−	128	82
pBL_{26}	Ser	−	133	74
pBL_{40}	Gln	−	129	107
pBL_{19}	Arg	−	18	18
pBL_{33}	Pro	+	2.2	24
$pBL_{26,40}$	Ser,Gln	+	0.4	13

GS244 (*metR*$^-$) was transformed with various plasmids containing either the wild-type *metR* gene (pBLR) or mutations in specific leucine residues (see legend to Fig. 1). The number designation of the plasmid corresponds to the leucine position mutated as described in the legend to Fig. 1.

when GS244 was transformed with plasmid pBLR, pBL_{26}, or pBL_{40}. However, there was no or very little increase in MetH activity in the transformants containing plasmid pBL_{19}, pBL_{33}, or $pBL_{26,40}$. Immunoblot analysis showed that there was very little difference in the total amount of MetR or mutant MetR proteins found in cell extracts from each of the transformants (data not shown). These results show that a replacement in the MetR protein of the leucine residue at amino acid 33 with proline, a helix breaker, (pBL_{33}), or a double mutation at amino acids 26 and 40 leads to a loss of MetR activity *in vivo*. This is reflected by low levels of both MetE and MetH activity and the inability of these transformants to grow in the absence of methionine.

Molecular Weight of MetR Protein. It is known that the eukaryotic DNA-binding proteins that contain the leucine zipper motif are found as homo- or heterodimers (for a review see ref. 27) and that the leucine zipper region is involved in dimer formation. As shown in Fig. 2, the purified MetR protein, when analyzed by gel filtration chromatography, appears to have a molecular weight of about 68,000, which is consistent with its being a dimer. This purified protein has previously been shown to be a DNA-binding protein and to specifically bind to a 24-bp region in the *metE–metR* intergenic region (16).

Truncation of the MetR Protein. In order to gain more information on the structure–function relationship of the MetR protein, plasmids pRSE562 and pMRIII (17), which contain the wild-type gene, were treated with *Sma* I/*Bgl* II and *Dra* I/*Mlu* I, respectively, and the liberated fragments were isolated and subcloned. These fragments contain a *metR* gene that would code for proteins lacking 135 and 229 amino acids from the MetR carboxyl terminus, respectively. Note that the *metR* fragments are ligated in frame with sequences of the plasmid vector, resulting in the synthesis of a fusion protein. The plasmids containing these truncated *metR* genes were transformed into GS244 and it was found that whereas the *Bgl* II transformant grew in the absence of methionine, the *Mlu* I transformant required methionine for growth. Both transformants, however, grew in the presence of vitamin B_{12} (data not shown). Extracts from these transformants were assayed for both MetE and MetH activity. Table 2 shows that cells transformed with pBglII, a plasmid in which the *metR* coding region was cut with *Bgl* II, contain about 50% of the MetE activity and 80% of the MetH activity compared to GS244 transformed with the wild-type plasmid (pBLR). However, the *Mlu* I construct (pMluI) showed no MetE activity but still retained about 90–100% of the wild-type MetH activity. Since it is known that homocysteine is required for expression of *metE* but not *metH*, these results suggest that the region between amino acids 88 and 182 of the protein might be involved in the homocysteine activation of *metE* expression.

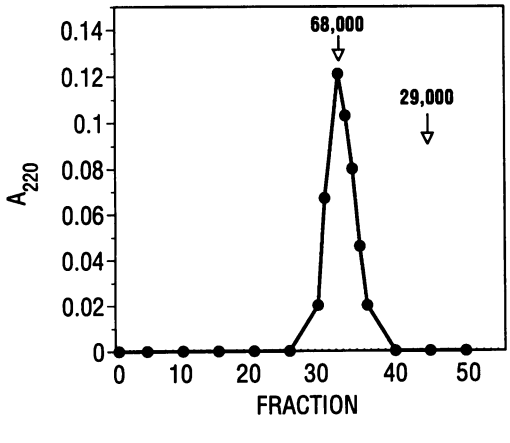

FIG. 2. Gel filtration of MetR protein. Purified MetR protein (14 μg) was applied to an AcA 44 Ultrogel column (0.5 × 42 cm) that had been equilibrated with 20 mM Tris Cl, pH 7.4/500 mM KCl/1 mM dithiothreitol. Elution (50 ml/hr) was with the same buffer, and fractions (≈0.60 ml) were collected and A_{220} was determined. Bovine serum albumin (M_r 68,000) and carbonic anhydrase (M_r 29,000) were used as molecular weight standards.

DISCUSSION

Previous studies both *in vivo* and *in vitro* have shown that the MetR protein is a positive transactivator of the *metE* and *metH* genes in *E. coli* and *S. typhimurium* and also autoregulates its own synthesis (9, 16–18, 28). In this study we have sequenced the *metR* gene from *E. coli* and have used site-directed mutagenesis and truncation of the gene to examine some structure–function relationships. The sequence of the *E. coli* protein derived from the DNA sequence is similar to that from *S. typhimurium* (26), except at the carboxyl end. The *S. typhimurium* gene, as reported, codes for a protein of ≈31 kDa whereas the *E. coli* gene codes for a protein of ≈34 kDa, which agrees well with the size determined by SDS/polyacrylamide gel electrophoresis. It is interesting that a small change in the DNA sequence has resulted in a large difference in the two proteins. The *E. coli* sequence shows an additional guanine residue at nucleotide 853 not seen in the *S. typhimurium* gene (26). This base insertion results in an extended open reading frame in the *E. coli* gene, accounting for the difference in the primary structure of the two proteins.

Although the MetR protein has been found by computer analysis to have characteristics similar to those of other bacterial activator proteins (29), it appears to be the first prokaryotic DNA-binding protein that belongs to the leucine zipper family (18). Similar to the eukaryotic proteins that are members of this group, MetR has four leucine residues spaced seven amino acids apart in a helical region of the protein. This would result in an exposed leucine at every

Table 2. Effect of truncation of the MetR protein on MetE and MetH activity *in vivo*

Plasmid	Activity, nmol of methionine per mg of protein	
	MetE	MetH
None	0.7	3.8
pBLR	165	58
pBglII	84	43
pMluI	0.9	64

The plasmids were used to transform GS244. The transformants were grown and assayed for MetE and MetH activity.

second turn of the helix in this region of the protein. It is now clear from studies with eukaryotic proteins such as GCN4 (30), c-Fos and c-Jun (31–33), C/EBP (34), etc., that this region is involved in homo- or heterodimer formation (27), which then facilitates the interaction of an adjacent region of the protein with specific sequences on the DNA.

Gel filtration analysis showed that the MetR protein is also a dimer. However, unlike the eukaryotic proteins, the leucine zipper region in MetR is close to the amino-terminal end of the protein, not more internal (27). Also, although there is no region in MetR that is as highly basic as the DNA-binding region of some leucine zipper proteins (27), there are two regions close to the putative leucine zipper in MetR that are basic. Thus the amino acids 4–10 and 43–57 contain net positive charges of four and five, respectively, and might constitute the MetR DNA-binding site.

Site-directed mutagenesis and truncation studies have provided evidence that the leucine zipper region of *E. coli* MetR is essential for activity and that another region on MetR is required for *metE* activation but not *metH* activation. In these experiments a *metR* mutant was transformed with plasmids containing the various constructs. The nontransformed mutant required methionine for growth and had <1% of the wild-type level of MetE activity and ≈20% of wild-type MetH activity. Under the growth conditions used, the lack of MetE activity accounts for the methionine auxotrophy in the *metR* mutant. Transformation of GS244 with a wild-type *metR* gene restored both MetE and MetH activity. A single substitution of each of the leucines in the zipper region yielded a wild-type phenotype except when leucine at amino acid 33 was replaced by proline, an amino acid known to disrupt α-helices. However, a transformant containing replacements of two leucines ($pBL_{26,40}$) in the *metR* gene showed a mutant phenotype, i.e., no growth in the absence of methionine, no MetE activity, and low MetH activity. Overall, there was a good correlation in these transformants between growth in the absence of methionine and high levels of MetE activity. The one exception was the transformant containing a substitution at position 19 (pBL_{19}). This mutant grew well in the absence of methionine but had about 15% of the wild-type level of MetE activity. It was concluded that this level of MetE activity, although significantly reduced from wild type, was sufficient for growth under these conditions.

If the leucine zipper region were involved in dimer formation, transformants containing plasmids pBL_{33} or $pBL_{26,40}$ would be expected to yield proteins that behave as monomers. However, although a significant effort was made to isolate these mutant proteins, it has not yet been possible to overproduce these proteins, as was previously done for wild-type MetR (16).

The results of the truncation of the MetR protein have provided valuable information on the possible binding site for homocysteine on MetR. Although MetR activates both *metE* and *metH* expression, homocysteine is a coactivator for *metE* expression but not *metH* expression (35, 36). A transformant in which about 40% of the 3′ end of the gene was removed behaved like the wild type. However, when an additional 94 amino acids were removed to yield a putative protein containing only 28% of the MetR sequence, there was almost complete loss of MetE activity but essentially no change in MetH activity. The most likely explanation of these results is that the region between amino acids 88 and 182 on the MetR protein contains the site where homocysteine interacts with the protein. Deletion of this region would yield the observed results, namely, loss of MetE activity but not MetH activity. Recently, Byerly *et al.* (37) have described MetR mutants in *E. coli* that have an altered homocysteine response. Our results predict that these mutations are in the region of amino acids 88–182 in the protein. Attempts to show stable binding of homocysteine to MetR have not been successful. Therefore direct proof of binding to this region has not been obtained.

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