JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5648–5653 Vol. 179, No. 17
0021-9193/97/$04.00+0

Protein Expression in Response to Folate Stress in *Escherichia coli*

ERIC Y. HUANG,† ANDREW M. MOHLER,‡ AND CHRISTOPHER E. ROHLMAN*
Department of Chemistry, Pomona College, Claremont, California 91711

Received 24 January 1997/Accepted 12 June 1997

Interruption of folate metabolism by trimethoprim results in the elevated expression of folate stress proteins in *Escherichia coli*. *E. coli* grown in culture medium supplemented with the folate-dependent metabolites glycine, methionine, and the purine nucleoside inosine shows reduced expression of folate stress proteins. The folate stress proteins include the universal stress protein, the ferric uptake regulatory repressor, and possibly, lipoamide dehydrogenase, the L protein component of the glycine cleavage enzyme complex.

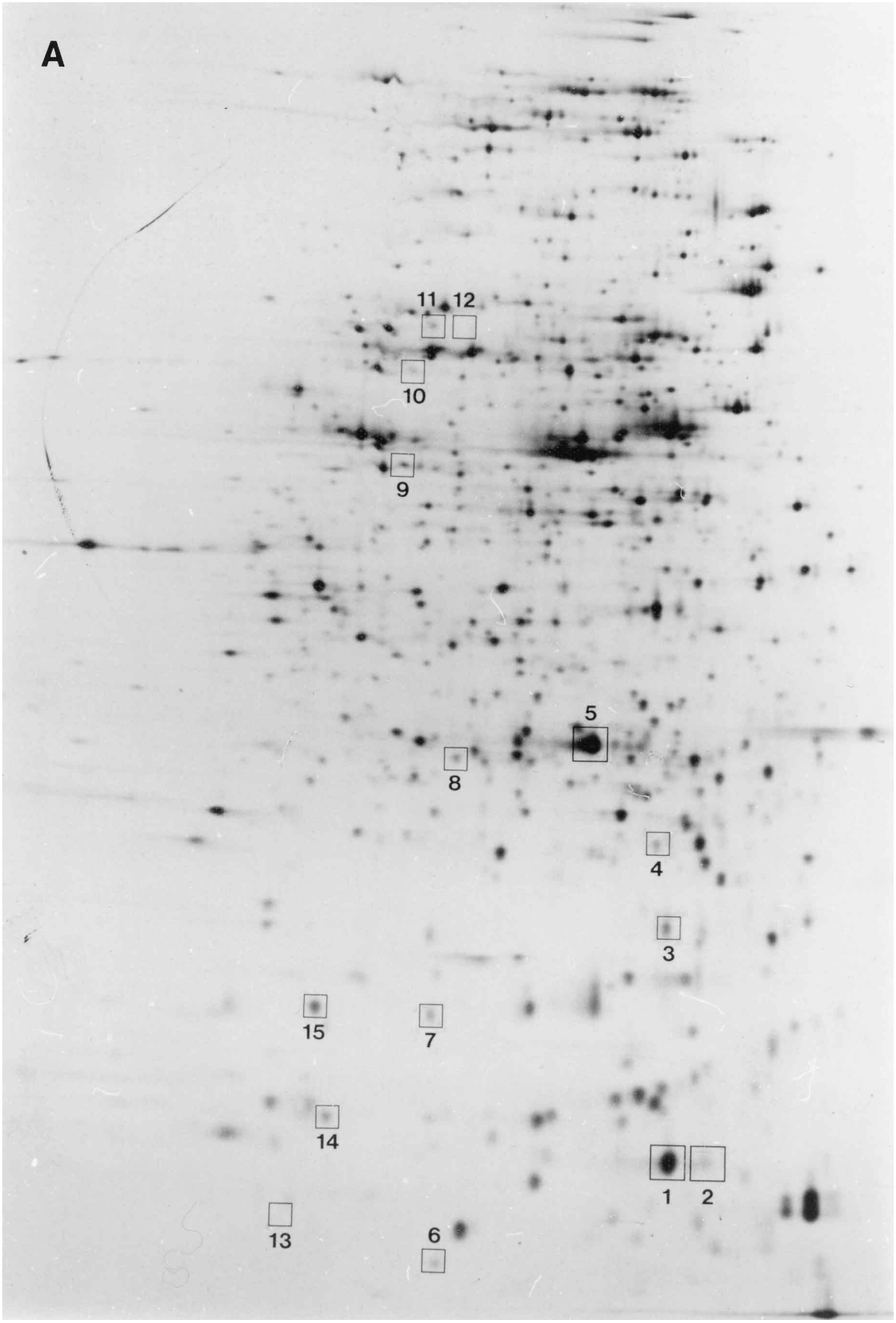
Tetrahydrofolate supplies methyl, methylene, and formyl carbon forms to the anabolic pathways of nucleotide, amino acid, and pantothenate biosynthesis. The antifolate dihydrofolate reductase inhibitor trimethoprim elevates the expression levels of putative stress proteins (11) in *Escherichia coli*. We wish to determine the relationship between the folate-dependent metabolites glycine, methionine, purine, and pyrimidine (1) and the putative stress protein induction. Modulation of the level of expression of trimethoprim-induced proteins by folate-dependent metabolites would suggest a connection between folate stress and the induced proteins. These proteins may represent alternative pathways in dihydrofolate reduction (4) or altered levels of one-carbon synthesis (6) and glycine cleavage (12). Characterization of this cellular response through large-format two-dimensional gel electrophoresis (10, 14) has confirmed the induction of previously identified folate stress proteins (11) and revealed additional candidate proteins.

Folate stress resulting from trimethoprim treatment elevates expression of a defined subset of proteins. Trimethoprim treatment resulted in the consistent induction of 21 proteins (not all indicated in Fig. 1), based upon analysis of at least three independently grown and labeled culture extracts. Trimethoprim treatment also caused the repression of several other proteins. Because equal counts from cell extracts were applied to the treated-cell and control cell gels, changes in these patterns reflect relative rather than absolute levels of proteins. Autoradiograms of the gels were both visually inspected and digitally scanned. Trimethoprim-induced proteins were characterized by weak (≥1-fold), moderate (≥3-fold), or strong (≥5-fold) induction relative to their level of expression prior to trimethoprim addition. Eighteen trimethoprim-induced proteins were identified as being moderately induced, and three exhibited strong induction after 60 min. As discussed below, results from this study indicate that 15 of these 21 trimethoprim-induced proteins are specifically associated with trimethoprim limitation of folate-dependent metabolites.

Trimethoprim reduces the fidelity of protein translation. A subset of proteins obtained from trimethoprim-treated cultures exhibited altered mobility, as observed in the first-dimensional isoelectric focusing of the two-dimensional gels (Fig. 1B). This "stuttering" phenomenon was most prevalent in the high-molecular-weight region of the gels and was greatly reduced in protein extracts supplemented with one-carbon metabolites. A similar phenomenon in cells which overexpress the universal stress protein, UspA, has been observed. UspA is induced by a range of stresses that cause growth rate repression, including carbon source limitation, cold and heat shock, and oxidation damage (8). Trimethoprim-treated cells also exhibit increased expression of UspA (proteins 1 and 2) along with a distinct set of stress proteins (14), which is likely a result of trimethoprim's limiting effect on both amino acid and nucleotide pools. It is possible that here and under other conditions that induce UspA, newly synthesized proteins are covalently modified, resulting in the observed isoelectric variants. It has been proposed that these altered pIs might result from UspA-dependent posttranslational modifications (9), which could include UspA-altered kinase activity. Alternatively, it has been suggested that perturbations of folate pools cause alterations in tRNA modification patterns and a subsequent reduction in the ribosome's ability to discriminate against mispaired tRNAs (2). These same alterations in tRNA modification could also affect the fidelity of aminoacyl tRNA synthetases. Either mischarging of aminoacyl tRNAs or increased tRNA mispairing at the ribosome would allow misincorpora-

FIG. 1. Protein induction in response to trimethoprim treatment. Strain W3110 was grown at 37°C in MOPS (morpholinepropanesulfonic acid) glucose minimal medium supplemented with thiamine (7, 11) radiolabeled with a 5-min pulse of [^{35}S]methionine followed by a 3-min cold methionine chase during mid-log-phase growth. Control samples were labeled 5 min prior to drug addition. An equal amount (counts per minute) of labeled protein was added on each gel. Gels were run as previously described (10, 11) with modifications (14). Gels were dried and placed in film cassettes for exposure to X-ray film. Gels were scanned with a Panasonic WV-BD400 CCD camera. Data were analyzed with Universal Imaging Corporation Image-1 3.93 software to measure relative protein levels. (A) Protein expression in strain W3110 at 5 to 0 min prior to trimethoprim addition. (B) Protein expression at 60 min following trimethoprim addition. The closed arrowhead indicates proteins exhibiting altered mobility. (C) Protein expression in cells grown with folate-dependent metabolites at 60 min following trimethoprim addition. Strain W3110 was grown in MOPS glucose minimal media with supplements of folate-dependent metabolites (1) which included glycine (400 μM), methionine (10 μM), inosine (500 μM), and thymine (400 μM) alone or in combination. Methionine was supplemented at 1/10 of the normal level for auxotrophs, adequate for log phase growth up to an optical density at 420 nm of 1, in order to facilitate incorporation of label.

* Corresponding author.
† Present address: University of Pennsylvania Medical School M.D.-Ph.D. Program.
‡ Present address: University of Colorado Medical School.


A
11
12
10
9
5
8
4
3
15
7
14
1
2
13
6


B
11
12
10
9
5
8
4
3
15
7
14
1
2
13
6


C
11
12
10
9
5
8
4
3
15
7
14
1
2
13
6

TABLE 1. Doubling times of strain W3110 in minimal and supplemented media[a]

Medium or supplement(s)	Doubling time (min):	
	Before trimethoprim addition	After trimethoprim addition
Glc min MOPS	62	341
Gly	62	281
Met	48	303
Met, Gly	50	252
Ino	84	304
Thy	60	328
Ino, Thy	111	246
Met, Gly, Ino	69	78
Met, Gly, Ino, Thy	63	63

[a] *E. coli* K-12 strain W3110 was grown aerobically as previously described (7, 11) at 37°C in MOPS (morpholinepropanesulfonic acid) glucose minimal medium supplemented either with thiamine (Glc min MOPS) or with the listed supplement(s). Overnight cultures were diluted into fresh media at an optical density at 420 nm (OD_{420}) of 0.05. After dilution, growth was continued until the bacteria were in mid-log-phase growth ($OD_{420} \approx 0.3$), at which point trimethoprim was added to a final concentration of 300 ng/ml. Growth was continually monitored at 0.5-h intervals for a period of between 3 and 4 h after drug addition. Growth curves were then plotted on a logarithmic scale, and by using first order linear regression, a slope for each concentration of drug was calculated. Doubling times are the averages of at least three trials.

tion of amino acids and alter the pI of the protein product. The longer open reading frames for high-molecular-weight proteins would be more susceptible to this process.

One-carbon metabolites modulate expression levels of folate stress proteins. We reasoned that those proteins that were induced in the absence of a particular supplement and not induced in the presence of that supplement represent candidates for being involved in a biosynthetic pathway connected to that nutrient. The addition of methionine (Met), glycine (Gly), and the purine nucleoside inosine (Ino) together suppressed stress protein induction and relieved the growth rate inhibition caused by trimethoprim (Tables 1 and 2). Inosine supplement slightly limited the growth rate prior to drug addition, as previously observed (1, 5, 11), but purine alone or in combination with thymine did not fully rescue cells from the drug's effect. Supplements of Met, Gly, Ino, and the pyrimidine base thymine (Thy) all resulted in reduced expression of 15 of 21 trimethoprim-induced proteins following drug treatment of the supplemented cultures (Table 2). Six of the original twenty-one proteins (not listed in Table 2) were expressed at the same level with or without supplements, suggesting that their expression levels are not dependent upon these one-carbon metabolites. Reduced expression of the 15 folate stress proteins in defined growth medium containing the folate-dependent metabolite Gly, Met, purine, or pyrimidine suggests that the levels of these nutrients are connected to the stress response.

The presence of thymine in addition to Met, Gly, and Ino completely suppressed induction of all the folate stress proteins except protein 1. This repression is apparent when induction levels in the trimethoprim-treated culture are compared to those in an unsupplemented control (Fig. 1A) as well as a control containing the four nutrients (data not shown). However, it appears that proteins other than the original 21 trimethoprim-induced proteins were also induced in the supplemented culture (Fig. 1C). One possible explanation is that nonauxotrophic strains supplemented with these nutrients experience a metabolic imbalance, even when faced with the restriction of the reduced folate pools caused by trimethoprim. The continued moderate induction of protein 1, the universal stress protein (UspA), supports this idea (8). It has been noted that while shortages of Gly, Met, Thy, and a purine source are the most crucial shortages experienced by cells treated with trimethoprim, other, less obvious metabolites also appear to become limiting (1). We have not tested whether pantothenate, which is also dependent upon folate for its biosynthesis, becomes limiting in the time frame of these studies. It is also possible that the 15 candidate proteins are growth rate regulated, like UspA (8). However, previous studies with other antifolate inhibitors support the claim that these proteins are specifically associated with trimethoprim's blockade of one-carbon metabolism (11). Overall these results strongly suggest that induction of the 15 identified folate stress proteins is dependent upon the cellular levels of the folate-dependent metabolites.

TABLE 2. Trimethoprim protein induction in defined growth media

Protein no.	Position in gel[a]		Protein induction in medium containing:[b]						
	Isolectric region	Coordinates (x, y)	Tm	Met	Gly	Ino	Gly, Ino	Met, Gly, Ino	Met, Gly, Ino, Thy
1	C	78.5, 29.5	M	W		W			M
2	C	83.0, 28.5	M						
3	C	78.5, 58	M		S				
4	D	71.3, 72.0	M						
5	E	66.5, 88	M				W		
6	G	42, 15	M						
7	G	41.5, 48.0	S		W	W	W		
8	G	45.5, 82.5	M	W					
9	G	41.5, 119.6	M						
10	G	41, 130	M	M					
11	G	43, 135.7	S	M					
12	G	49.4, 136.0	M						
13	H	16.5, 25	M						
14	H	27, 35	S	M	W		W		
15	H	22.5, 50	M	M			W		

[a] Coordinates and region refer to the standard reference gel shown in Fig. 1 of reference 14.

[b] Data shown is a compilation of the results obtained with three or more extracts. The highest level of protein induction occurred with Tm. All samples were taken 60 min after trimethoprim treatment. Levels of induction: W, weak; M, moderate; S, strong. Met, methionine; Gly, glycine; Ino, inosine; Thy, thymine; Tm, trimethoprim.

TABLE 3. Folate stress proteins found in the *E. coli* gene-protein database[a]

Protein no.	A-N	RRM	Coordinates (F1X, F1Y)	MWc	pIc	MWg	pIg	Gene	Protein
1	C013.5	R0135	78.5, 29.5	15,842	5.21	13,048	5.44	*uspA*	UspA
2	C013.4	R2296	83, 28.5	15,842	5.21	12,744	5.35	*uspA*	UspA isoform II
3	C018.0	R2206	78.5, 58			19,446	5.44		
6	G010.7	R2366	42, 15			9,906	6.18		
7	G015.8	R2229	41.5, 48	16,792	6.05	17,792	6.19	*fur*	Ferric uptake regulation repressor
8	G025.8	R2144	45.5, 82.5			24,571	6.11		
9	G041.4	R1882	39.5, 119.5	41,430	6.3	42,824	6.24	*carA*	Carbamoyl phosphate synthase, alpha subunit
9	G041.3	R2436	43.5, 119.5	43,282	6.21	42,824	6.15	*ackA*	Acetate kinase
10	G048.1		41, 130			50,103	6.2		
11	G050.5	R2975	43, 132.5	50,554	6.12	52,122	6.16	*lpdA*	Dihydrolipoamide dehydrogenase
11	G050.6		41, 133.5			52,976	6.2		
11	G054.6	R2560	43.5, 135.5			54,779	6.15		
12	G060.0		50.5, 138.5	65,551	5.99	57,769	6.01	*dnaG*	DnaG primase
13	H011.7		16.5, 25			11,713	6.7		

[a] Protein numbers refer to Table 2 of reference 14. The abbreviations are those used in the *E. coli* gene-protein database: A-N, alpha-numeric; RRM, response/regulation map name; F1X and F1Y, x and y coordinates of the standard reference gel shown in Fig. 1 of reference 14; MWc, calculated molecular weight; pIc, calculated isoelectric point; MWg, molecular weight estimate from gel; pIg, isoelectric point estimate from gel.

Identification of folate stress proteins. We have begun efforts to identify the 15 folate stress proteins described in this study. The gene-protein database, edition 6 (14), has provided potential correlates for 11 of 15 folate stress proteins and seven potential identifications (Tables 2 and 3). Confidence is high for proteins 1 and 2, the universal stress protein and its isoform, respectively. Also, protein 7 is likely the *fur* gene product, the ferric uptake regulation repressor. Fur expression is negatively regulated by the Fe-Fur complex and positively regulated by cyclic AMP-catabolite gene activator protein (3). It is not known if trimethoprim treatment might lead to an increase of either cyclic AMP or catabolite gene activator protein levels, but increased Fur expression does correlate with reduced FepA (ferric enterochelin receptor) synthesis in cells overexpressing UspA (9). Some ambiguity still remains in the assignment of the remaining eight proteins where the database offers reference points, and in these cases the potential candidates from the current gene-protein index are listed. Among the more exciting possibilities is that protein 11 may be dihydrolipoamide dehydrogenase, which serves as the L protein component of the glycine cleavage enzyme system (13). This would suggest that *E. coli* recruits the glycine cleavage system during trimethoprim-induced folate stress. Experiments are under way to evaluate this hypothesis.

In summary, glycine, methionine, inosine, and thymine modify the pattern of protein expression in *E. coli* under trimethoprim-induced folate stress, suggesting that these nutrients are connected to the stress response. In addition, trimethoprim causes changes in cellular metabolism which decrease translational fidelity and/or posttranslational modification of several proteins not necessarily associated with the folate stress response. This altered pattern of protein migration is analogous to that observed during the increased expression of UspA, the universal stress protein.

We voice appreciation for the helpful discussions with Ruth VanBogelen, Frederick Neidhardt, and all the members of the Neidhardt laboratory.

A.M.M. was supported by a grant to Pomona College from the Howard Hughes Medical Institute. This work was supported by grants to C.E.R. from the Irvine Foundation and from the Research Corporation.

REFERENCES

1. **Amyes, S. G. B., and J. T. Smith.** 1974. Trimethoprim action and its analogy with thymine starvation. Antimicrob. Agents Chemother. **5:**169–178.
2. **Basso, J., and M. B. Herrington.** 1994. Changes in translational accuracy of *Escherichia coli* when folate metabolism is perturbed. Microbios **77:**231–237.
3. **de Lorenzo, V., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur protein and CAP modulate transcription of the *fur* gene in *Escherichia coli*. Eur. J. Biochem. **173:**537–546.
4. **Hamm-Alvarez, S. F., A. Sancar, and K. V. Rajagopalan.** 1990. The presence of reduced folates in *Escherichia coli* dihydrofolate reductase mutants. J. Biol. Chem. **265:**9850–9856.
5. **Jensen, K. F.** 1983. Metabolism of 5-phosphoribosyl 1-pyrophosphate (PRPP) in *Escherichia coli* and *Salmonella typhimurium*, p. 1–25. *In* A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides, and nucleobases in microorganisms. Academic Press, Inc., London, England.
6. **Nagy, P. L., A. Marolewski, S. J. Benkovic, and H. Zalkin.** 1995. Formyltetrahydrofolate hydrolase, a regulatory enzyme that functions to balance pools of tetrahydrofolate and one-carbon tetrahydrofolate adducts in *Escherichia coli*. J. Bacteriol. **177:**1292–1298.
7. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
8. **Nyström, T., and F. C. Neidhardt.** 1992. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. Mol. Microbiol. **6:**3187–3198.
9. **Nyström, T., and F. C. Neidhardt.** 1996. Effects of overproducing the universal stress protein, UspA, in *Escherichia coli* K-12. J. Bacteriol. **178:**927–930.
10. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:**1133–1142.
11. **Rohlman, C. E., and R. G. Matthews.** 1990. Role of purine biosynthetic intermediates in response to folate stress in *Escherichia coli*. J. Bacteriol. **172:**7200–7210.
12. **Stauffer, L. T., S. J. Fogarty, and G. V. Stauffer.** 1994. Characterization of the *Escherichia coli gcv* operon. Gene **142:**17–22.
13. **Steiert, P. S., L. T. Stauffer, and G. V. Stauffer.** 1990. The *lpd* gene product functions as the L protein in the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol. **172:**6142–6144.
14. **VanBogelen, R. A., K. Z. Abshire, A. Pertsemlidis, R. L. Clark, and F. C. Neidhardt.** 1996. Gene-protein database of *Escherichia coli* K-12, edition 6, p. 2067–2117. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Renikoff, M. Riley, M. Schaechter, and M. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.