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Regulatory Mutants of Dihydrofolate Reductase in *Escherichia coli* K12

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Summary. Trimethoprim inhibits dihydrofolate reductase. Mutations conferring trimethoprim-resistance on *E. coli* K12 result in either an altered reductase with decreased affinity for the drug, or in 2–30 fold higher levels of the enzyme. Studies of the latter class of mutants indicate that dihydrofolate reductase is regulated by a diffusible molecule, and is probably under negative control. The regulatory mutants, some of which are temperature-sensitive, act *cis*.

Introduction

Dihydrofolate reductase catalyses the reduction of dihydrofolic acid to tetrahydrofolic acid, which participates in many biosynthetic reactions leading to the synthesis of proteins and polynucleotides. Competitive inhibitors of the enzyme such as trimethoprim, aminopterin and methotrexate (Hitchings and Burchall, 1964) are commonly used antifolate drugs. Drug-resistant mutants of bacteria and eukaryotes often have greatly increased levels of dihydrofolate reductase (see for example Sirotnak and McCuen, 1973; Littlefield, 1969; Kashket et al., 1964).

Very little is known about the regulation of expression of dihydrofolate reductase, even in bacteria. Sirotnak and co-workers have concluded from studies of aminopterin-resistant mutants of *Diplococcus pneumoniae* that the control is transcriptional (Sirotnak and McCuen, 1973) and that dihydrofolate reductase participates in its own regulation (Sirotnak, Hachtel and Williams, 1968). However the inability to construct cells of this organism diploid for the pertinent region precludes the dominance studies necessary to define the regulatory mechanism.

Trimethoprim-resistant mutants in *Escherichia coli* K12 have been mapped at about 0.5 min, between *pdxA* and *pyrA* (Breeze et al., 1975). In this paper we describe the types of trimethoprim-resistant mutants which can be generated in *E. coli* K12. Some mutations lower the affinity of dihydrofolate reductase for trimethoprim, while others increase the level of dihydrofolate reductase in the cell.

Materials and Methods

Chemicals

Folic acid, trimethoprim, NADPH and dithionite were purchased from Sigma. Lederle Laboratories generously donated pure methotrexate.

Dihydrofolic acid was prepared according to Blakley (1960) as modified by N. Harding (personal communication). All steps were done under nitrogen. A suspension of 10 g ascorbic acid and 400 mg folic acid in 25 ml H_2O was titrated to pH 7.0 with 30% NaOH, then mixed slowly with 6 g sodium dithionite. After dissolution of the dithionite the mixture was stirred for 15 min at 20° C. N NaOH was added to maintain the pH between 6.0 and 7.0. The solution was then cooled to 4° C in an ice bath, brought to pH 2.8 with careful addition of 1 N HCl, then stirred 5 min to allow complete precipitation of the dihydrofolic acid. The precipitate was centrifuged at $1{,}000 \times g$ for 5 min at 4° C, washed three times at 4° C with 1 mM HCl which had been degassed, then equilibrated with nitrogen, The dihydrofolic acid was lyophilized under nitrogen and stored dessicated in vacuo at −20° C.

Media. B broth, M9, H plates and minimal glucose plates have been described by Landy et al. (1967). Minimal medium contains M9, 2 mM $MgSO_4$, 0.5% glucose, 5 μg/ml thiamin (B1), and amino acids as described in the text at 25 μg/ml each.

Mutagenesis and Mutant Isolation

Spontaneously-arising trimethoprim-resistant mutants were obtained by plating $1–5 \times 10^8$ cells of a fresh overnight culture on minimal glucose plates (supplemented with any required amino acid at 25 μg/ml) and between 1 and 20 μg/ml trimethoprim. We have

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Table 1. Bacterial strains

Name	Relevant genotype	Origin
AB259	HfrH	B. Bachmann
AB1157	F⁻ *thr ara leu proA argE his* Str^R	Cambridge Collection
AB2463	AB1157 *recA13*	B. Bachmann
AB2463/F101	AB2463/F′*thr*⁺ *leu*⁺	B. Bachmann
AB2463/F104	AB2463/F′*thr*⁺ *leu*⁺ *pro*⁺	B. Bachmann
D1	HfrCav *metB*	Cambridge Collection
E40	Hfr P4X *recA*	Cambridge Collection
RS7	D1 *fol13*	
RS10	D1 *fol18*	
RS16	D1 *fol38*	
RS22	D1 *fol50*	
RS35	D1 *fol60*	
RS50	D1 *fol60 rev5*	
RS70	F⁻ *thr his fol60*	RS35 × AB1157
RS71	F⁻ *thr fol60 recA*	E40 × RS70
RS79	F⁻ *thr his fol60 rev5*	RS50 × AB1157
RS81	F⁻ *thr his fol18–74*	RS85 × AB1157
RS82	F⁻ *thr fol60 rev5 recA*	E40 × RS79
RS83	F⁻ *thr his fol50*	RS22 × AB1157
RS84	F⁻ *thr his fol13*	RS7 × AB1157
RS85	D1 *fol18–74*	
RS87	F⁻ *thr fol50 recA*	E40 × RS83
RS89	D1 *fol38–73*	
RS90	F⁻ *thr fol13 recA*	E40 × RS84
RS91	F⁻ *thr fol18–74 recA*	E40 × RS81
RS94	F⁻ *thr pro his fol38–73*	RS89 × AB1157
RS95	F⁻ *pro his fol*⁺ (D1) Str^R	
RS99	F⁻ *thr fol38–73 recA*	E40 × RS94
RS100	F⁻ *pro recA* Str^R *fol*⁺ (D1)	
RS104	F⁻ *pro his thr leu recA* Str^R	Spontaneous *arg*⁺ of AB2463

termed these mutants *fol*, the name used for similar mutants in *S. typhimurium* (Sanderson, 1972).

Most mutants, however, were isolated after treatment with N-methyl-N′-nitro-N-nitrosoguanidine (NG) according to the method of Abelson et al. (1970). After mutagenesis the cells were grown 8–10 generations to allow segregation and expression of the mutant alleles. Mutants were selected as described above.

Strain Construction

Strains of *Escherichia coli* used are shown in Table 1. Most *fol* mutants were isolated in D1, then introduced into AB1157 by mating overnight in broth and selecting for *met*⁺ *his fol thr* and/or *pro* recombinants. The *recA* allele was introduced by mating E40 with the female *fol* strains and selecting *his*⁺ *fol thr* (or *pro*) recombinants. Generally 6–30% of these were also *recA*, as determined by sensitivity to ultraviolet irradiation.

E. coli K12 episomes F101 (F′ *thr*⁺ *leu*⁺) or F104 (*thr*⁺ *proA*⁺) were transferred into the F⁻ *recA fol thr* (or *proA*) strains from AB2463/F101 or AB2463/F104 by overnight mating, then selecting for *his*⁺ *thr*⁺ or *his*⁺ *pro*⁺ recombinants.

Episomes Carrying Mutant fol Alleles

Mutant *fol* alleles were introduced onto F101 by growing together overnight in broth an episome donor (e.g. AB2463/F101), a female *rec*⁺ *fol* strain (e.g. RS70) and a suitable female *recA* strain (e.g. RS104) and selecting for F⁻ *recA*/F′ *fol* recombinants. Other markers were verified by conventional techniques.

Growth

For enzyme assays the D1 strains were grown overnight in B broth or in appropriately supplemented minimal medium. There is little effect of growth conditions on enzyme levels. The F⁻ *fol* strains that were constructed as described above were grown overnight in appropriately supplemented minimal medium. The episome-carrying derivatives of these strains were grown under the same conditions but without threonine or proline, in order to maintain diploid cells. Under these conditions more than 99.9% of the cells were diploid.

Enzyme Preparation

Dihydrofolate reductase was isolated as described by Poe et al. (1972). Cells were centrifuged 10,000 × *g* at 4° C for 5 min, washed twice with 0.1 M Tris-HCl pH 7.4 and once with assay buffer (50 mM Tris-HCl pH 7.2, 50 mM KCl, 10 mM B-mercaptoethanol, 1 mM EDTA) at 4° C, and resuspended in assay buffer. The washed cells were lysed by sonication. Dihydrofolate reductase is labile to prolonged sonication; care was taken to use short bursts of sonication while swirling the cells in an ice-water bath. After centrifugation at 25,000 × *g* at 4° C for 20 min, the supernatant fluid was carefully decanted and assayed.

Enzyme Assays

Dihydrofolate reductase was assayed by the spectrophotometric method of Poe et al. (1972). The conversion of dihydrofolate to tetrahydrofolate and NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nM with an extinction coefficient of 11,650 at pH 7.2 at 23°. The enzyme was assayed at 20° (unless noted otherwise) in assay buffer that included 0.1 mM NADPH and 0.1 mM dihydrofolate. Parallel reactions were always run to determine the rate of dihydrofolate-independent oxidation of NADPH. One unit of enzyme is defined as the amount of enzyme which causes a decrease of 1 A_{340}/min under standard conditions. This corresponds to a conversion of 86 nmoles substrate per ml. Since the enzyme has a turnover rate of approximately 500 moles substrate/mole enzyme (Fig. 2) this is approximately equivalent to 0.17 nmoles enzyme. Enzyme specific activities are the averages of 3–9 independent determinations.

Inhibition of Dihydrofolate Reductase by Trimethoprim

Dihydrofolate reductase is competitively inhibited by methotrexate with an apparent Ki of 4 nM (Williams et al., 1973). Preliminary experiments indicated that trimethoprim was equally effective. It was thus necessary to do all drug inhibition studies with as little enzyme as possible. We routinely used 0.01 units enzyme/ml, which corresponds to about 1.7 nM dihydrofolate reductase. Enzyme was added to the standard assay mixture containing different concentrations of trimethoprim, and the decrease in A_{340} was monitored on a strip chart recorder. After about 5 min the enzyme and drug had equilibrated and the relative inhibition was noted. This corresponds to the competitive inhibition noted by others, and not to the so-called "stoichiometric" inhibition by methotrexate described below. The trimethoprim concentration which gives half-maximal inhibition under our conditions is not strictly the Ki, and is referred to as I50.

Methotrexate Titration of Dihydrofolate Reductase

Dihydrofolate reductase was incubated 10 min at 20° in assay buffer with 0.1 mM NADPH and different concentrations of methotrexate, then mixed with 0.1 mM FH_2 and assayed as described above. Under these conditions methotrexate binds the enzyme stoichiometrically in a 1:1 complex over the first 3–5 min of the reaction (Williams et al., 1973; R.S. unpublished observations). The smallest amount of methotrexate which causes complete inhibition of the enzyme is equal to the amount of enzyme present (Williams et al., 1973).

Protein Concentration

Protein concentration was determined by Biuret's method (Jacobs et al., 1956).

Results

a) Dihydrofolate Reductase Activity in Trimethoprim-Resistant Mutants

Strain D1 can grow on minimal glucose plates which contain trimethoprim at 0.1 μg/ml but not at 1.0 μg/ml. Twenty-two trimethoprim-resistant mutants were isolated in D1 after treatment with NG and selection on plates containing 1 μg/ml trimethoprim. Dihydrofolate reductase activity was measured in the presence and absence of 40 nM trimethoprim, a concentration which inhibits the wild-type enzyme by 83% in crude extracts. Of the 22 mutants, 10 had dihydrofolate reductase specific activities varying from 2-fold to 30-fold more than the wild-type strain, 10 had dihydrofolate reductase activities inhibited only 33–58% by 40 nM trimethoprim, and 2 had no apparent change in dihydrofolate reductase activity. The last two have not been characterized further.

The affinity of dihydrofolate reductase for trimethoprim was compared more closely for enzymes isolated from wildtype and mutant strains which apparently had an altered sensitivity to the drug. The data presented in Figure 1 demonstrate that the enzyme isolated from D1 *fol*$^+$ has an I50 of about 4 nM trimethoprim, while the enzyme isolated from D1 *fol 38* has an I50 of about 100 nm. Similarly, the *fol 18* enzyme has an I50 of about 100 nM (data not shown). These data demonstrate that one type of trimethoprim-resistant mutant ("structural" mutant) contains dihydrofolate reductase molecules with decreased affinity for the drug.

The mutants having increased dihydrofolate reductase activity could contain either more enzyme molecules, or enzyme molecules with higher turnover numbers than wildtype dihydrofolate reductase. Since dihydrofolate reductase has one methotrexate binding site these alternatives may be tested by comparing the rates of reduction of FH_2 per methotrexate binding site for enzymes from *fol*$^+$ and mutant (*fol 50*) strains. D1 *fol 50* contains 25-fold more enzyme activity than D1 *fol*$^+$. The results displayed in Figure 2 show that *fol*$^+$ dihydrofolate reductase has a turnover number of about 640 molecules FH_2/min/methotrexate binding site, while the enzyme from *fol* 50 has a turnover number of 530 molecules FH_2/min/methotrexate binding site. Assuming that dihydrofolate reductase from both wildtype and mutant strains have the same number of methotrexate binding sites these results demonstrate that D1 *fol 50* contains about 25-fold more enzyme molecules than D1 *fol*$^+$. Thus a second type of trimethoprim-resistant mutant ("regulatory" mutant) contains an increased number of dihydrofolate reductase molecules. These results agree with those of Sirotnak et al. (1968).

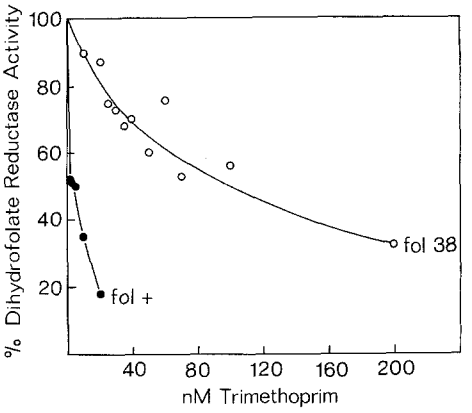

Fig. 1. Trimethoprim inhibition of dihydrofolate reductase from D1 *fol*$^+$ (●) and D1 *fol 38* (○)

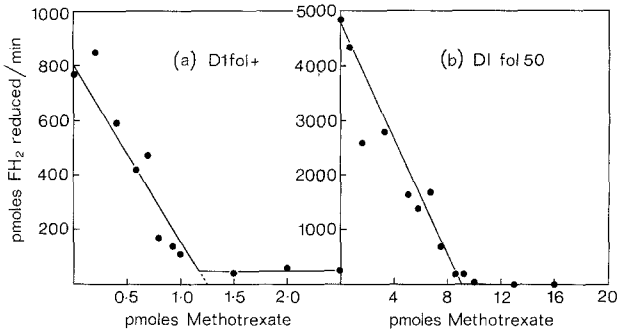

Fig. 2a and b. Methotrexate titration of dihydrofolate reductase from **a** D1 *fol*$^+$ and **b** D1 *fol 50*

b) Temperature-sensitive Regulatory Mutants

It seemed desirable to have temperature-sensitive *fol* mutants. D1 was mutagenized with nitrosoguanidine, and plated on minimal glucose plates containing 2 μg/ml trimethoprim. About 6% of the mutants examined

grew at 30° but not 42° on similar plates. Surprisingly, none of these mutants grew at 42° on minimal glucose plates in the presence or *absence* of trimethoprim. Eight of these mutants were grown in B broth at 30° and assayed for enzyme activity; all eight were found to be regulatory mutants with enzyme levels 5–25 fold more than wildtypes cells. One of these mutants, *fol60*, was studied forther.

D1 *fol 60* expresses 25–30 times more enzyme at 30° than does D1 *fol*$^+$. It grows at 42° in some batches of B broth and on some batches of H plates. Both contain Difco Bacto-Tryptone. We have not been able to identify the necessary growth requirement, but it does not appear to be an amino acid, purine, pyrimidine, a common vitamin or any combination of these nutrients.

One possible explanation was that either the expression of dihydrofolate reductase or the enzyme itself was temperature-sensitive. However, we have detected no difference in vitro in thermolability between *fol*$^+$ and *fol 60* enzymes. Hence it seemed unlikely that the enzyme itself was temperature-sensitive. We next determined whether expression of the enzyme was temperature-sensitive in D1 strains carrying various *fol* alleles grown in B broth. The results presented in Table 1 show that strains which are not appreciably temperature-sensitive (i.e. D1 *fol*$^+$, *fol 50* and *fol 18–74*) contain about half as much enzyme at 42° as at 30°. D1 *fol 60* contains about one quarter the specific activity at 42° as at 30°. Significantly, the specific activity of dihydrofolate reductase in D1 *fol 60* at 42° is intermediate between those of D1 *fol*$^+$ and D1 *fol 50*, both of which grow in minimal medium at 42°. It appears that neither dihydrofolate reductase nor its expression are likely to be responsible for the inviability of this strain at 42°.

This raised the possibility that the temperature-sensitivity of D1 *fol 60* might be totally unrelated to the *fol* locus. Guerola et al. (1971) showed that nitrosoguanidine treatment induces multiple, closely linked mutations. D1 *fol 60* might carry a mutation in the *fol* regulatory allele and a second, temperature-sensitive mutation in an unrelated locus. If the *fol 60* mutation is not the site of the temperature-sensitive lesion then spontaneous temperature-stable revertants should have enzyme levels similar to those of D1 *fol 60* at both 30° and 42°. Conversely, if the *fol 60* mutation is itself temperature-sensitive then at least some of the revertants should have enzyme levels altered from those of *fol 60*. To test this we isolated 19 spontaneous revertants of D1 *fol 60* which could grow on minimal medium at 42°. After purification, these were grown in B broth at 30° and 42° and assayed for dihydrofolate reductase activity.

The results in Table 2 indicate that all 19 revertants expressed dihydrofolate reductase differently from *fol 60*. Most of the revertants fall in four classes, based upon the pattern of expression of dihydrofolate reductase. Revertants 4, 8, 9, 14 and 19 are in the first category and have approximately wild-type levels of the enzyme at both 30° and 42°. In the second category revertants 2, 10, 13 and 18 contain highly elevated levels of the enzyme at both 30° and 42°. For example, *fol 60 rev 2* has dihydrofolate reductase specific activities of 0.33 and 0.44 units/mg at 30° and 42°, respectively. In category 3, revertants 3, 7 and 11 contain about 10 fold more enzyme at 30° and about 2–3 fold more enzyme at 42° than does wild-type D1. For example, *fol 60 rev 3* has enzyme specific activities of 0.44 and 0.041 units/mg at 30° and 42°, respectively. Category 4 is composed of revertants 5, 12 and 17, which contain 5–20 fold more dihydrofolate reductase at 30° than wild-type D1, but have enzyme levels less than or equal to wild-type levels at 42°. For example *fol 60 rev 5* has an enzyme specific activity of 0.25 at 30° but only 0.006 at 42°. The revertants in category 5 have a miscellany of phenotypes. For example, *fol 60 rev 1* does not grow on minimal medium at 30°, while *fol 60 rev 6* has only 0.002 and 0.001 units/mg dihydrofolate reductase at 30° and 42°, respectively. This is about 6% of the wild-type specific activity.

In summary, the data in Table 2 show that all temperature-stable revertants of D1 *fol60* have altered levels of dihydrofolate reductase compared

Table 2. Dihydrofolate reductase levels in D1 strains carrying various *fol* mutants

Class	Alleles	Dihydrofolate reductase specific activity	
		30°	42°
Controls	Wild Type	0.040	0.016
	50	1.0	0.58
	18–74	0.24	0.13
	60	1.0	0.25
1.	60 *rev4*, 60 *rev8*, 60 *rev9* 60 *rev14*, 60 *rev19*	0.017–0.062	0.013–0.032
2.	60 *rev2*, 60 *rev10* 60 *rev13*, 60 *rev18*	0.27–0.58	0.28–0.52
3.	60 *rev3*, 60 *rev7*, 60 *rev11*	0.35–0.45	0.041 0.065
4.	60 *rev5*, 60 *rev12*, 60 *rev17*	0.20–0.24	0.006–0.015
5.	60 *rev1*	No growth	0.01
	60 *rev6*	0.002	0.001
	60 *rev15*	0.008	0.04
	60 *rev16*	No growth	0.04

Table 3. Dihydrofolate reductase levels in Haploid and Merodiploid Strains

Haploid strain	fol allele	DFRase Sp. Act.	Diploid strain	fol alleles	DFRase Sp. Act.
AB2463	+	0.027	AB2463/F101	+/+	0.060
RS100	+	0.025	RS100/F104	+/+	0.063
RS91	18.74	0.12	RS91/F101	18.74/+	0.14
RS82	60 *rev5*	0.32	RS82/F101	60 *rev5*/+	0.32
RS99	38.73	0.42	RS99/F101	38.73/+	0.32
RS90	13	0.46	RS90/F101	13/+	0.42
RS87	50	0.56	RS87/F101	50/+	0.45

to D1 *fol60*. This indicates that the increased enzyme level and the temperature-sensitive phenotype of D1 *fol60* are different manifestations of a single mutational event. Consonant with this, we have been unable to separate genetically these two characteristics by recombination. Also, *fol 60 rev 5* and *fol 60 rev 12* remained temperature-sensitive for enzyme regulation when introduced into AB1157.

c) Expression of Regulatory Mutants in fol Merodiploids

Initially we characterized the expression of the enzyme in merodiploid cells which contained a chromosomal regulatory mutant *fol* allele and an episomal fol^+ allele. The dihydrofolate reductase specific activities of various haploid strains are presented in the left half of Table 3, while the specific activities of the corresponding diploids strains are presented in the right half.

AB2463 is haploid fol^+ and has a dihydrofolate reductase specific activity of 0.027 units/mg. RS100, which contains the fol^+ locus of D1 introduced by recombination, has a very similar level. Merodiploid derivatives of these strains with episomal fol^+ alleles have elevated activities which correspond to the additional 1–2 episomal genes per cell. RS91, which carries *fol 18–74*, has a specific activity of 0.12 units/mg, and its merodiploid derivative has a slightly higher activity of 0.14 units/mg. Strain RS82, which carries *fol 60 rev 5*, and its merodiploid derivative RS82/F101 have the same specific activity of 0.32 units/mg.

The three haploid strains with even higher dihydrofolate reductase levels (RS99, RS90 and RS87) have merodiploid derivatives with slightly (but reproducibly) depressed enzyme levels. For example the merodiploid RS87/F101 has only 80% as much enzyme as the haploid RS87.

The two salient features of this analysis are that: 1) Dihydrofolate reductase specific activity increases with an increase in *fol* gene dosage for *fol* alleles expressing relatively little enzyme and 2) *fol* regulatory mutants expressing high levels of enzyme are neither fully dominant nor fully recessive. Episomal fol^+ alleles reduce the expression of chromosomal *fol* regulatory mutants by at least 10–25%.

Table 4. Dihydrofolate reductase levels in strains carrying *fol60*

Strain	Chrom. allele	Epis. allele	DFRase Sp. Act.
RS71	60		1.07
RS71/F101	60	+	0.77
RS104	+		0.035
RS104/F101fol60	+	60	1.93
RS71/F101fol60	60	60	2.87

The expression of dihydrofolate reductase in strains carrying *fol 60* was studied in more detail. For technical reasons, the strains were assayed at 26°. The results are presented in Table 4, RS71 is a female strain with this allele and has an enzyme specific activity of 1.07 units/mg. The corresponding heterozygous merodiploid derivative RS71/F101 fol^+ has a specific activity of 0.77 units/mg. We also constructed a strain with a chromosomal fol^+ allele and an episomal *fol60* allele. This strain, RS104/F101 *fol60*, has a specific activity of 1.93 units/mg. The fol^+ allele alone in RS104 expresses 0.035 units/mg dihydrofolate reductase. RS71/F101 *fol 60* is diploid for *fol 60*, and has an enzyme specific activity 2.87 units/mg. It is evident that *fol 60* is slightly repressed by fol^+, and that an increase in *fol 60* gene dosage is accompanied by an approximately commensurate increase in dihydrofolate reductase levels. RS71/F101 *fol60* and RS104/F101 *fol60* have about thrice and twice as much enzyme, respectively, as RS71. Thus, although there is a slight *trans* effect the data suggest that the *fol* regulatory mutations act *cis*.

d) fol Regulatory Mutations Act cis

To determine whether the regulatory mutants act *cis* or *trans* we used strains in which the enzymes encoded by chromosomal and episomal alleles are distinguishable by their sensitivities in vitro to trimethoprim. For example, RS119 carries a chromosomal fol^+ allele with an I50 of 6–7 nM trimethoprim, and an episomal *fol 38* allele with an I50 of about 100 nM trimethoprim. Dihydrofolate reductase was prepared from RS119 and its sensitivity in vitro to trimethoprim was determined. The data presented in Figure 3 show that the trimethoprim-sensitivity of the enzyme from RS119 is intermediate between that of fol^+ and *fol 38*.

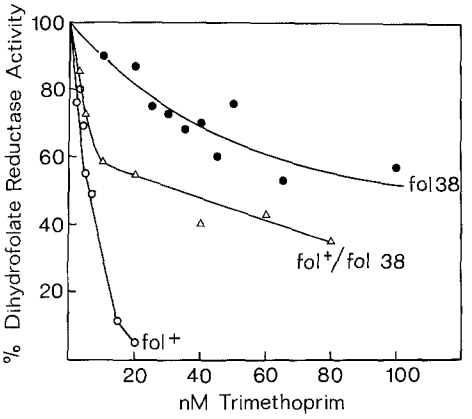

Fig. 3. Trimethoprim inhibition of dihydrofolate reductase from RS16 (*fol 38*); AB259 (*fol*$^+$); and RS119 (*fol*$^+$/*fol 38*)

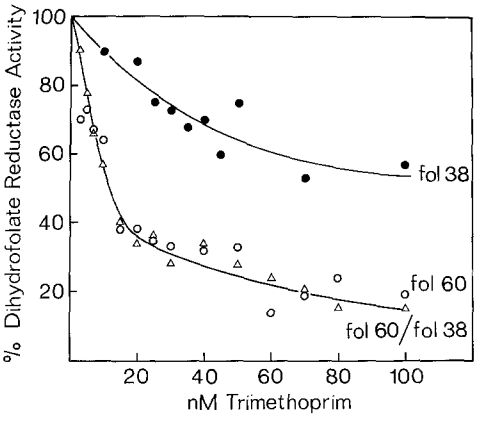

Fig. 4. Trimethoprim inhibition of dihydrofolate reductase from RS16 (*fol 38*); RS71 (*fol 60*); and RS71/F′ *fol 38* (*fol 60*/*fol 38*)

For example, 20 nM trimethoprim inhibits the *fol*$^+$ enzyme by 95%, the *fol38* enzyme by about 20%, and the enzyme from RS119 by about 55%. These data indicate that about 60% of dihydrofolate reductase in RS119 is encoded by the *fol 38* allele. This agrees satisfactorily with the fact that there are probably 1–2 episomal *fol* genes per cell.

We next tested RS71/F101 *fol38*, which carries a chromosomal *fol60* allele with an I50 of 10 nM trimethoprim and an episomal *fol 38* allele. The trimethoprim-sensitivities of the enzymes from the strain and from RS71 are shown in Figure 4. Clearly there is very little difference in the drug-sensitivity of the enzymes from the haploid RS71 (*fol 60*) and the merodiploid RS71/F101 *fol38* (*fol60*/F *fol38*). This shows that virtually all the enzyme in this strain is encoded by *fol 60*, and hence that *fol 60* causes a high level of enzyme expression by a cis-acting mechanism.

Similar experiments demonstrated that *fol 73* and *fol 74* also act cis (data not shown). These are spontaneous mutations selected from D1 *fol 38* and D1 *fol 18* respectively, on the basis of increased resistance to trimethoprim in vivo. Thus all three *fol* regulatory alleles examined appear to act *cis*.

Discussion

a) Mutations Conferring Trimethoprim-resistance

This paper describes two mechanisms of trimethoprim-resistance in *E. coli* K12. Both affect dihydrofolate reductase. One class of mutants contains dihydrofolate reductase molecules with decreased affinities for trimethoprim, while the second class contains mutants with elevated enzyme activity.

The strongest mutants, *fol 50* and *fol 60*, express about 1.0 units/mg dihydrofolate reductase in a D1 background. Selection of trimethoprim-resistant mutants on plates containing as much as 50 μg/ml drug does not lead to mutants which express more enzyme than this. (R. Sheldon, unpublished results). This suggests that *fol 50* and *fol 60* are fully constitutive mutants. Since the enzyme has a molecular weight of about 22,000 daltons and a turnover number of about 500 moles substrate/mole enzyme/min, one can calculate that in fully constitutive strains the enzyme accounts for about 0.4% of cell protein. This compares favourably with mutants isolated in other prokaryotic species (Sirotnak and McCuen, 1973; Gundersen et al., 1972).

b) Control of Dihydrofolate Reductase

The results indicate that dihydrofolate reductase is controlled by a diffusible regulator, and that mutants causing increased expression of the enzyme act *cis*.

The data in Table 3 show that enzyme levels are higher in haploid strains such as RS87 carrying *fol* mutant regulatory alleles than in the corresponding heterozygous merodiploids such as RS87/F101. That this effect is not seen in strains with less pronounced mutant alleles may be due to the larger contribution of the episomal genes to the dihydrofolate reductase pool.

No trans-acting mutants have yet been isolated in haploid strains. One plausible explanation is that the regulatory molecule has other essential functions, and hence that mutations in the regulatory gene would be lethal in haploid strains.

It is not clear why the *trans* regulation is only 10–30% effective. It might be that the regulatory molecule has reduced affinity for the mutant *cis* regulatory sites. Conversely, it is possible that dihydrofolate

reductase is controlled by several different regulators, each of which is only partly effective. If the concentration of the regulator coded in the *fol* region is limiting it is clear that doubling the gene dosage would cause increased repression.

The data shown in Figure 4 demonstrate that *fol 60* is a *cis*-acting regulatory mutant. Both *fol 73* and *fol 74* also act *cis*. It is surprising that *fol 60* is temperature-sensitive. The data in Table 2 clearly show that the temperature-sensitivity and mutant regulatory properties of *fol 60* are related, since spontaneous temperature-stable mutants of *fol 60* have altered patterns of enzyme expression. It should be noted that the data do not define the level of action of the regulatory site.

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