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Biochemistry

Sulfonamide resistance mechanism in *Escherichia coli:* R plasmids can determine sulfonamide-resistant dihydropteroate synthases

(*Citrobacter/Klebsiella pneumoniae*/multiple drug resistance)

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ABSTRACT **Several natural isolate *E. coli* strains highly resistant to sulfonamides and antibiotics are shown to contain a sulfonamide-resistant dihydropteroate synthase (2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-diphosphate:4-aminobenzoate 2-amino-4-hydroxydihydropteridine-6-methenyltransferase, EC 2.5.1.15) in addition to the normal sensitive enzyme. The resistant dihydropteroate synthases examined are determined by an R plasmid and are smaller and less heat stable than the normal sulfonamide-sensitive enzyme. One synthase resistant to any sulfonamide tested, and to sulfanilic and arsanilic acids, was still inhibited by several non-sulfonamide analogs of *p*-aminobenzoate. *Citrobacter* and *Klebsiella pneumoniae* strains also show similar mechanisms of sulfonamide resistance.**

Sulfonamide resistance in *Escherichia coli* and related enteric rod bacteria has been common for many years and is normally associated with multiple resistances to various antibiotics (1–4). These resistance genes are usually carried on plasmids (5–7]. Sulfonamide resistance is currently a problem of considerable clinical importance, especially in urinary tract infections. Twenty-five to 80% of *E. coli* strains found in these infections are normally resistant to 100–1000 times the normal minimum inhibitory concentration of sulfonamide-sensitive strains (8). We will deal with 15 urinary tract strains of *E. coli* and the closely related *Citrobacter* highly resistant to sulfamethoxazole.

In this report we show that the most common sulfonamide resistance mechanism in *E. coli* and *Citrobacter*, and perhaps in *Klebsiella pneumoniae*, is the presence of an R-plasmid-determined sulfonamide-resistant dihydropteroate synthase (DHPS; 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine-diphosphate:4-aminobenzoate 2-amino-4-hydroxydihydropteridine-6-methenyltransferase, EC 2.5.-1.15). This is the enzyme which forms dihydropteroate from a pyrophosphorylated, partially reduced pteridine and *p*-aminobenzoic acid (PABA) on the path to dihydrofolate (see *Materials and Methods*). The mechanism that we report for sulfonamide resistance is reminiscent of that recently reported for R-plasmid-determined trimethoprim resistance (9, 10).

MATERIALS AND METHODS

Bacterial Strains. Twelve *E. coli* and three *Citrobacter* strains, resistant to 1000 μg or more of sulfamethoxazole, were obtained mostly from the urinary tract of patients in hospitals and in private practice (see Fig. 1). All strains were originally classed as *E. coli* by the donor laboratories. One strain was from the human upper respiratory tract; four strains were from unknown human sites; one strain was from a dog. From North Carolina came five strains; Texas, 4; New York, 3; Florida, 1; Australia, 1; France, 1. Seven sulfonamide-sensitive human urinary tract *E. coli* strains are included for comparison. All these strains were the gift of S. R. M. Bushby of these laboratories. A sulfonamide-sensitive *E. coli* K12 strain, J62-1 (nalidixic-acid-resistant and auxotrophic for proline, histidine, and tryptophan), was the gift of N. Datta. This strain is denoted "recipient" and was used to receive sulfa-resistant R plasmids from our wild-type strains. Wild-type strains, either sulfa-sensitive or sulfa-resistant, are denoted with "wt" before the strain number. J62-1 strains containing an R plasmid from a wild-type strain are denoted "C", for cross, followed by the strain number that was the source of the sulfa-resistant R plasmid. Thus C5166 is our K12 strain with an R factor from wt5166. Six out of the 15 strains of *E. coli* and *Citrobacter* transferred sulfonamide resistance to this *E. coli* strain.

Other Resistance Markers. Standard Kirby-Bauer techniques were used with drug-laden discs on Mueller-Hinton agar containing almost confluent growth of strains (11). All potential donors, crosses, and the recipient were tested for resistance to 14 drugs. All sulfonamide-resistant strains, whether transferable or not to the K12 recipient, had one to seven other resistances, with an average of four. All strains but two showed different resistance patterns. The strain reported on most extensively, wt5166, was resistant to sulfonamide and ampicillin (and to carbenicillin; data not shown).

R Plasmid Transfer. Overnight cultures of donor and recipient grown in Mueller-Hinton broth were diluted 50-fold and 500-fold, respectively, into a single tube of fresh broth and the mixture was then grown overnight without shaking (12, 13). This culture was then plated on Mueller-Hinton agar containing 200 μg/ml of sulfathiazole and 100 μg/ml of nalidixic acid. Resistant colonies were replated twice on the same medium and the crosses were tested for the markers of the recipient. All donors were fully or partially nalidixic-acid-sensitive.

DHPS Preparations. Strains were grown to late exponential phase in Mueller-Hinton broth and were washed with cold 30 mM sodium phosphate buffer, pH 7.0. The bacteria were broken by sonication and then incubated with DNase and RNase at about 100 μg/ml each, at 0° for 15 min. The sonicate was then centrifuged at 20,000 × *g* for 30 min at 0–4°, and the supernates were assayed as such or were applied to an 85 × 1.5 cm column of Sephadex G-100 and eluted with 10 mM sodium phosphate buffer, pH 7.0 (14), followed by assay.

Abbreviations: DHPS, dihydropteroate synthase; PABA, *p*-aminobenzoic acid.

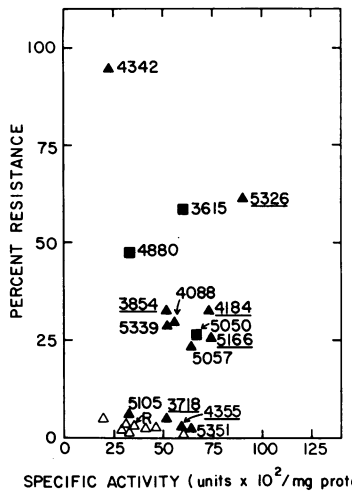

FIG. 1. Dihydropteroate synthase activity in wild-type *E. coli* and *Citrobacter* strains. Sulfonamide-resistant strains (▲) and sulfonamide-sensitive strains (△) of *E. coli* are shown with the K12 recipient strain denoted R. Sulfonamide-resistant *Citrobacter* strains (■) are also shown. Percent resistance is defined in the *text*. Values shown for resistant strains are the average of data from two or more batches of that strain. For a given strain the range of values of specific activity and percent resistance is at most ≤ ±20% of the average value for that strain with the high percent resistance organisms. The range is ≤ ±50% of the average specific activity and ≤ ±100% of the average percent resistance for the four low percent resistant strains that are sulfonamide-resistant. Underlined strains could transfer sulfonamide resistance to *E. coli* K12.

Assay of DHPS. The assay is based on that of others (14–16). The substrate 2-amino-4-hydroxy-6-pyrophosphorylmethyl-7,8-dihydropteridine (hydroxymethyldihydropteridine pyrophosphate), prepared essentially as in ref. 15, was present at 10 μM; [*carboxy*-^{14}C]PABA (10 Ci/mol), 20 μM except where noted; $MgCl_2$, 5 mM; Tris·HCl pH 8.3, 40 mM. Enzyme extract was added. The final total volume was 200 μl. The assay was run at 37° for 15 min to 1 hr. One hundred microliters were spotted on Whatman 3 MM paper and chromatograms were run descending for 45 min in phosphate buffer, pH 7.0, 0.1 M (0.04 M KH_2PO_4, 0.06 M Na_2HPO_4). Papers were dried and counted in a liquid scintillation counter. Protein was measured using a bovine serum albumin standard (17). Sulfonamide-insensitive DHPS was routinely measured in the presence of 10^{-4} M sulfathiazole. One unit of dihydropteroate synthase activity is 1 nmol/min.

Hydroxymethylpteridine was prepared by B. Roth, E. Aig, and B. Rauckman of these laboratories. The homopteroates were the gift of the The National Cancer Institute through the courtesy of R. Kisliuk, Tufts University.

RESULTS

Sulfonamide-resistant DHPS activity in crude extracts

DHPS activity per unit protein was measured in 20,000 × *g* × 30 min supernatant fractions of sonicated late exponential phase bacteria. These data for wild-type *E. coli* and *Citrobacter* strains are plotted on the abscissa of Fig. 1. We have calculated the ratio of DHPS activity measured in the presence of 10^{-4} M sulfathiazole to that measured in the absence of inhibitor. This ratio, multiplied by 100, is plotted on the ordinate of Fig. 1 as "percent resistance." There is considerable sulfa-resistant DHPS enzyme activity in many, but not all, sulfa-resistant strains. The specific activity of total (uninhibited) enzyme in the resistant strains averages only slightly more than that of sulfa-sensitive strains. The small "resistant" activity in the sensitive strains (and a few resistant strains) represents the truly sensitive activity remaining when only five times as much sulfonamide, 10^{-4} M, is present as PABA substrate, 2×10^{-5} M. Four resistant strains appear to have extracts no more resistant than the sensitive *E. coli*. The resistance mechanism in these strains may differ from that in the majority of strains, or the resistant enzyme in these strains may require different experimental conditions for detection.

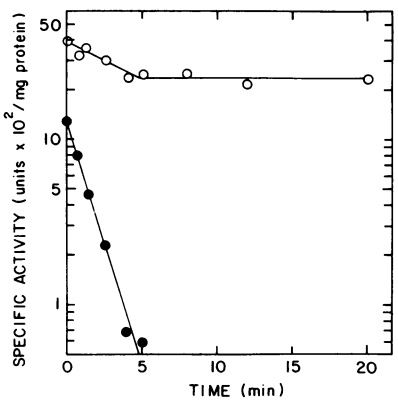

FIG. 2. Heat sensitivity of DHPS activity in *E. coli* wt5166 crude extract. Extract was heated at 50°; aliquots were removed at various times and were assayed for DHPS activity in the presence (●) or absence (○) of 10^{-4} M sulfathiazole.

Heat inactivation

The sulfa-resistant enzyme in crude extracts was more sensitive to heat than the sulfa-sensitive enzyme in all strains that we tested (wt3615, wt3854, wt4184, wt4880, and wt5166). The effect of heating wt5166 extract at 50° is shown in Fig. 2. The loss of total activity in uninhibited extracts approximates that of the sulfonamide-resistant activity. The heat resistance of *E. coli* strain B DHPS (14) and the heat sensitivity of *Lactobacillus plantarum* DHPS have been noted previously (16).

Lack of induction of DHPS enzymes

Strains in the high percent resistant enzyme class of Fig. 1 (wt4088, wt5166, wt5326) and in the low percent resistant enzyme class (wt3718 and wt5105) were grown exponentially in the presence and in the absence of 100 μg/ml of sulfathiazole for at least six generations. These 10 cultures were then assayed for DHPS in the presence and in the absence of sulfonamide. No significant induction of either resistant or sensitive enzyme was seen (data not shown).

In strain wt5166, grown without sulfonamide, the specific activity of the sulfa-resistant enzyme was relatively constant throughout the culture growth cycle, while that of the sulfa-sensitive enzyme decreased in the stationary phase. Thus the percent resistance shown in crude extracts of strain wt5166 was 70–75% in late stationary cells compared with 25–30% in early or late exponential growth.

Sephadex separation of variant DHPS enzymes

Fig. 3 shows the Sephadex G-100 profiles of sensitive and resistant DHPS in five strains. Fig. 3A shows the results for the K12 recipient strain. No resistant enzyme is seen. In Fig. 3B

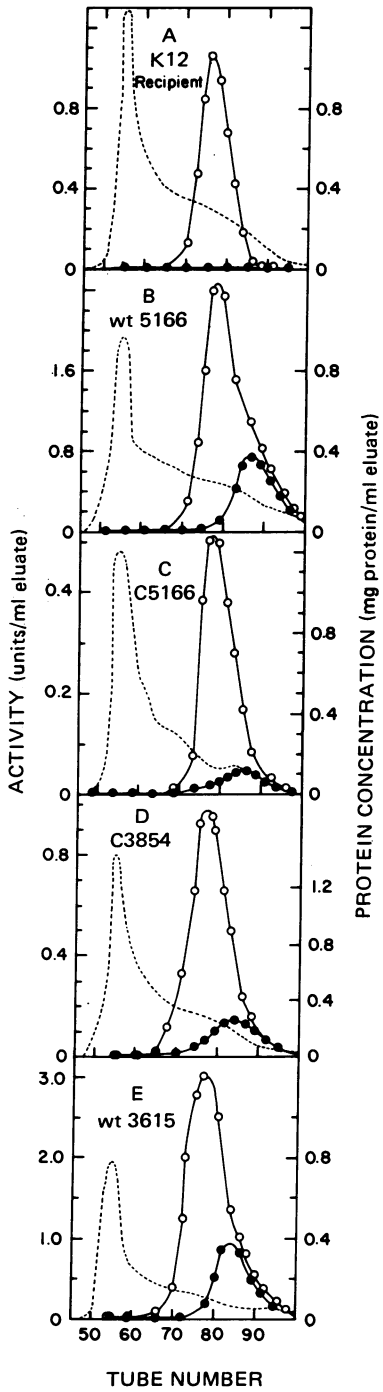

FIG. 3. Separation of *E. coli* and *Citrobacter* DHPS enzymes on Sephadex. One-half milliliter of extract from several strains was applied to an 85 × 1.5 cm Sephadex G-100 column in the cold room (4°) and eluted with 10 mM sodium phosphate, pH 7.0. Fractions of 0.85 ml were collected and assayed in the presence (●) and absence (○) of 10^{-4} M sulfathiazole. Protein concentration (- - - - -) is also shown. Protein applied to columns A-E was 22.5, 8.5, 15.2, 18.8, and 11.9 mg, respectively. Strain wt3615 is a *Citrobacter*, the others are *E. coli*. For molecular weight determinations 150 mM KCl was added to the phosphate buffer eluting fluid.

strain wt5166 has considerable sulfonamide-resistant DHPS activity whose peak appears 8 to 10 tubes after the peak of sulfonamide-sensitive DHPS activity. When the R plasmid of wt5166 is transferred into the K12 recipient the pattern of Fig. 3C results. Another transferable R plasmid in the K12 background yields Fig. 3D. Fig. 3E shows a *Citrobacter* strain whose resistance is not transferable to our K12 strain. A sulfonamide-resistant urinary tract strain of *Klebsiella pneumoniae*, wt3724, showed a Sephadex G-100 profile similar to that in Fig. 3E. This *Klebsiella* strain, when analyzed as the strains were for Fig. 1, showed a specific activity of 0.62 units/mg of protein in the absence of sulfonamide and 22% resistance. This is similar to *E. coli* wt5057 shown in Fig. 1.

Running the column with buffer plus KCl (see Fig. 3 legend) and calibrating it with ovalbumin, chymotrypsinogen, myoglobin, and alkaline phosphatase (18) yields values of about 49,000 daltons for the molecular mass of the sulfonamide-sensitive DHPS from strains K12, wt5166, and wt3615. This is not significantly different from the 50,000 dalton value reported by others for the sulfonamide-sensitive DHPS of *E. coli* strain B (14). The sulfonamide-resistant enzymes of wt5166, C5166, C3854, and wt3615 are similar to one another and are about 45,000 daltons.

The K_m for *p*-aminobenzoic acid, determined on the partially purified enzymes of *E. coli* wt5166, was 7×10^{-7} M for both sensitive and resistant enzymes.

Effect of other inhibitors on resistant and sensitive DHPS

In Fig. 4 the concentrations of sulfathiazole necessary to inhibit 50% of the activity (I_{50}) of the two DHPS enzymes of wt5166 are shown. The I_{50} values for sulfathiazole for the two enzymes are 1000-fold different. Using these same sensitive (S) and resistant (R) enzyme fractions, large and/or highly acidic PABA analogues such as sulfathiazole, sulfanilamide (and other sulfa drugs not shown), sulfanilic acid, and arsanilic acid all show a high degree of cross resistance. On the other hand, small PABA analogues such as *p*-aminosalicylic acid (PAS), and 2-chloro-4-aminobenzoic acid yield similar I_{50} values for both sensitive and resistant enzymes. With *p*-aminobenzoic acid amide the spread is greater and with *p*-hydroxybenzoic acid there is a significant reversal of sensitivities. This same reversal is seen in product inhibition by dihydropteroic acid. The inhibition by homopteroates was seen previously by S. R. Webb of these laboratories with *E. coli* B DHPS (personal communication). Homopteroic acid is an analog of pteroic acid with an additional methylene group between the pteridine and *p*-aminobenzoic acid moieties. Dihydrohomopteroic acid is the 7,8-dehydro compound.

p-Nitrobenzoic acid was inactive with both enzyme fractions although its toxicity to whole cells is reversed by PABA (19). Pneumococci show this same toxicity of *p*-nitrobenzoic acid to whole cells but not to extracted DHPS (20). Hence, this inhibitor may act on a target other than DHPS, and may be carried into the cell on the PABA transport system.

DISCUSSION

We have shown that there is sulfonamide-resistant dihydropteroate synthase (DHPS) activity in the majority of those *E. coli* and *Citrobacter* strains that are highly resistant to sulfonamides. Further, we have demonstrated that in several strains this resistant activity is caused by a sulfonamide-resistant DHPS enzyme that is present in addition to the major sulfonamide-sensitive DHPS, and that differs from the latter enzyme in size, heat stability, and sensitivity to various PABA analogues. Finally we show that R plasmids determine these resistant enzymes in some and perhaps most strains even though not all have a capability for transfer of resistance to *E. coli* K12.

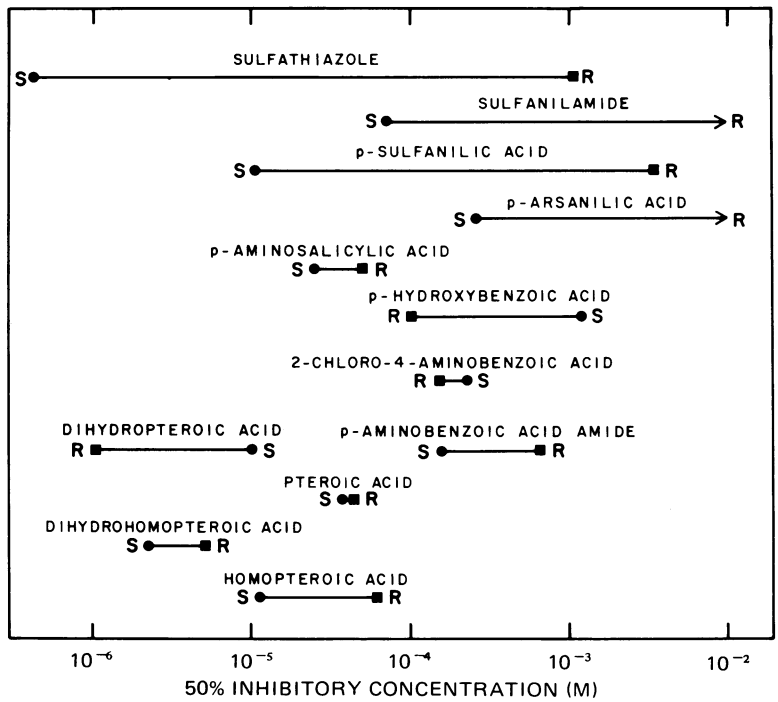

FIG. 4. Resistance of *E. coli* wt5166 DHPS enzymes. Sensitive enzyme (S) was taken from tubes 72–75 of the column shown in Fig. 3B and resistant enzyme (R) was taken from tubes 90–93 of that column. PABA concentration was lowered to 2 μM for these assays. The PABA analogues were recrystallized prior to use. Dihydrofolic acid at 2×10^{-4} M gave 42% inhibition of the sensitive enzyme and 26% inhibition of the resistant enzyme. Folic acid at 10^{-4} M gave about 10% inhibition of both enzymes. *p*-Nitrobenzoic acid at 10^{-2} M gave no inhibition of either enzyme. Arrows mean that the 50% inhibitory concentration for the resistant enzyme was greater than 10^{-2} M for sulfanilamide and *p*-arsanilic acid. (Folic acid is pteroylglutamic acid; dihydrofolic acid is 7,8-dihydropteroylglutamic acid.)

While we have shown a new mechanism for sulfonamide resistance in several natural isolates of *E. coli*, that does not mean there could not be other routes to sulfonamide resistance in various *E. coli* strains in addition to, or alternative to, a new insensitive, additional target enzyme. We have not yet explained the resistance mode of several strains shown in this first group of strains that we have examined. We have not yet examined lower resistance *E. coli* strains (i.e., minimum inhibitory concentration < 1000 μg/ml).

Although we have investigated only strains of *E. coli*, *Citrobacter*, and *Klebsiella pneumoniae*, R plasmids of these genera are transferable to a variety of other bacteria such as the closely related *Salmonella* and *Shigella* and the more distantly related *Proteus*, *Serratia*, *Pseudomonas*, *Vibrio*, *Pasteurella*, *Chromobacterium*, *Rhizobium*, and *Agrobacterium* (12, 13, 21). In addition, of course, these genera as well as other genera may be expected to have their own R plasmids of more (or less) restricted infectivity with similar sulfonamide resistance mechanisms.

Rather little has been reported by others on mechanisms of sulfonamide resistance in natural isolates of bacteria. Previous work with an R plasmid sulfonamide resistance in *E. coli* showed a 3-fold increase in the permeability barrier to sulfonamides (22). It seems unlikely that this alone could account for a 200 to 500-fold increase in resistance (1); hence this decreased permeability may be a mechanism ancillary to a main mechanism of resistance. One sulfonamide-resistant natural isolate of *Staphylococcus* greatly increased its PABA production, which could explain the resistance (23). Natural isolates of *Neisseria gonorrhoeae* and *Pneumococcus* may also use this latter mechanism (24, 25).

Trimethoprim, a broad spectrum antibacterial agent discovered in these laboratories, inhibits dihydrofolate reductase of many bacteria (26, 27) and its action on cells is synergistic with sulfonamides even with some highly sulfonamide-resistant strains (28–31). In R-plasmid-determined trimethoprim resistance a trimethoprim-insensitive dihydrofolate reductase is present, in addition to the sensitive enzyme (9, 10). We thus see a new mechanism, in clinical resistance to sulfonamide and to trimethoprim, in which the gene for a drug-insensitive target is brought into the cells of a previously drug-sensitive species, perhaps from a species that has been naturally resistant to the drug for eons without ever having seen that drug.

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