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TWO DIFFERENT EFFECTS OF MUTATION ON THE BINDING OF FOLATE ANTAGONISTS BY A MICROBIAL DIHYDROFOLATE REDUCTASE

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SUMMARY

Two types of mutant dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) with altered inhibitor binding have been identified with antifolate-resistant strains of *Diplococcus pneumoniae*. One class (mutant Type A) exhibits a small decrease in affinity for 4-aminofolate analogs (about 3-fold higher average K_i). The estimated K_i for binding of aminopterin and amethopterin by the wild-type enzyme is somewhat less than 10^{-10}. There is also a small decrease in affinity for dihydrofolate (1.5–2-fold higher K_m) and an increase in affinity for NADPH (2–4-fold lower K_m). The mutations which determine this class of enzyme are distributed at 4 distinct sites within the dihydrofolate reductase structural gene. A second class (mutant Type B) of mutant enzyme shows a marked decrease in affinity for 4-aminofolate analogs (50–100-fold higher K_i) and a shift in pH optimum for dihydrofolate reduction from 7.3 to 6.7. The mutations, which determine these enzymic effects, are clustered at one region of the structural gene. Binding of folate to the wild-type enzyme was almost 10^6-fold less than that of the 4-aminofolates, aminopterin and amethopterin. Binding of folate by mutant and wild-type enzymes was not dependent on pH within the range of 5.9 to 9.5. Binding of aminopterin by the wild-type enzyme was independent of pH within the range of 5.9 to 7.4, but decreased at pH levels above and below this range. The binding by a B mutant enzyme shows a somewhat greater dependence on pH within the same range. A discussion of these results in terms of the possible mode of binding of the pyrimidine moiety of these pteridine analogs is presented. Two related 2,4-diaminopyrimidines (NSC-110 180 and NSC-110 191) bearing both aminobenzoyl and aspartyl moieties were bound to the mutant and wild-type enzymes to nearly the same extent as the 4-aminofolates. The 5-aryl diaminopyrimidine, trimethoprim ($K_i = 3.7 \cdot 10^{-9}$) was bound better than the related pyrimethamine ($K_i = 2.46 \cdot 10^{-8}$) to A mutant and wild-type enzymes. Binding to a B mutant enzyme was reduced only 5-fold for pyrimethamine, but 100-fold for trimethoprim.

INTRODUCTION

A variety of chemical and steric factors have been examined[1–17] as to their importance for binding of 4-aminofolate analogs to dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3). The binding of both the substrate, dihydrofolate, and folate, as well as the 4-amino analogs appears to involve the interaction of dihydrofolate reductase with the pyrimidinyl, aminobenzoyl and glutamyl moieties[18]. The much stronger binding of the inhibitors[18] reflects the markedly more effective complexing of the 2,4-diaminopyrimidine at the active site.

In all of the work reported so far (see reviews by Baker[18] and Blakley[19]), the effect of structurally altering the inhibitor on binding to a particular dihydrofolate reductase has been studied. Another approach, which might conceivably yield useful information as to this enzyme–inhibitor interaction, would utilize mutant enzyme proteins altered in respect to binding. We have been studying a number of modified dihydrofolate reductase proteins from antifolate-resistant strains of *Diplococcus pneumoniae*. Some of our results obtained recently, which are specifically related to both substrate and inhibitor association, are reported here.

MATERIALS AND METHODS

Strains

Wild-type dihydrofolate reductase was obtained from a rough variant of the parental *D. pneumoniae* strain (R_6 variant from R. D. Hotchkiss, Rockefeller University). The various mutant enzymes were obtained from amethopterin-resistant strains, which bear a different individual mutation in the structural gene for dihydrofolate reductase at the AME I chromosomal locus[20–22]). Class A mutations are designated *ame*^{r}-3, -14, -71, -74, -75, -77, -78, -88, -89, -94, -97 and -98. Class B mutations are designated *ame*^{r}-4, -5, -62, -64, -66, -67, -68, -69, -70, -102, -103, -104, -105, -108, -109 and -110. The methods of strain isolation, the genetic characteristics of most of the mutations and the details of recombinational analysis by DNA-mediated transformation have already been described[20,21]. In addition to the enzymic effects described here, Class A mutations also determine quantitative effects on enzyme synthesis[23–26].

Reagents

Dihydrofolate was prepared from folic acid by the method of Blakley[27] and spectrophotometrically tested for purity. NADPH was purchased from Calbiochem. Folic acid, amethopterin (methotrexate) and aminopterin were generously supplied by Lederle Laboratories. Pyrimethamine (Daraprim) and trimethoprim were gifts of the Wellcome Research Laboratories. Two related 2,4-diaminopyrimidines (NSC-110 180 and NSC-110 191) were provided by Parke-Davis and Co. Amethopterin and aminopterin were chromatographically purified before use[28]. All other chemicals were reagent or analytical grade.

Enzyme assay

Dihydrofolate reductase activity in crude cell-free extracts was determined by a modified procedure of Osborn and Huennekens[29]. The methods of inhibition ana-

lysis used in these studies were given in earlier reports[23,24] and are essentially those described by Werkheiser[30] and Lineweaver and Burk[31].

RESULTS

Properties of wild-type and mutant forms of dihydrofolate reductase

The mutant forms of dihydrofolate reductase from *D. pneumoniae* are subdividable into two major categories on the basis of their interaction with both NADPH and dihydrofolate, and the 4-amino analogs of folic acid. The properties of these enzymes are compared to those of the wild-type enzyme in Table I. One class

TABLE I

PROPERTIES OF MUTANT AND WILD-TYPE DIHYDROFOLATE REDUCTASE FROM *D. PNEUMONIAE*

Dihydrofolate reductase activity in crude cell-free extracts was determined from the decrease in absorbance at 340 nm at 25 °C following the addition of 0.06 mM dihydrofolate, 0.065 mM NADPH and enzyme extract to 0.05 M potassium phosphate buffer with 12.8 mM 2-mercaptoethanol. Inhibition studies were carried out in the same system by adding drug 4 min prior to the addition of dihydrofolate. The values for K_m or K_i determined from double reciprocal plots were based on 2 to 3 replicate experiments for each enzyme.

Source	*No. of mutants*	*pH optimum*	$K_m \times 10^5$ *NADPH (M)*	$K_m \times 10^6$ *Dihydrofolate (M)*	$K_i \times 10^8$ *Amethopterin*** *(M)*
Wild-type	—	7.3	4.7	3.5	<0.01***
A mutant	12	7.3	1.1–2.1	4.9–7.8	0.01***
B mutant	16	6.7	4.2–4.7	3.3–3.9	0.35–0.41
Recombinant* (A and B)	12	6.7	1.0–2.1	5.1–7.7	1.1 –1.25

* Twelve recombinant strains were prepared in a cross between a B mutant (*ame*^{r}-5) and all of the A mutants.

** 4-Amino-10-methylpteroylglutamic acid.

*** Both wild-type and A mutant enzymes titrate amethopterin (Figs 1 and 2), therefore, a dissociation constant can only be estimated. That some dissociation of drug occurs with the wild-type enzyme (Fig. 1) and to a greater extent with the A mutant enzymes (Fig. 2) is evident from the inhibition obtained at drug concentrations above the molar equivalent concentration.

of mutant enzyme (mutant Type A) exhibits a finite decrease in affinity for both dihydrofolate (1.5–2-fold increase in K_m) and the inhibitor, amethopterin. Because of the tight binding of drug to both wild-type and A mutant enzymes, a dissociation constant (K_i) for the inhibitor–enzyme interaction can only be estimated. However, the effect of the A mutations on this interaction is readily apparent from the decreased ability of the mutant enzyme to titrate inhibitor (see Fig. 1). All of the mutant enzymes in this class also appear to exhibit a corresponding increase in affinity for the cofactor NADPH (the K_m for binding by each mutant enzyme decreased from 2–4-fold when compared to the wild-type enzyme) and are unaltered in respect to the pH optimum for dihydrofolate reduction. A preliminary study of the inhibition characteristics of some of these enzymes has been reported[22]. Moreover, they appear to exist partially and to a varying extent in an associated form having a mol. wt of 60 000 as compared to a mol. wt of 20 000 for the wild-type enzyme. Experimental details relating to this unusual physical effect are reported elsewhere[32,33]

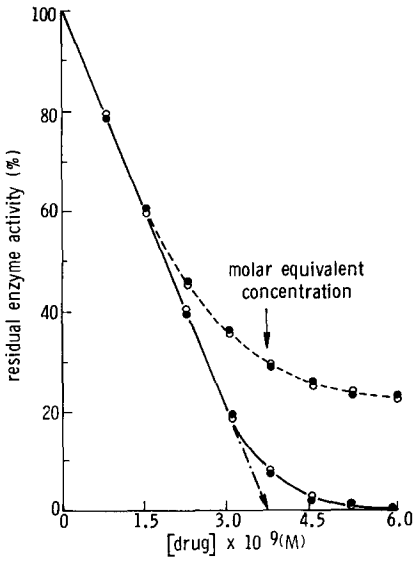

Fig. 1. The inhibition of wild-type and mutant dihydrofolate reductase from *D. pneumoniae* by folate analogs. The method of enzyme assay is given in Table I. Varying amounts of each analog are added to the reaction tube 4 min before the addition of dihydrofolate; wild-type enzyme, unbroken line; A mutant (*ame*^{r}-89) enzyme, dashed line; amethopterin (●), aminopterin (○).

A second class of mutant dihydrofolate reductase proteins (mutant Type B in Table I) exhibit a pronounced decrease in affinity for 4-aminofolate analogs. The dissociation constant for amethopterin binding by these proteins is about 50–100-fold higher than the estimated constant for binding by the wild-type. In contrast to Type A mutant proteins, no difference in either dihydrofolate or NADPH binding is observed with these enzymes. However, in each case there is an identical shift in pH optimum for dihydrofolate reduction to the acid side (from pH 7.3 to pH 6.7). None of the physical properties characteristic of the A mutant proteins have been associated with this second class of enzymes. One mutant Type B enzyme has been partially described in a previous communication[24] from this laboratory.

Related genetic information on the corresponding A and B type mutations is of some value in interpreting the biochemical data. The effects on the dihydrofolate reductase protein observed in both mutant classes can be attributed[21–25] in every case to single point mutations in the structural gene. The distribution within the gene of mutations determining A and B class dihydrofolate reductase proteins is shown in Fig. 2. The individual mutational sites were determined from an extensive recombinational analysis[21,22]. Mutations determining Class A enzymic effects are distributed among 4 distinct regions of the gene. The position of some of these mutations within each region may involve the same site. However, in most cases, they do not appear to involve identical mutations, since they are often distinguishable on the basis of other genetic and biochemical criteria.

Mutations determining Class B enzymic effects map at one region of the dihydrofolate reductase structural gene. Although the method of recombinational analysis[21] does not readily distinguish between mutational sites which are less than 1

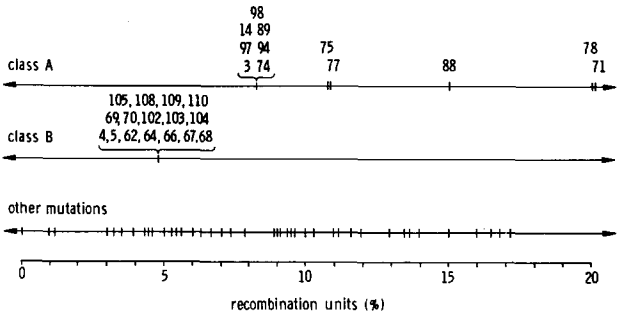

Fig. 2. The distribution of mutation sites in the dihydrofolate reductase structural gene. Sites of A and B mutations are indicated by numbers. The distribution of other mutational sites is also shown. Recombination experiments by DNA mediated transformation were carried out as two point crosses between two mutant strains, or by the extent of cotransfer of two mutations to a wild-type recipient. See Materials and Methods for appropriate references to the actual procedures.

recombination unit apart, all of these mutations may actually be identical, since they are genetically[25] (also Sirotnak, F. M., unpublished results) and biochemically (Table I) indistinguishable.

Dihydrofolate reductase from recombinant strains bearing both A and B mutations exhibits interesting properties (Table I). In one group, the pH optimum is characteristic of a B mutant enzyme and binding of dihydrofolate and NADPH resembles an A mutant enzyme. The joint effect of the A and B mutations on inhibitor binding is approximately a factoral function of each individual mutational effect. The K_i for binding of amethopterin by these recombinant enzymes is increased by about 3-fold over binding by the B mutant enzyme.

Quantitative differences in binding related to the 4-amino substitution in folate

By a comparison of both folate and aminopterin inhibition of *D. pneumoniae* dihydrofolate reductase, it was possible to analyze quantitatively the effect on binding of the amino *versus* the hydroxyl substitution at Position 4 of the pyrimidine

TABLE II

THE BINDING OF FOLATE ANALOGS TO MUTANT AND WILD-TYPE DIHYDROFOLATE REDUCTASE FROM *D. Pneumoniae*

Methods of enzyme assay are given in Table I. The values for K_i are based on 2 or 3 replicate determinations.

*Inhibitor**	$K_i \times 10^8$ *for inhibition (M)*			
	*Wild-type***	*A mutant***	*B mutant*	*Recombinant (A and B)****
Folate	4400	4500	4800	4700
Aminopterin	<0.01	0.01	0.39	1.21
Amethopterin	<0.01	0.01	0.41	1.19

* Folate, pteroylglutamic acid; aminopterin, 4-aminopteroylglutamic acid; amethopterin; 4-amino-10-methylpteroylglutamic acid.

** Values for K_i which are $0.02 \cdot 10^{-8}$ M or lower were estimated.

*** Prepared in a cross between A mutant, *ame*^{r}-89 and B mutant, *ame*^{r}-5.

moiety. The results obtained with wild-type and representative A and B mutant enzymes and a recombinant enzyme are summarized in Table II. The dissociation constant (K_i) obtained for folate inhibition of wild-type dihydrofolate reductase is $4.4 \cdot 10^{-5}$ M. Assuming that the K_m value for dihydrofolate ($3.5 \cdot 10^{-6}$ M) at least approximates a dissociation constant, there is about a 10-fold difference in binding in favor of the reduced from. The 4-amino analog, aminopterin, like amethopterin almost completely titrates this enzyme (Fig. 1). With an estimated K_i for inhibition somewhat below 10^{-10} M, this is almost a 10^6-fold increase in binding attributable to the 4-amino substitution. Both A and B mutant enzymes which were tested, bind folate to about the same extent as the wild-type enzyme. Similar to that observed with amethopterin, binding of aminopterin was somewhat less by the A enzyme (Fig. 1) and 50–100-fold less with the B enzyme (Table II). A study of drug binding to the recombinant enzymes also offers a means of more accurately quantitating the effect of A mutations. With the representative recombinant form used here, there was a 3–4-fold increase in K_i for aminopterin when compared to the B mutant enzyme.

The effect of pH on binding of folate and aminopterin to dihydrofolate reductase

The effect of the B mutations on inhibitor binding is of particular interest, since they appear to modify the interaction of the 2,4-diaminopyrimidine moiety at the active site of the enzyme. Not only is there a shift in pH optimum for catalytic activity (Table I), but these mutations clearly discriminate between amino and hydroxyl substitutions at Position 4 on the pyrimidine ring of the pteridine. We have examined the effect of pH on binding of both folate and aminopterin to the wild-type and a B mutant enzyme by an enzyme inhibition analysis. The results are given in Fig. 3. There appears to be no difference in binding of folate to either enzyme over a pH range of 5.75 to 9.05. Maximum binding of aminopterin to the wild-type enzyme, as indicated by the degree of inhibition, occurred between pH 5.9 and 7.4. The same

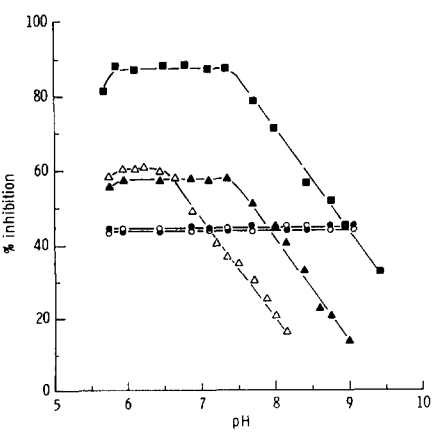

Fig. 3. The effect of variation in pH on the inhibition of B mutant and wild-type dihydrofolate reductase from *D. pneumoniae* by folate and aminopterin. The enzyme assay is described in Table I. The following buffers were used; 0.05 M sodium acetate–acetic acid at pH 5.7 to 5.9, 0.05 M Na_2HPO_4–NaH_2PO_4 at pH 5.9 to 8.0, 0.05 M Tris–HCl at pH 7.8 to 9.4. Wild-type enzyme with folate at $5.5 \cdot 10^{-4}$ M (●); aminopterin at $2 \cdot 10^{-9}$ M (▲) and aminopterin at $3.7 \cdot 10^{-9}$ M (■). B mutant (*ame*^{r}-5) enzyme with folate at $5.5 \cdot 10^{-4}$ M (○) and aminopterin at $1.3 \cdot 10^{-8}$ M (△).

effect on binding was noted when the aminopterin concentration was equal to ($3.7 \cdot 10^{-9}$ M) or below ($2 \cdot 10^{-9}$ M) the molar equivalent enzyme concentration (Fig. 1) in the assay tube. It was of some significance to note the result at a molar equivalent concentration, since at this concentration some dissociation of aminopterin from the enzyme is observed. Inhibition of enzyme activity appears to decrease below this range of pH, although adequate measurements were not possible because of the decreased enzyme activity and dihydrofolate solubility at this pH. Above pH 7.4, inhibition decreases linearly with increasing pH.

Binding of aminopterin to the B mutant enzyme (Fig. 3) is maximum when the pH is between 5.9 and 6.4. Inhibition of enzyme activity also appears to decrease below this range. As the pH is increased above 6.4, inhibition decreases in the same manner as that observed for the wild-type enzyme.

The binding of 2,4-diaminopyrimidines to mutant and wild-type dihydrofolate reductase

The binding by wild-type and mutant dihydrofolate reductase of 2,4-diamino-5-benzylpyrimidines, two of the so called small-molecule antifolates[14], has also been examined. The inhibition by these compounds is of interest, since the mode of binding[14,18] is somewhat different than of the folate analogs. The results of studies with two of these inhibitors, pyrimethamine and trimethoprim, are given in Table III.

TABLE III

THE BINDING OF SUBSTITUTED PYRIMIDINES BY MUTANT WILD-TYPE DIHYDROFOLATE REDUCTASE

Methods of enzyme assay are given in Table I. The values for K_i are based on 2 to 3 replicate determinations.

*Inhibitor**	$K_i \times 10^8$ *for inhibition (M)*			
	*Wild-type***	*A mutant***	*B mutant*	*Recombinant (A and B)****
Pyrimethamine	2.46	2.20	12.10	12.59
Trimethoprim	0.37	0.38	43.20	40.50
NSC-110 180	0.01	0.02	1.60	4.50
NSC-110 191	0.01	0.02	1.30	4.10

* Pyrimethamine, 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine; trimethoprim, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine; NSC-110 180, *N*-[*p*-[[*p*-(2,4-diamino-5-pyrimidinyl)benzyl]amino]benzoyl]-L-aspartic acid; NSC-110 191, *N*-[*p*-[[*p*-(2,4-diamino-6-methyl-5-pyrimidinyl)benzyl]amino]benzoyl]-L-aspartic acid.

** Values for K_i which are $0.02 \cdot 10^{-8}$ M or lower were estimated.

*** Prepared in a cross between A mutant, *ame*^{r}-89 and B mutant, *ame*^{r}-5.

Neither derivative was bound by the wild-type enzyme as effectively as the pteridines. Trimethoprim, however, was bound more effectively (7-fold) than was pyrimethamine. Binding of both pyrimidines by the A mutant enzyme was identical to binding by the wild-type. Binding to the B mutant enzyme was reduced, but to quite a different extent for each derivative. Whereas pyrimethamine binding to this enzyme was reduced about 5-fold when compared to binding by the wild-type enzyme, trimethoprim binding was reduced almost 120-fold. Net binding of both derivatives to the mutant enzyme now differs by 4-fold in favor of pyrimethamine.

We have also examined the binding to the *D. pneumoniae* enzyme of two dia-

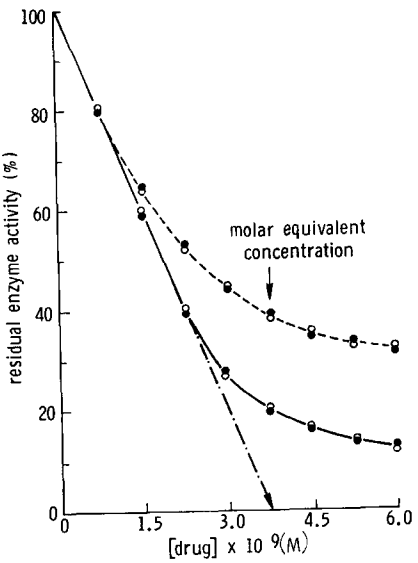

Fig. 4. The inhibition of wild-type and mutant dihydrofolate reductase from *D. pneumoniae* by substituted 2,4-diaminopyrimidine analogs. See Table I and Fig. 1 for experimental details and Table III for chemical nomenclature; wild-type enzyme, unbroken line; A mutant (*ame*^{r}-89) enzyme, dashed line; NSC-110 180 (●) and NSC-110 191 (○).

minopyrimidine derivatives modified by virtue of the attachment of both aminobenzoyl and L-aspartyl moieties. This permits a more adequate assessment of binding involving a 2,4-diaminopyrimidine when compared to the 2,4-diaminopteridine of amethopterin and aminopterin. Binding of two of these compounds available to this study, NSC-110 180 and NSC-110 191 are shown in Fig. 4 and Table III. Both compounds are very effective inhibitors of this dihydrofolate reductase. Binding to the wild-type enzyme is at a titration level only slightly less than the corresponding pteridinyl derivative. Binding to the other enzymes is reduced to about the same relative extent as is binding of the pteridines.

DISCUSSION

The strong binding of 4-aminofolate analogs by dihydrofolate reductase has been well documented by a number of workers (see reviews by Baker[18] and Blakley[19]). In the case of dihydrofolate reductase from wild-type *D. pneumoniae*, the binding of such an analog, either aminopterin, or the N^{10}-methyl derivative, amethopterin, is at least 500 000-fold greater than that of folate.

The interaction between *D. pneumoniae* dihydrofolate reductase and the 2,4-diaminopteridine inhibitors is affected in at least two different ways by mutation in the structural gene. The A mutational effects are most easily explained as a conformational alteration affecting the steric relationship between binding regions on the enzyme and the corresponding contact points on the substrate or inhibitor. This idea is in agreement with the genetic data and also with the increase in binding of NADPH also observed with these mutant enzymes. Similar effects on binding result when one

of a number of sites on the enzyme molecule, which appear to be crucial to structural integrity, are affected, by mutation. The opposing effects on substrate and cofactor binding are understandable, since a single conformational change which sterically interferes with one catalytically related association could enhance a second.

In connection with these effects on substrate and inhibitor binding, just discussed, the apparent association between enzyme molecules[32,33] also mediated by Class A mutations should be considered. This type of homopolymeric association might also interfere with substrate and inhibitor binding by making the binding sites on each molecule less accessible. There are a number of observations, however, which argue against this explanation for the decreased binding observed with Type A mutant enzymes. In the first place, although the binding of inhibitor by these enzymes is similar, the amount of association among them varies considerably[33]. Secondly, one would not expect to find a corresponding increase in the binding of NADPH. Thirdly that these mutant enzymes are under a fair degree of conformational stress can be construed from their marked instability[22]. Finally, the binding of the "small molecule" 5-arylpyrimidines does not appear to be affected by these mutations. This observation fits more readily with the idea of a generalized conformational alteration, since the binding of these small molecules, which involves only two closely positioned sites, would be less subject to steric distortion. In this connection, the results, showing that the binding of the larger 2,4-diaminopyrimidine derivatives, NSC-110 180 and NSC-110 191, were also affected by A mutations, is of some significance.

The lack of any effect by A mutations on folate binding is not readily understood. This might be related to the very weak binding of this 4-hydroxy analog (folate binding exhibits the largest K_i of any analog examined) reflecting the relative absence of interaction at the active site. Certainly, if the basis for decreased antifolate binding to these mutant enzymes is due solely to the greater inaccessibility of sites in an associated molecular form, then one would expect a similar effect on folate binding.

The B mutations appear to affect the active site directly, since they are qualitative in nature, *i.e.* they shift the pH optimum for dihydrofolate reduction and have a differential effect on binding which clearly discriminates between amino and hydroxyl substitutions at Position 4 of the pyrimidine ring. No other physical or catalytic effect has been associated with these mutations.

The absence of any effect of pH on binding of folate by the *D. pneumoniae* wild-type enzyme is in agreement with findings reported earlier[12] with an Ehrlich ascites enzyme. A somewhat different result showing partial pH dependence has been reported[18] following studies with a pigeon liver dihydrofolate reductase. On the other hand, the binding of aminopterin at varying pH by the wild-type enzyme is quite different from that reported in previous studies with the mammalian[12] and the avian[18] enzymes. In both of these studies, binding exhibited an optimum at pH 5.9 and decreased sharply when the pH was lowered or increased. Greater binding of 4-amino substituted folates as compared to folates has been attributed to the greater suitability of the former for either hydrogen bond[8,10,13] or ionic bond[4,7,18] formation at the active site. The results with the *D. Pneumoniae* enzyme showing no difference in binding between pH 5.9 and 7.4 are difficult to explain if one assumes that binding of the 2,4-diaminopyrimidine occurs solely by an ionic mechanism. Binding in this case should be maximum in the rage of the pK value (approx. 5.5) and diminish in

relation to the degree of protonation at higher pH. Protonation of aminopterin decreases 22-fold to about 1% in the pH range of 5.9 to 7.4[18]. Moreover, the decrease in binding that is observed occurs within a pH range (7.4–9.0) in which the inhibitor is essentially unprotonated. At the same time, folate ($pK \cong 2.5$), which exhibits no dependence on pH for binding, is almost completely unprotonated within the pH range of 5.9 to 9.0.

In contrast to the results obtained with the wild-type enzyme alone, the results on the effect of the B mutation (*ame*^{r}-5) on the pH dependence of aminopterin binding seem to agree more with an ionic mode of bonding of the 2,4-diaminopyrimidine ring to the enzyme. Two observations appear important in respect to this interpretation. First, the mutational effect is a differential one in that there is no effect on folate binding. This would appear to localize the effect to the contact site for Position 4 of the pyrimidine. Secondly, the decrease in binding of aminopterin determined by the mutation is only 50–100-fold, whereas the difference in binding of folate and aminopterin by the wild-type enzyme is $5 \cdot 10^5$–10^6-fold. If the difference in binding of these two analogs is due[8–10,13] only to the existence of an additional hydrogen bond between the 4-amino substituent and the enzyme, then the mutation would have to act by preventing the formation of this bond. This would be expected to result in a decrease in aminopterin binding to the level of folate binding.

A possible explanation for these apparently contradictory findings is that hydrogen bonding involving the 4-amino position of the pyrimidine ring occurs with the wild-type enzyme. The mutational change could interfere with hydrogen bond formation, but at the same time provide an electron donor site suitable to ionic bond formation. A more definitive understanding of the mode of bonding at this site will require additional investigation. It appears obvious, at least that any explanation must take into consideration a large diversity in binding kinetics among different enzymes.

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