The purity of the compounds was checked by ascending TLC on Merck's precoated silica gel plates (0.25 mm) with fluorescent backing.

The IR spectra were taken on a Perkin-Elmer 399 spectrophotometer in KBr. The C=O stretching lies between 1705 and 1710 cm^{-1}.

^1H NMR spectra (data reported in δ) were recorded on a Bruker 270 MHz spectrometer with Me_4Si as internal reference.

The ^1H NMR analysis of the reported compounds (Table III) showed the prochiral α-CH_2 on the 3-(*N*-alkylamino) chain as an AB system approaching an AX system, with chemical shifts lying at about δ 3.5 and 3.0 ppm (J_{AB} = 12 Hz). The difference between the chemical shifts of these geminal protons can be caused by the diamagnetic anisotropy of the carbonyl group deshielding the proton lying at lower field, while the other proton would be located in the shielding region of ring current of phenyl on C(2) of the thiazolidinone system,[7] as is supported by the X-ray structure of **7** (Figure 1a).

The C(5)-methylenic protons, H_A and H_B, of the thiazolidinone ring appeared as an AB system with a geminal proton coupling J_{AB} of 16 Hz. The A proton, lying at lower field, showed a small coupling (J = 2 Hz) with the C(2)-H signal. This long-range coupling takes place through the eclipsing lone pair of the sulfur atom with the C-H_A bond of the thiazolidinone ring. The H_B proton appeared at higher field owing to the shielding effect of the nearly coplanar sulfur orbital.[8,9]

General Procedure for the Preparation of 2-(Substituted phenyl)-3-[3-(*N*,*N*-dimethylamino)propyl]-1,3-thiazolidin-4-ones (1-7 and 9-15). An equimolar mixture (0.01 mol) of substituted benzaldehydes and *N*,*N*-dimethylpropylamine (0.01 mol) in dry benzene (50 mL) was refluxed until no more water was collected in a Dean-Stark trap. α-Mercaptoacetic acid (0.01 mol) was added, dropwise, to this crude mixture, and the reaction was carried out at reflux temperature until stoichiometric amount of water was collected.

The mixtures, cooled and evaporated in vacuo, afforded pale yellow oils, which were dissolved in anhydrous ethanol (20 mL). Ether (20 mL), saturated with HCl, was added to these solutions. White powders were collected and recrystallized with absolute ethanol, to yield the thiazolidin-4-one hydrochlorides (**1-7, 9-15**).

N,*N*-Dimethylethylamine and pyridine-4-carboxaldehyde were used, respectively, in the preparation of **1a** and **1b**.

2-(4-Aminophenyl)-3-[3-(*N*,*N*-dimethylamino)propyl]-1,3-thiazolidin-4-one (8). 2-(4-Nitrophenyl)-3-[3-(*N*,*N*-dimethylamino)propyl]-1,3-thiazolidin-4-one (**7**) (0.005 mol) was dissolved in an ethanol-water mixture (50 mL, 3:2, v/v). Iron filings and glacial acetic acid (1 mL) were added, and the mixture was refluxed for 3 h. It was cooled and filtered, made alkaline by sodium carbonate, and extracted ($CHCl_3$). The organic layer, evaporated in vacuo, afforded the crude base which was converted to the corresponding hydrochloride.

Guinea Pig Ileum in Vitro Assay for H_1-Receptor Histamine Antagonism. The assay was performed on ileum of either sex (weighing ~250 g) Percentage inhibition for guinea pig[5] was calculated on the response caused by 0.5 μM histamine in the absence of drugs. Six observations were carried out for each drug concentration. The results, IC_{50}, listed in Table II, are reported as concentration of drug causing 50% inhibition of the submaximal contractions induced by histamine.

The dissociation constant (K_B), for pA_2 value calculations ($pA_2 = -\log K_B$), was evaluated according to the method of Schild,[10] from the equation $K_B = B/(x - 1)$, where x is the respective ratio of concentrations of histamine needed to produce half-maximal responses in the presence and absence of different concentrations (B) of antagonists. pA_2 values, reported in Table II, are the average of five observations.

Acknowledgment. This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica.

Registry No. **1**, 141955-94-4; **1** base, 141956-11-8; **1a**, 141956-09-4; **1a** base, 141956-26-5; **1b**, 141956-10-7; **1b** base, 141956-27-6; **2**, 141955-95-5; **2** base, 141956-12-9; **3**, 141955-96-6; **3** base, 141956-13-0; **4**, 141955-97-7; **4** base, 141956-14-1; **5**, 141955-98-8; **5** base, 141956-15-2; **6**, 141955-99-9; **6** base, 141956-16-3; **7**, 141956-00-5; **7** base, 141956-17-4; **8**, 141956-01-6; **8** base, 141956-18-5; **9**, 141956-02-7; **9** base, 141956-19-6; **10**, 141956-03-8; **10** base, 141956-20-9; **11**, 141956-04-9; **11** base, 141956-21-0; **12**, 141956-05-0; **12** base, 141956-22-1; **13**, 141956-06-1; **13** base, 141956-23-2; **14**, 141956-07-2; **14** base, 141956-24-3; **15**, 141956-08-3; **15** base, 141956-25-4.

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Communications to the Editor

Probing the Molecular Basis of Resistance to Pyrimethamine by Site-Directed Mutagenesis

Rational drug design is based on understanding the principles of molecular recognition which govern receptor-ligand interactions. Recently, investigations of the mechanism of action and design of selective inhibitors for dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; DHFR) have been greatly facilitated by advances in X-ray crystallography, recombinant-DNA technology, and molecular modeling techniques.[1-3] The enzyme, by catalyzing the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to tetrahydrofolate (H_4F), is primarily responsible for the maintenance of essential intracellular cofactor pools of key importance for biosynthetic reactions requiring one-carbon unit transfer. Consequently, the synthesis of highly specific anti-folates has been spectacularly successful in producing powerful therapeutic agents.[3] For example, pyrimethamine is 1400-fold more active against the malaria enzyme isolated from *Plasmodium berghei* than the rat liver enzyme.[4] The basis for this specificity has been

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Table I. Steady-State Parameters for *E. coli* Wild-Type and *E. coli* Mutant Dihydrofolate Reductases

	wild-type[a]	T46S	T46N	T46A
V, s^{-1}	12.3	7.2 ± 1.1	8.5 ± 0.5	13.5 ± 1.1
$K_m(H_2F)$, μM	0.7	0.87 ± 0.3	1.25 ± 0.37	1.1 ± 0.05
V/K, μM^{-1} s^{-1}	17.6	8.28	6.8	12.3
$K_m(NH)$, μM	5.0	3.0 ± 0.5	3.2 ± 0.4	
$pK_a(V)$	8.4	8.48	<4.0	7.13
^DV pH 6.0	1.0	1.1 ± 0.05	2.19 ± 0.01	2.3 ± 0.1
^DV pH 9.0	2.7	2.9 ± 0.1	nm	3.4 ± 0.4

[a] *E. coli* parameters were taken from Fierke et al.[14] nm = not measured.

postulated to result from species variation along the active-site surfaces.[5]

Unfortunately, shortly after implementation, the therapeutic efficacy of pyrimethamine was jeopardized by the emergence of resistant strains of *Plasmodium falciparum*. The resistance was not caused by dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene amplification or via a reduction in the drug permeability of the cells.[6] Resistant strains from various sources, showed a 400–1000-fold increase in the ID_{50} for pyrimethamine.[7] The cDNAs for DHFR from these strains were isolated, and sequence analysis revealed an asparagine for serine substitution at residue 108 in all cases.[8,9] Sequence alignment of *P. falciparum* DHFR with *Escherichia coli* and vertebrate DHFRs revealed that serine-108 corresponds to strictly conserved threonine-46 (Figure 1). Since an X-ray structure of the malarial DHFR does not exist, structures of the bacterial and vertebrate DHFR–ligand complexes have been used to analyze the structural basis for resistance[9] owing to the remarkable conservation observed in DHFR tertiary structures despite sequence homologies as low as 30%. Consequently, we sought to quantify the ability of *E. coli* DHFR to serve as a useful model for the malarial enzyme, substituting by oligonucleotide mutagenesis position 46 with serine, asparagine, and alanine, and evaluating the respective kinetic parameters for these mutant enzymes.

Mutagenesis at position 46 was carried out by cassette mutagenesis and randomly selected clones screened by dideoxy sequencing.[10,11] Each mutant was purified by methotrexate affinity chromatography.[12]

Table II. Dissociation Rate Constants (s^{-1}) of Ligands and Rates of Hydride Transfer (s^{-1}) for Mutant and Wild-Type *E. coli* Enzymes

		E. coli[a]	T46S	T46N	T46A
NADPH	E	3.5	60 ± 7	56 ± 5	28 ± 1
$NADP^+$	E·H_4F	200	230 ± 10	253 ± 51	274 ± 7
H_2F	E	22	76 ± 6	56 ± 5	28 ± 1
H_4F	E	1.4	3.6 ± 0.6	14 ± 2	12.2 ± 0.5
	E·N	2.4		13 ± 2	
	E·NH	12	10 ± 2	18 ± 4	52 ± 5
k_H		950	120 ± 10	25 ± 5 (pH 6.0)	20 ± 2

[a] *E. coli* parameters were taken from Fierke et al.[14]

All kinetic measurements were obtained at 25 °C in buffers of constant ionic strength containing 50 mM 2-morpholinoethanesulfonic acid (Mes), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM sodium chloride (MTAN buffer, pH 5–10) or 25 mM Mes, 50 mM Tris, 25 mM sodium acetate, and 100 mM sodium chloride (MTEN buffer, pH 4–5).[13] Steady-state kinetics and pre-steady-state kinetics were determined by absorbance and fluorescence measurements (Tables I and II).[14]

Thermodynamic dissociation constants (K_d) for pyrimethamine from the mutant DHFRs were measured by fluorescence titration (Table III).[15] Enzyme concentrations used were either at or slightly less than the K_d of the ligand. K_i values listed in Table III for pyrimethamine and the mutants were determined by Dixon plot analysis at pH 7.0.[16]

Kinetic Characterization. The complete kinetic mechanisms for bacterial and vertebrate DHFRs have been elucidated and shown to contain the following key features: (1) H_4F release limits steady-state turnover at neutral pH, (2) the preferred pathway for H_4F release is from the mixed ternary E·NH·H_4F complex, and (3) the overall reaction strongly favors H_4F formation due in part to the high value associated with the internal equilibrium (K_{int} = 100–1000) for the reactive ternary complexes.[14,17,18] Therefore, the impact of mutant DHFRs on turnover can be attributed to the perturbation of one or more steps within the kinetic mechanism.

Replacement of threonine 46 with serine caused a 40% reduction in the pH-independent V while the $K_m(H_2F)$ was maintained (Table I). This resulted in a 2-fold reduction in the V/K_m. The steady-state kinetic deuterium isotope effects (^DV at pH 6.0) were little changed, since the rate

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Table III. Thermodynamic Dissociation Constants K_d (nM) and Inhibitor Constants for Pyrimethamine to Mutant and Wild-Type DHFRs

constant	*E. coli*	T46S	T46N	T46A	*P. falc.*	S108N
K_d(NH)	330[c]	4300 ± 200	1500 ± 100	189 ± 20		
K_d(H_2F)	220[c]	2400 ± 300	2000 ± 100	243 ± 30		
K_d(pyr)	13 ± 2.0	1.5 ± 1.4	16 ± 6.0	1.4 ± 0.9		
K_i(pyr)	10[a]	5.2 ± 0.5	20 ± 3.0	1.2 ± 0.1	0.19[b]	2.0[b]
K_d/K_i(pyr)	1.3	0.29	0.8	1.2		

[a] Taken from Stone et al.[16] [b] Taken from Chen et al.[6] [c] Taken from Fierke et al.[14]

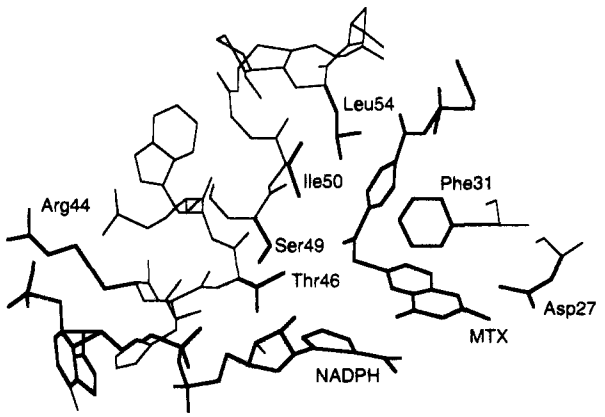

Figure 1. Active site of *E. coli* DHFR containing bound NADPH and MTX.[1]

of the hydride-transfer step (120 s^{-1}), although decreased, still exceeds the loss of H_4F from E·NH·H_4F (10 s^{-1}). Nevertheless, the increase by greater than 1 order of magnitude (Table III) in the dissociation constants of the substrate and cofactor from their respective binary complexes is indicative of differences between the mutant and wild-type enzyme complexes.

Perturbation of substrate binding or cofactor binding or both are consistent with the location of the side chain at the interface of the ligand binding sites. Projection of the threonine γ-methyl into the folate binding site contributes to maintenance of the necessary hydrophobic surface. Loss of this moiety is reflected in both decreased ligand affinity and a reduction in the rate of the chemical step. However, this substitution is not reflected in the product complexes as evidenced by the unperturbed off rates for $NADP^+$ and H_4F from various binary and ternary complexes, suggesting different interactions dominate for these intermediates.

Surprisingly, the values for V, K_m, and consequently V/K_m for the threonine-46 to alanine mutant were close to those obtained for the wild-type enzyme. However, unlike the T46S mutant, the apparent pK_a of the enzymatic reaction decreased by 1.3 pK_a units (Table I). This change is explained by the substantial shift in the rate-limiting step from the assisted product off-rate to the chemical step, as indicated by the 2-fold increase in the deuterium isotope effect on V (^DV at pH 6.0). The observed nearly 50-fold reduction in the rate of hydride transfer (Table II) is in accord with the observed deuterium isotope effect. This reduction translates into a loss of greater than 2 kcal/mol in stabilization energy for the hydride-transfer step. A 5-fold increase in the off-rate of H_4F from the E·NH·H_4F product complex is also consistent with the loss of a critical interaction which compensates for the reduced rate of hydride transfer. The K_d and consequently K_m for H_2F remain unchanged so that V/K_m is close to that of the wild-type enzyme.

Unlike threonine, the side chain of asparagine contains a hydrogen-bond donor and acceptor as well as an additional methylene. These characteristics should allow the side chain to maintain the threonine γ-hydroxyl contacts while simultaneously probing the positions sensitivity to steric bulk. The T46N mutant exhibited a slight decrease in V from 12.3 to 8.5 s^{-1} and an unchanged K_m(H_2F). Analysis of the K_d values for the cofactor and substrate revealed a 4.5–10-fold decrease in the stability of the E·NH and E·H_2F complexes. This trend is reflected in the increase in the ligand off-rates from their respective binary complexes. However, the substrate ternary complex, as represented by the K_m values for both H_2F and NADPH, exhibited no significant alteration.

Current rationalization of protein mutational effects maintains that nondeleterious substitutions exert their influence in an independent and additive fashion.[19] Therefore, changes in one part of a binding site should be localized to that region of the active site. However, it was discovered recently that although separated by a distance of approximately 25 Å, the *E. coli* arginine-44 to leucine mutant destabilized the catalytically essential aspartate-27 by 2.0 pK_a units (Figure 1).[20] This result demonstrated that point-site mutations that do not directly affect ligand binding or significantly alter the tertiary structure of the enzyme can transmit perturbations over long distances. This behavior is also found for the T46N mutant. Marginal changes in the off-rates for $NADP^+$ from the E·N·H_4F complex and for H_4F from the E·NH·H_4F complex and the unperturbed K_m values for the cofactor and substrate are in agreement with the largely nondeleterious effect of the T46N mutant. Nevertheless, the pK_a of the enzymatic reaction was dramatically decreased by at least 2.4 units. A similar decrease in the pK_a of approximately 2 pK_a units was observed with the *P. falciparum* S108N mutant.[6] An explanation for this dramatic effect cannot be rationalized in terms of a direct stabilization of aspartate-27 by asparagine because the side chains are separated by at least 10 Å. The similarity of the effects on the pK_a for the enzymatic reaction by the *E. coli* T46N mutant and *P. falciparum* S108N mutant, regardless of the active site, provides evidence for compartmentalization of the DHFR active site and provides evidence of the transferability of subtle long-range perturbations between species.

Effects on Inhibitor Binding. The dissociation constants for pyrimethamine for the *E. coli* T46S, T46A, and T46N mutants reflect the trends observed for inhibitor binding. Consistent with the increased sensitivity to pyrimethamine observed for *P. falciparum*, the binding of pyrimethamine to the T46S mutant was enhanced by 1 order of magnitude. A similar increase in the binding affinity was observed for the T46A mutant, implicating the γ-methyl and not the γ-hydroxyl as necessary for tight binding. Furthermore, in accord with the mutation in the malarial enzyme, a 10-fold difference in the dissociation constants for the T46N and T46S mutants was observed

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with the T46N showing decreased affinity for pyrimethamine.

In order to probe perturbations of the more physiologically relevant E·I·NH complex, the K_i values were determined for the inhibitor with the mutants. In only one case does the cofactor impart a synergistic decrease in the binding affinity for the inhibitor. Although the K_i of pyrimethamine for the T46S mutant is a factor of 3.5 greater than the K_d, it still remains lower than the K_i for the wild-type enzyme. Compared to T46S, the T46N mutant exhibits a modest 4-fold increase in the K_i which corresponds to the increase observed for the S108N mutant over wild-type malarial enzyme. Overall the malarial and bacterial DHFRs respond similarly to active-site perturbation with respect to pyrimethamine binding.

Conclusions. Although, significant perturbations by the mutant DHFRs are observed for individual rate constants along the kinetic pathway, their impact on the steady-state parameters is minimized by their compensatory nature. These results are consistent with the transferable nature of kinetic effects observed for other DHFR mutations.[21]

The ability of pyrimethamine to distinguish between the bacterial and malarial enzymes is largely due to the threonine γ-methyl, with little impact on the overall catalytic effectiveness of the enzyme by mutations at this site. Recent isolation of mildly resistant *P. fariciparum* isolates containing the S108T mutation are in agreement with these findings.[9] A reduction in affinity of the malarial enzyme by a factor of 10 or less, accurately reflected in this *E. coli* protein set, has been shown to confer physiologically relevant resistance to the parasite.[6] Consequently, in the absence of X-ray structural data for the malarial DHFR-thymidylate synthase binary enzyme, *E. coli* DHFR can serve as a useful model of the malarial enzyme. The parasites' strategy of using catalytically silent mutations in order to become less sensitive to pyrimethamine might be circumvented in the future by an appropriate antifolate design.

Acknowledgment. We thank Dr. Patricia Jennings, for preparing the mutant DNA, and Kaye Yarnell, for the expert typing of the manuscript.

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† Supported by NIH research Grant GM24129—C.R.W. was an NIH Postdoctoral Fellow.

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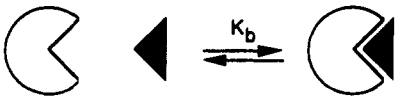
Use of Affinity Capillary Electrophoresis To Measure Binding Constants of Ligands to Proteins[1]

This paper outlines the use of affinity capillary electrophoresis[2,3] (ACE) as a technique for measuring binding constants of proteins for ligands. We illustrate this use with a model system comprising carbonic anhydrase B (CAB, EC 4.2.1.1, from bovine erythrocytes) and 4-alkylbenzenesulfonamides.[4] The principle of the method is illustrated schematically in eq 1.



(1)

Mass	M	m	M + m
Net Charge	Z	± z	Z ± z
Electrophoretic Mobility μ	~ Z / $M^{2/3}$		~ (Z ± z) / $(M + m)^{2/3}$

The electrophoretic mobility μ of a protein is related to its mass (M) and net charge (Z) by a relationship of the approximate form $\mu \sim Z/M^{2/3}$.[5] If the protein binds a charged ligand of relatively small mass, the change in μ due to the change in mass [from $M^{2/3}$ to $(M + m)^{2/3}$] is small relative to the change in μ due to the change in charge (from Z to $Z \pm z$). Thus, the protein–ligand complex will migrate at a different rate than the uncomplexed protein.[6] By measuring migration times (t) as a function of the concentration of charged ligand present in the buffer, it is possible to estimate K_b. These measurements are best carried out by measuring changes in t relative to another protein having a similar value of migration time

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(5) This expression is approximate. Other functional forms have been suggested: Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. Correlation of Electrophoretic Mobilities From Capillary Electrophoresis With Physicochemical Properties of Proteins and Peptides. *Anal. Biochem.* **1991**, *197*, 197–207. Grossman, P. D.; Colburn, J. C.; Lauer, H. H. A Semiempirical Model for the Electrophoretic Mobilities of Peptides in Free-Solution Capillary Electrophoresis. *Anal. Biochem.* **1989**, *179*, 28–33. Deyl, Z.; Rohlicek, V.; Adam, M. Separation of Collagens by Capillary Electrophoresis. *J. Chromatogr.* **1989**, *480*, 371–378. Offord, R. E. Electrophoretic Mobilities of Peptides on Paper and Their Use in the Determination of Amide Groups. *Nature* **1966**, *211*, 591–593.

(6) If μ_b and t_b are the values of electrophoretic mobility and migration time of the protein–ligand complex, and μ_0 and t_0 are these values for free protein, eq i allows an estimate of the change in mobility or migration time for given values of Z and z.

$$(\mu_b - \mu_0)/\mu_0 = (t_b - t_0)/t_0 \approx [(z/Z) + 1][M/(M + m)]^{2/3} - 1 \approx z/Z \quad \text{(i)}$$

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