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Crystallographic Analysis Reveals a Novel Second Binding Site for Trimethoprim in Active Site Double Mutants of Human Dihydrofolate Reductase†,‡

Vivian Cody^{*,§,||}, Jim Pace[§], Jennifer Piraino[§], and Sherry F. Queener[⊥]

[§]Structural Biology Department, Hauptman Woodward Medical Research Institute, 700 Ellicott St. Buffalo, New York 14203

^{||}Structural Biology Department, School of Medicine and Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260

[⊥]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Abstract

In order to produce a more potent replacement for trimethoprim (TMP) used as a therapy for *Pneumocystis pneumonia* and targets dihydrofolate reductase from *Pneumocystis jirovecii* (pjDHFR), it is necessary to understand the determinants of potency and selectivity against DHFR from the mammalian host and fungal pathogen cells. To this end, active site residues in human (h)DHFR were replaced with those from pjDHFR. Structural data are reported for two complexes of TMP with the double mutants Gln35Ser/Asn64Phe (Q35S/N64F) and Gln35Lys/Asn64Phe (Q35K/N64F) of hDHFR that unexpectedly show evidence for the binding of two molecules of TMP: one molecule that binds in the normal folate binding site and the second molecule that binds in a novel subpocket site such that the mutated residue Phe64 is involved in van der Waals contacts to the trimethoxyphenyl ring of the second TMP molecule. Kinetic data for the binding of TMP to hDHFR and pjDHFR reveal an 84-fold selectivity of TMP against pjDHFR (K_i 49 nM) compared to hDHFR (K_i 4093 nM). Two mutants that contain one substitution from pj- and one from the closely related *Pneumocystis carinii* DHFR (pcDHFR) (Q35K/N64F and Q35S/N64F) show K_i values of 593 and 617 nM, respectively; these K_i values are well above both the K_i for pjDHFR and are similar to pcDHFR (Q35K/N64F) and Q35S/N64F (305 nM). These results suggest that active site residues 35 and 64 play key roles in determining selectivity for pneumocystis DHFR, but that other residues contribute to the unique binding of inhibitors to these enzymes.

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[‡]Coordinates and crystallographic structure factors for mutant human DHFR TMP complexes have been deposited in the Protein Data Bank under the accession codes 3S3V and 3N0H.

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^{*}To whom correspondence should be addressed. cody@hwi.buffalo.edu. Phone: 716 898-8614. Fax: 716 898-8660.

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The numbering used throughout this paper for the human DHFR sequence is based on the first position being Val-1 rather than Met-1 as observed in the gene sequence listing. This numbering scheme has been used in previous publications of the kinetic and structural data for hDHFR and is being used here for continuity.

Introduction

Trimethoprim (TMP, Fig. 1) in combination with sulfamethoxazole is a potent antibacterial agent and is the first line therapy for pneumonia caused by *Pneumocystis jirovecii*, a common opportunistic fungal pathogen in immunosuppressed patients (Benfield et al, 2008; Hughes, 1991; Hughes et al, 1974; Kovacs et al, 2001; Totet et al, 2004). The mechanism of action of TMP is the inhibition of dihydrofolate reductase (DHFR), an enzyme that participates in the recycling of folates by reducing dihydrofolate to tetrahydrofolate in bacteria, pneumocystis and mammalian cells.

Selectivity of TMP action is based upon significant differences in the structures of the DHFR from various organisms. When used against bacteria, TMP is highly selective, with K_i values for hDHFR over 3000 times higher than the K_i for bacterial DHFR (Birdsall et al 1983). Selectivity is not as great for pjDHFR; the ratio of hDHFR/pjDHFR K_i values for TMP is below 100 (Cody et al 2009). The relatively weak action of TMP against pjDHFR makes the selection of resistant mutants likely. Indeed, many mutations of pjDHFR are now being noted world-wide, although as yet there is no definitive evidence for frank resistance (Ma et al 1999; Raman et al, 2008; Nahimana et al, 2004.). Drug design to produce more potent replacements for TMP depend upon understanding determinants of both potency and selectivity of antifolate binding to DHFR from mammalian host cells and from the fungal pathogen, *Pneumocystis*. To approach this problem, we have explored the effects of artificially inserting pj-like residues into hDHFR, with the dual goal of understanding how to develop compounds of greater potency towards pjDHFR and with better selectivity (i.e., hold potency toward hDHFR the same or reduce it). We have also inserted analogous mutations to make the active site of hDHFR more like *P. carinii* (pc) DHFR; *P. carinii* is the strain of *Pneumocystis* that infects rats and the DHFR from this strain has been widely studied (Chan et al, 2005; Gangjee et al, 2010).

We herein report the first crystal structures of TMP bound as binary complexes with two active site double mutants of hDHFR (Q35S/N64F; Q35K/N64F) that make the hDHFR active site more like pjDHFR or more like pcDHFR. These data are compared with the wild type hDHFR binary complex with TMP (Cody, 2002), the wild type hDHFR ternary complex with TMP and NADPH (PDB entry 2w3a), and the previously determined structure of pcDHFR ternary complex with NADPH and TMP (Champness et al, 1994). These structural data are also compared with similar studies of the hDHFR single and double mutants and the potent TMP derivative, PY957 (Fig. 1) (Cody, et al, 2009).

EXPERIMENTAL PROCEDURES

Expression and Purification of DHFR

The expression and purification of recombinant hDHFR and its active site mutants were carried out as previously described (Cody et al, 2009).

Crystallization and data collection

hDHFR-Q35S/N64F-NADPH-TMP and hDHFR-Q35K/N64F-NADPH-TMP – Crystals were grown at 14°C by vapor diffusion on glass cover slips. The protein was incubated with a 10:1 molar excess of NADPH and TMP over ice for one hour prior to crystallization and the protein concentrated to 9.0 mg/mL and dissolved in 100 mM K_2HPO_4 , pH 6.9, 30% saturated $(NH_4)_2SO_4$ and set up with 10 μ L drops over a reservoir of 100 mM K_2HPO_4 , pH 6.9, 60% saturated $(NH_4)_2SO_4$, and 3% v/v EtOH. Both mutant hDHFR crystals were cryoprotected with Paratone-N oil (Hampton Research, Aliso Viejo, CA).

hDHFR-TMP—Crystals were grown at 20°C by vapor diffusion on glass coverslips. The protein concentration was 9.0 mg/mL in 100 mM phosphate buffer, pH 7.5, with 10 μ l drops over a reservoir of 61% saturated $(\text{NH}_4)_2\text{SO}_4$.

Data were collected at the Stanford Synchrotron Research Lightsource (SSRL) facility using the remote access protocol (Cohen et al, 2002; Gonzalez et al, 2008; McPhillips et al, 2002) on beamline 9-2 for the hDHFR Q35K/N64F double mutant complex of hDHFR and on a Rigaku RaxisIVc imaging plate system with MaxFlux optics for the Q35S/N64F double mutant complex with hDFHR. Crystals of the wild type hDHFR-TMP binary complex were mounted in a capillary and data collected at room temperature on an RaxisIIc area detector. Diffraction statistics are shown in Table 1 for these complexes.

Structure Determination

The structures of the two mutant hDHFR complexes were solved by molecular replacement methods using the coordinates for wild-type human DHFR (1U72) (Cody, et al, 2005) in the program Molrep (CCP4, 1994). Inspection of the resulting difference electron density maps was made using the program COOT (Emsley & Cowtan, 2004) running on a MacG5 workstation and revealed both structures were binary inhibitor complexes. To monitor the refinement, a random subset of all reflections was set aside for the calculation of R_{free} (5%). The model and parameter file for TMP was prepared using the Dundee PRODGR2 Server Website (<http://davpc1.bioch.dundee.ac.uk/programs/prodrg>) (Schuettelkopf et al., 2004). The final cycles of refinement were carried out using the program Refmac5 in the CCP4 suite of programs (CCP4, 1994). The Ramachandran conformational parameters from the last cycle of refinement were generated by RAMPAGE (Lovell et al, 2002) and showed that the majority of residues in both complexes have the most favored conformation and none are in the disallowed regions (Table 1). Coordinates and structure factors for the mutant hDHFR structures have been deposited with the Protein Data Bank (accession numbers 3S3V and 3N0H).

The structure of the hDHFR-TMP binary complex was solved by molecular replacement using the coordinates of hDHFR MTXT ternary complex (Cody et al., 1992). The crystals grew in a rhombohedral system, space group R3, with hexagonal indexing, with lattice parameters of $a=b=85.66$, $c=77.61$ Å, data were collected to 1.90 Å resolution. R_{merge} was 5.2% for 2σ data for 13623 unique reflections. Refinement was carried out using the restrained least squares program PROLSQ (Hendrickson et al, 1980; Finzel, 1987) in combination with the model building program CHAIN (Sacks, 1988). $R = 21.3\%$. Since the original frames are no longer available, the data could not be reprocessed to complete the refinement with current software.

Kinetic analysis

Standard DHFR assays were conducted at 37°C with continuous recording of change of OD at 340 nm. The assay contained 41 mM sodium phosphate buffer at pH 7.4, 2-mercaptoethanol 8.9 mM, 150 mM KCl, and saturating concentrations of NADPH (117 μ M) and dihydrofolic acid (DHFA; 15 to 75 μ M). Initial linear rates of enzyme activity were measured; rates were linear under standard conditions for 1-5 minutes, depending upon the enzyme being assayed. Activity was linearly related to protein concentration under these conditions of assay.

K_m values were determined by holding either substrate or cofactor at a constant, saturating concentration and varying the other over a range of concentrations. K_m values were calculated by fitting the data to the Michaelis-Menten equation with or without substrate inhibition, using nonlinear regression methods to select the best statistical fit (Prism 4.0).

The value of k_{cat} was determined from the V_{max} value and the protein concentration ($k_{\text{cat}} = V_{\text{max}}[\text{Etot}]$). Etot was determined by methotrexate titration (Cody et al, 2009).

K_i values were determined by measuring inhibition of the reaction at two or more concentrations of substrate (DHFA). For the competitive inhibitor TMP in this study, K_i could be calculated from Dixon plots ($1/v$ versus $[I]$) or directly from the equation:

$$K_i = IC_{50} / (1 + S/K_m)$$

Statistical comparisons were performed with InStat 2.03, using the conservative nonparametric Welch t-test because variances among groups were not equal. Kinetic data reported in Table 2 differ from that reported in Cody et al, 2009, in that the new data sets include only results of assays performed within the past four years; thus, Table 2 includes control values that were run in parallel with the mutant samples so that the comparisons do not rely on historical controls.

RESULTS

Structure and Ligand Binding Conformation of TMP

Overall, the crystal structures of the two double mutant hDHFR enzyme complexes and that of the wild type hDHFR resemble those previously reported (Cody et al, 2009; Cody & Schwalbe, 2006) and preserve the hydrogen bonding network involving structural water, the conserved residues Thr136, Trp24, Glu30 and the N1 nitrogen and 2-amino group of TMP. Similarly, the 4-amino group of TMP maintains contact with the conserved Ile7 and Tyr121.

The Q35K/N64F double mutant replaces two hDHFR active site residues with two residues found in pcDHFR; the Q35S/N64F double mutant contains one substitution from pjDHFR (Q35S) and one from pcDHFR (N64F). Structural results for the TMP binary complexes with these double mutants Q35S/N64F and Q35K/N64F of hDHFR reveal the expected binding of TMP with full occupancy in the substrate pocket but also reveal the unexpected binding of a second TMP molecule with 50% occupancy as reflected in the refinement of their thermal parameters (Fig. 2). In both mutant structures the difference electron density maps indicated extra density that was not water or crystallization additives in the buffer. Further analysis of the density as the refinement progressed indicated that it could be identified as TMP with partial occupancy (Fig. 2a,b). When this molecule was added to the refinement, the thermal parameters behaved reasonably. Refinement of the Q35K/N64F hDHFR TMP complex showed that the thermal parameters for the backbone atoms of DHFR ranged from 8.0-15.0 with their side chains somewhat higher (11.0-22.0). The thermal parameters for the fully occupied TMP ranged from 9.0-14.0 while those for the partially occupied TMP ranged from 16.0-20.0. A comparison of the initial electron density prior to the addition of the second TMP are shown (Fig. 2a,b) as well as the electron density from an omit map removing both ligands (Fig. 2c).

The second molecule of TMP is positioned in a shallow pocket near the γ -glutamate binding site for folate substrates, and is unrelated to the NADPH binding site (Fig. 3). In both mutant structures, the 2,4-diaminopyrimidine ring of the second TMP interacts with a sulfate anion from the crystallization buffer. The second TMP is also involved in a series of hydrophobic contacts with the trimethoxybenzyl ring of the first TMP, Phe31 and the mutated Phe64 position in this structure (Fig. 4). This binding pocket is also near that occupied by the unusual side chain conformation observed for the TMP derivative PY957 (Fig. 1) in the hDHFR ternary complex with the same Q35S/N64F double mutant (Cody et al, 2009) (Fig. 5). These results suggest that the combined influence of the hydrophobic residues at

positions Phe31 and Phe64 of the mutant hDHFR enzyme provides sufficient van der Waals energy to stabilize binding in this unusual site.

The conformation of TMP is defined by two torsion angles about the methylene bridge, θ_1 and θ_2 (C4-C5-C7-C1')/(C5-C7-C1'-C2') (Fig. 1), as well as the methoxybenzyl groups adopting an in-plane or out-of-plane conformation. Analysis of TMP conformations observed in DHFR structures shows that they cluster in two broad torsion angle ranges: those with θ_1 near 180-210°/50-90° and for θ_2 near 260-280°/95-105° (Table 3; Fig. 6) (Cody & Schwalbe, 2006; Cody et al, 2006). The cluster near 260-280°/95-105° is less populated with only a few outliers with θ_2 near 50-65° for the two bridge angles. The bridging angles for the two molecules of TMP observed in the binary complexes with the Q35S/N64F and the Q35K/N64F double mutants of hDHFR differ significantly from each other (points 20/21 and 22/23, Fig. 6) and have torsion angles near the extremes of the cluster at 270° and either 50° or 120° (Table 3), whereas the conformations of the two molecules in the asymmetric unit of the ternary complex of TMP and NADPH with hDHFR (PDB 3w2a) (points 24/25, Fig. 6) cluster together, and differ significantly from those observed in the binary TMP complex with wild type hDHFR (Cody, 2002) and the ternary complex with TMP and NADPH with pcDHFR (Champness et al, 1994) (Table 3).

This analysis exemplifies TMP's flexibility in adapting to the local environment in the enzyme target. The observation that conformational subsets can be discerned within a narrow range suggests that this flexibility may be inherent in its selectivity and specificity of binding to various forms of DHFR. Additionally, the clustering of conformational descriptors between binary and ternary DHFR complexes suggests that there is a correlation between TMP conformation and DHFR selectivity that is as yet not clearly understood.

Kinetics

Single mutations that convert hDHFR to a more pj-like active site, i.e., Q35S and N64S significantly lower the hDHFR K_i for TMP from 4093 nM to 1355 and 738 nM, respectively; the K_i of the double mutant Q35S/N64S at 429 nM is lower than those of the single mutants but all three mutant enzymes have K_i values significantly higher than the pjDHFR value (49 nM) (Table 2). Mutants converting human active site residues to pcDHFR residues (Q35K and N64F) show K_i values for TMP that are similar to each other (286 and 375 nM, respectively), and similar to the value for pcDHFR (K_i 305 mM). The double mutant Q35K/N64F does not show synergy (K_i 584 nM). Mutants carrying one pj-like active site residue and one pc-like residue (Q35K/N64S and Q35S/N64F) have K_i values for TMP (593 nM and 617 nM, respectively) that are not different from the double mutant Q35K/N64F bearing two pc-like active site residues.

K_m values for substrate and cofactor were measured to fully characterize all mutants (Table 2). In general, the K_m value for DHFA for all mutants remained indistinguishable from that of hDHFR. Likewise, the K_m values for NADPH tend to be similar between the mutants and hDHFR, except for the value of Q35K (12.5 μ M) which is significantly higher than that of the human enzyme (4.5 μ M), although it did not reach the value of 23 μ M seen for pcDHFR.

As reported previously (Cody et al, 2009) values for k_{cat} for the mutants and for control hDHFR all lie in a relatively narrow range (27 to 48 s^{-1}); the new mutants Q35K/N64S and Q35S/N64F also show k_{cat} values (36 ± 1 and 21 ± 2 , respectively) similar to the hDHFR value (26 ± 2). The calculated value of catalytic efficiency (k_{cat}/K_m for DHFA) was previously reported to be highest for pcDHFR and two of the mutants bearing pc-like residues, Q35K and Q35K/N64F. The same pattern holds with these studies in that Q35K/N64S has a higher catalytic efficiency (51 $s^{-1} \mu M^{-1}$) than hDHFR (37 $s^{-1} \mu M^{-1}$), but the value for Q35S/N64F (30 $s^{-1} \mu M^{-1}$) is in the same range as the hDHFR control.

DISCUSSION

This report of structural data for the binding of TMP (Fig. 1) to the Q35S/N64F and Q35K/N64F double mutants of hDHFR is the first to show two molecules of TMP bound to a form of hDHFR. These data showed one TMP bound in the normal ligand site and the other bound in an alternate binding pocket not previously known to be occupied by antifolates (Fig. 3). These data suggest that the presence of the N64F mutation, in conjunction with the natural Phe31 of hDHFR, contribute sufficient hydrophobic contacts to stabilize the binding of a second molecule of TMP trapped in this alternative site. The crystal structure of the Q35S/N64F double mutant of hDHFR bound to the inhibitor PY957 (Fig. 1) also revealed an unexpected binding conformation in which the carboxyalkoxy side chain flipped out of its normal binding pocket to occupy this alternate binding site (Fig. 5)(Cody, et al, 2009). These studies illustrate the plasticity of the DHFR molecule and reveal synergistic effects of these mutations on binding site interactions. This point has bearing on our understanding of the effects of mutations in the gene for pJDHFR which are now being observed in patients receiving therapy for pneumocystis pneumonia (Raman et al, 2008).

In theory the binding of a second molecule of TMP by the two mutant enzymes could be visible kinetically. For example, if two molecules of TMP bind randomly and independently to two sites, the replot of the TMP concentration against the slopes of plots of $1/v$ vs $1/S$ will be parabolic (concave upward) rather than linear. In four experiments with Q35S/N64F and one experiment with Q35K/N64F we determined that the replot of the slope of double reciprocal plots versus TMP concentration gave linear plots (r square values of 0.981 and 0.932) rather than parabolic plots; these curves were not different from those generated using native sequence hDHFR (data not shown). These results may not be unexpected in that the second binding site is only 50% occupied, suggesting that even weaker binding than seen in the primary site; such weak interactions may be difficult to observe kinetically. Moreover, the structural data suggests interactions between the two molecules of TMP, which would not lead to the same kinetic patterns seen with independently bound molecules of inhibitor. Thus, the kinetic data reinforce the structural data.

Although it can be argued that the binding of the second TMP molecule is an artifact of crystallization which was carried out with an excess of TMP, or that the interpretation of the electron density is problematic, that such binding has only been observed for those hDHFR mutants that contain the N64F substitution pattern would suggest that this is not a random event. Furthermore, similar results were observed for the N64F double mutant of hDHFR bound to the TMP derivative PY957, in which the carboxylate side chain bound near this site rather than interacting with Arg70. These data suggest that although these interactions are weak, they are not random.

These observations are of interest as they can be viewed within the context of fragment-based drug design strategies that seek to identify subpockets within the overall active site (Murray & Blundell, 2010; Chen & Shoichet, 2009; Wendt et al, 2007; Mpamhanga et al, 2009). The use of fragment-based drug design strategies to modify ligand specificity has become a more established protocol in drug discovery and recent examples have revealed that alternate binding modes present in closely related analogues can be exploited to design compounds with nanomolar potency and favorable physicochemical properties (Murray & Blundell, 2010; Mpamhanga et al, 2009). Other fragment-based design examples revealed the presence of alternate binding sites distinct from the normal ligand site that were exploited for the design of therapeutics that acted by allosteric disruption of binding (Wendt et al, 2007). Thus, the creation of a subpocket that utilizes the Phe31-Phe64 hydrophobic properties illustrates one of the effects of mutational substitutions that could alter ligand binding. Such effects are not readily predicted and may be of importance in the design of

novel inhibitors that can overcome the effects of drug-resist mutations in the pathogen p_jDHFR. The observations of an alternate binding pocket in the N64F mutants of hDHFR suggest that a correlation exists between this variant and its interactions with the native residue (F31) of the active site. The fortuitous creation of a binding pocket near the active site as a result of these mutational changes suggest that further study of those variants that have arisen in patient populations as a result of TMP treatment could produce similar synergies or be involved in the transmission of long-range effects that influence selectivity and potency of TMP for p_jDHFR.

In summary, the strong interactive effects of mutations at positions 35 and 64 of hDHFR suggest that compounds can be designed to show not only selectivity and potency to discriminate between wild-type enzymes from host and from pathogen, but that activity may also be designed toward expected mutants of p_jDHFR. These results also suggest that active site residues 35 and 64 play key roles in determining selectivity of TMP for pneumocystis DHFR, but converting these two residues alone does not produce a hDHFR that is identical to p_jDHFR in its response to TMP or in the K_m value for substrate or cofactor. The contribution of other residues within DHFR to these properties is critical to understanding selectivity and potency for new drug design.

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Abbreviations

DHFR	dihydrofolate reductase
DHFA	dihydrofolic acid
p_j	<i>Pneumocystis jirovecii</i>
pc	<i>Pneumocystis carinii</i>
IC₅₀	concentration giving 50% inhibition
K_i	equilibrium dissociation constant for inhibitor and enzyme

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Fig. 1.
Schematics of antifolates under study.

Fig. 2.

a. Difference electron density map (Fo-Fc, 3σ green) for hDHFR Q35S/N64F double mutant binary complex without the contributions of the second TMP molecule. 2b. The same map with the density map (2Fo-Fc, 1σ blue) showing the fit of the second TMP molecule to the density. 2c. Difference electron density omit map with both TMP molecules removed from the final cycle of refinement. X is water. The data are similar for the Q35K/N64F double mutant binary complex with TMP and hDHFR.

Fig. 3. Surface rendering of hDHFR MTX-NADPH (Cody et al, 2005) showing the binding of MTX (yellow) compared with that of the second copy of TMP (violet) in the structure of the Q35S/N64F double mutant hDHFR binary complex (cyan). This hydrophobic pocket is formed by the side chains of Phe31 and Phe64 (cyan). Figure drawn with PyMol (DeLano).

Fig. 4. Hydrophobic interactions of Phe31 and Phe69 (green) in the crystal structure of the double mutant Q35S/N64F hDHFR binary complex with two molecules of TMP in position 1 at full occupancy (yellow) and in position 2 at half occupancy (violet). Figure drawn with PyMol (DeLano).

Fig. 5.

Comparison of Q35S/N64F double mutant hDHFR binary complex with TMP bound in two positions (green), Q35S/N64F double mutant of hDHFR ternary complex with NADPH and PY957 (cyan) (Cody et al, 2009), and pcDHFR ternary complex with NADPH and TMP (violet) (Champness et al, 1994). Figure drawn with PyMol (DeLano).

Fig. 6.

Plot of the bridging torsion angles for TMP (Fig. 1) bound to DHFR. Points 1-19 are taken from Cody et al, 2006 while points 20-25 are from Table 3. Ternary crystal complexes with NADPH are filled (red) diamonds and the binary crystal complexes are filled (blue) circles. The open diamonds are NMR structures and the open circles are NMR binary structures. There are only two exceptions to a suggested correlation between the presence or absence of the cofactor and the TMP torsion angle.

Table 1

Crystal Properties and Refinement Statistics for TMP binary hDHFR complexes.

Properties	hQ35K/N64F-TMP	hQ35S/N64F-TMP	hDHFR-TMP
PDB accession	3S3V	3N0H	
Space Group	H3	H3	H3
Lattice constants			
a	84.7	84.0	85.7
b	84.7	84.0	85.7
c	78.7	78.5	77.6
Beamline	SSRL 9-2	Raxis-IV	Raxis-IIc
Resolution	1.53 (1.61)	1.92 (2.00)	1.90
Wavelength	0.975	1.5418	1.5418
Rmerge	0.09	0.08	0.05
Completeness	97.5 (99.7)	98.1(97.4)	96.2
Observed Reflections	69508	39906	30988
Unique Reflections	30987	15381	14844
I/ σ (I)	6.3 (5.2)	12.6	6.7
Multiplicity	2.2 (2.2)	2.6	2.0
Reflections used	29446	14615	13623
R-factor	0.195	0.19	0.21
Rfree	0.22	0.22	0.24
Total protein and ligand	1723	1662	1528
Total water	140	91	31
Average B-factor	14.9	17.6	23.2
Error in Luzzati plot	0.16	0.22	
Rms deviation from ideal			
Bond length	0.03	0.02	0.05
Bond angle	2.72	2.11	3.50
Ramachandran plot			
Most favored	99.5	96.2	95.2
Additional allowed	0.5	3.8	4.8
Disallowed	0.0	0.0	0.0

^aThe values in parentheses refer to data in the highest resolution shell.^b $R_{\text{Sym}} = \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_i I_{h,i}$, where $\langle I_h \rangle$ is the mean intensity of a set of equivalent reflections.^c $R\text{-factor} = \sum |F_{\text{Obs}} - F_{\text{Calc}}| / \sum F_{\text{Obs}}$, where F_{Obs} and F_{Calc} are observed and calculated structure factor amplitudes.^d R_{free} -factor was calculated for R-factor for a random 5% subset of all reflections.

Table 2

Kinetic constants for TMP against pjDHFR, pcDHFR, and wild type and mutant hDHFR.

Enzyme	TMP, nM K_i	Km DHFA, μ M	Km NADPH, μ M
pjDHFR	49 ± 8^I (n=18)	2.2 ± 0.2^I (n=13)	19 ± 1^I (n=4)
pcDHFR	$305 \pm 29^{I,2}$ (n=15)	4.9 ± 0.3^I (n=5)	16 ± 2^I (n=4)
hDHFR	4093 ± 1007 (n=7)	0.7 ± 0.1 (n=8)	4.5 ± 1.2 (n=3)
Mutant h→pjDHFR			
hDHFR Q35S	$1355 \pm 172^{I,2}$ (n=3)	0.7 ± 0.2 (n=4)	3.8 ± 1.4 (n=3)
hDHFR N64S	$738 \pm 140^{I,2}$ (n=3)	0.9 ± 0.3 (n=3)	6.3 (n=1)
hDHFR Q35S/N64S	$429 \pm 43^{I,2}$ (n=4)	0.4 ± 0.1 (n=2)	5.3 ± 1.5 (n=3)
Mutant h→pcDHFR			
hDHFR Q35K	$286 \pm 33^{I,2}$ (n=4)	0.5 ± 0.1 (n=4)	$12.5^I \pm 0.6$ (n=3)
hDHFR N64F	$375 \pm 64^{I,2}$ (n=8)	0.8 ± 0.2 (n=5)	7.5 ± 3.1 (n=3)
hDHFR Q35K/N64F	$584 \pm 104^{I,2}$ (n=8)	0.6 ± 0.1 (n=8)	5.7 ± 1.1 (n=3)
hDHFR cross→pc/pj			
hDHFR Q35K/N64S	$593 \pm 44^{I,2}$ (n=5)	0.7 ± 0.2 (n=6)	6.6 ± 1.8 (n=3)
hDHFR Q35S/N64F	$617 \pm 64^{I,2}$ (n=10)	0.7 ± 0.2 (n=5)	5.6 ± 0.5 (n=3)

¹Value is statistically different from the value for native human DHFR²Value is statistically different from the value for pjDHFR

Table 3

Bridge conformation for TMP DHFR complexes. See Fig. 1 for torsion angle definitions; Fig. 6 for position on plot.

DHFR Complex	C4-C5-C6-C1'	C5-C7-C1'-C2'	Reference
h-Q35S/N64F-TMP (A) #20	275°	117°	This paper
h-Q35S/N64F-TMP (B) #21	274	53	This paper
h-Q35K/N64F-TMP (A) #22	289	117	This paper
h-Q35K/N64F-TMP(B) #23	280	61	This paper
h-TMP #15	201	66	Cody, 2002
h-TMP-NADPH (1) * #24	263	97	PDB 2w3a
h-TMP-NADPH (2) *	263	100	PDB 2w3a
#25 pc-TMP-NADPH #4	176	70	Champness et al 1994

* There is no publication for this PDB entry.