Effects of Allosteric Mutations on Dihydrofolate Reductase

Marian Okondo

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Effects of Allosteric Mutations on Dihydrofolate Reductase

By

Marian Okondo

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Thesis Committee:

Thesis Sponsor: Dr. Nina Goodey

Committee Member: Dr. Jim Dyer

Committee Member Dr. David Konas
ABSTRACT

Dihydrofolate reductase (DHFR) is an essential enzyme necessary in the synthesis of DNA precursors. DHFR acquires resistance to numerous known anti-folates and this has led to the continuous need to discover novel and selective DHFR inhibitors. Allosteric mutations on *Bacillus stearothermophilus* (*Bs.*) DHFR were previously found to alter inhibitor binding and specificity. In this work, we investigate how these mutations affect the conformational motions of DHFR associated with inhibitor binding. Two distal allosteric mutations, isoleucine at position 86 to alanine (I86A) and tyrosine at position 127 to alanine (Y127A) were separately introduced to C73A/S131C, a previously modified wild type DHFR to give C73A/I86A/S131C and C73A/Y127A/S131C DHFRs. The modified C73A/S131C DHFR contains a site for attachment of fluorescent label, N-[2-(1-maleimidyl) ethyl]-7-(diethyl amino) coumarin-3-carboxamide (MDCC). Development of C73A/S131C<sub>MDCC</sub> DHFR allowed for a method to study two native conformations of DHFR that could otherwise not be studied by intrinsic fluorescence alone. Stopped-flow instrumentation together with fluorescence is used to study the kinetics of protein structural motions. This approach allowed us to examine the conformational changes associated with methotrexate (MTX) and pyrimethamine (PYR) binding, for the mutants and compare them to C73A/S131C DHFR. Intrinsic tryptophan fluorescence of the three DHFR constructs (C73A/S131C, I86A, Y127A) revealed a different conformational change upon PYR binding to one conformer compared to MTX binding. To study the second conformer, MDCC is covalently attached to the cysteine at position 131 on the DHFR (C73A/S131C<sub>MDCC</sub>, I86A<sub>MDCC</sub>, Y127A<sub>MDCC</sub>) and all three enzymes show a conformational change, different from that observed by intrinsic fluorescence upon inhibitor binding that are inhibitor specific. This study provides insight on the conformational changes associated with inhibitor binding. How the conformational changes are inhibitor specific. As well as how the mutants’ (I86A and Y127A) affect the conformational changes compared to C73A/S131C. Understanding what happens during inhibitor binding and the role of allosteric mutations on ligand binding specificity can inform design and repurposing of selective DHFR drug compounds.
EFFECTS OF ALLOSTERIC MUTATIONS ON DIHYDROFOLATE REDUCTASE ENZYME

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of Masters of Science Chemistry

By
MARIAN OKONDO
Montclair State University Montclair, NJ

2015
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ABBREVIATIONS

DHFR: Dihydrofolate Reductase Enzyme

Bs. DHFR: *Bacillus stearothermophilus* DHFR

WT: Wild type *Bs. DHFR*

MDCC: N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide fluorescent tag

C73A/S131C DHFR: Modified WT DHFR

C73A/S131C<sub>MDCC</sub> DHFR: MDCC labeled C73A/S131C DHFR

I86A: DHFR construct with Isoleucine replaced at position 86 with Alanine on C73A/S131C

I86A<sub>MDCC</sub>: MDCC labeled I86A DHFR

Y86A: DHFR construct with Tyrosine replaced at position 127 with Alanine on C73A/S131C

Y127A<sub>MDCC</sub>: MDCC labeled Y127A DHFR

DHF: 7,8-dihydrofolate

THF: 5,6,7,8- tetrahydrofolate

Allosteric Mutations: Mutations not located in the active site of DHFR but affect enzyme function

LB: Luria Bertani

Amp<sub>100mg/mL</sub>: Ampicillin at a concentration of 100mg/mL

diH<sub>2</sub>O: Distilled Water

IPTG: isopropyl-β-D-thiogalactopyranoside for inducing expression

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

NaCl: Sodium Chloride

DTT: Dithiothreitol
MTX: Methotrexate

PYR: Pyrimethamine

$k_{cat}$: The number of substrate molecules each substrate site converts to a product per unit time

$K_i$: The Inhibitor concentration needed to produce half maximum inhibition

E: A conformer of Bs. DHFR

E’: Another conformer of Bs. DHFR

$k_1$ and $k_{-1}$: Association and Dissociation rate of E

$k_4$ and $k_{-4}$: Association and Dissociation rate of E’

$k_2$ and $k_{-2}$: Conformational change rates

MTEN buffer: (50mM 2-(N-morpholino) ethanesulfonic acid, 25mM tris (hydroxymethyl)-aminomethane, 25mM ethanolamine and 100mM NaCl) at pH 7
Introduction

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme, which catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), an essential molecule used for the synthesis of important metabolites including DNA precursors. For this reason, DHFR is a well-established drug target for the treatment of cancers and some infectious diseases. However, DHFR acquires resistance to a number of known inhibitors such as methotrexate (anticancer), pyrimethamine (protozoan infections) and trimethoprim (antibacterial) to mention a few. Amino acid residue mutations at highly conserved positions on DHFR are thought to be responsible for the resistance. Understanding the role these residues play in inhibitor binding, catalysis and specificity is important in the rationale of drug design.

Enzymes exhibit flexibility and conformational changes upon ligand binding and catalysis. A "network" of amino acid residues in and away from the active site may allosterically regulate and form the "global protein dynamics" that play a role in enzyme catalysis. In this context, global protein dynamics refers to the overall motions exhibited by all the atoms present in DHFR. DHFR is one of the most studied enzymes for enzyme flexibility and it is known that the active site residues are involved in ligand binding. Still, an extensive number of experimental, computational and theoretical studies, have provided evidence to show, besides active site residues, coupled motions of residues proximal and distal from the active site play a role in ligand binding and catalysis.

In published work, using a computational method, Goodey and co-workers were able to predict residues within and away from the active site that not only affected
enzyme catalysis but were also found to be involved in inhibitor specificity. To validate the distal residues predicted, determinant regions on \textit{Bacillus stearothermophilus} (\textit{Bs}) DHFR were identified; allosteric mutations were introduced and found to affect inhibitor binding and specificity \textsuperscript{21}.

In this work, we try to understand the effect of these allosteric mutations on ligand binding and specificity by studying the overall motions of \textit{Bs}. DHFR containing single allosteric mutations and comparing them to the wild type. We introduce two distal allosteric mutations; these mutations are similar to those introduced in the determinant region study\textsuperscript{21}, isoleucine in position 86 to alanine (I\textsubscript{86}A) and tyrosine in position 127 to alanine (Y\textsubscript{127}A), separately, to a modified \textit{Bs}. DHFR (C\textsubscript{73}A/S\textsubscript{131}C DHFR) to give C\textsubscript{73}A/I\textsubscript{86}A/S\textsubscript{131}C and C\textsubscript{73}A/Y\textsubscript{127}A/S\textsubscript{131}C\textsuperscript{a}.

The C\textsubscript{73}A/S\textsubscript{131}C DHFR was previously modified to develop a novel method for studying temperature dependent conformational changes associated with inhibitor binding\textsuperscript{22} using stopped flow instrumentation together with intrinsic tryptophan fluorescence and extrinsic fluorescence. The introduction of a cysteine at position 131 provided a specific site for covalent attachment of the fluorescent label N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC). MDCC does not respond to the absence or presence of an inhibitor, rather to the conformational motions in its environment that cannot be detected by intrinsic tryptophan fluorescence alone \textsuperscript{22}.

This method allowed us to study the conformational motions of the mutants during methotrexate (MTX) and pyrimethamine (PYR) binding and compare them to C\textsubscript{73}A/S\textsubscript{131}C. Here, we report on the mechanism of binding of PYR to DHFR as well as

\textsuperscript{a} C\textsubscript{73}A/I\textsubscript{86}A/ S\textsubscript{131}C and C\textsubscript{73}A/Y\textsubscript{127}A/S\textsubscript{131}C will be referred to as I\textsubscript{86}A and Y\textsubscript{127}A respectively
the conformational changes associated with PYR binding a study that has not been done before.

Some of the questions we try to answer are as follows: 1) Do the allostERIC mutations affect the conformational motions associated with inhibitor binding? 2) Are the conformation motions, inhibitor specific? 3) Do the allostERIC mutations change the conformational equilibrium of DHFR prior to inhibitor binding and what is the significance of these effects on inhibitor binding and specificity?
**Materials and Methods**

**1. Construction of I86A mutant gene**

1.1 *QuickChange Mutagenesis*

I86A mutation was introduced to modified C73A/S131C Dihydrofolate reductase enzyme (DHFR) DNA (DNA was from Sarah Cho, a student in Dr. Goodey’s Lab) by mutagenesis, using the QuickChange II Site-Directed Mutagenesis kit. (Table 1)

<table>
<thead>
<tr>
<th>Volume</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10μL</td>
<td>5X reaction buffer</td>
</tr>
<tr>
<td>0.26μL (10ng)</td>
<td>C73A/S131C DHFR DNA</td>
</tr>
<tr>
<td>2.5μL</td>
<td>I86A 5'GGAAGTCAAGCAATGGGCCGCATCG (forward Primer)</td>
</tr>
<tr>
<td>2.5μL</td>
<td>I86A 5'GCGATGCGCCCATGCTTGAACCT (reverse primer)</td>
</tr>
<tr>
<td>1μL</td>
<td>dNTP mix</td>
</tr>
<tr>
<td>33.74μL</td>
<td>Double Distilled (dd) H₂O</td>
</tr>
</tbody>
</table>

*Table 1: Protocol for the sample reactions to perform QuickChange II Site-Directed Mutagenesis for the introduction of I86A to C73A/S131C DHFR.*

Three PCR sample reaction mixtures were made and 0.5μL of Pfu turbo DNA polymerase was added. For the control reaction, no DNA was added. The samples and control were placed in the thermo-cycler (Fig 1) and left overnight after the reaction was over. Reaction parameters are included in (Table 2) and were based on recommendations suggested in the QuickChange II Site-directed Mutagenesis protocol for optimal results.
Figure 1: A picture of the thermo cycler used for the PCR mutagenesis reaction. Eppendorf Mastercycler gradient thermo cycler using round top PCR tubes setting was used.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C</td>
<td>30s</td>
</tr>
<tr>
<td>22</td>
<td>98°C</td>
<td>10s</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30s</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Store</td>
<td>4°C</td>
<td>Overnight</td>
</tr>
</tbody>
</table>

Table 2: QuickChange II Site Directed Mutagenesis cycling parameter methods for introducing I86A mutation to C73A/S131C DHFR.

Dpnl digestion was performed the next day, by adding 1μL of Dpnl restriction enzyme (10 U/μL) directly to each reaction and incubated for 1 h at 37°C.

1.2 Transformation of Digestion reaction in XLBlue Super Competent Cells

To 100μL of XLBlue cells super competent cells (made competent by Sarah Cho), 1μL of the digestion reaction was directly added and the reaction mixture placed on ice for 30 min. The cells were heat shocked for 2 min at 42°C and placed on ice for 2 min. Luria Bertani (LB) broth (900μL) was added and the cells were incubated for 45 min at
37 °C. The cells were pipetted on an LB<sub>amp100mg/mL</sub> plate and spread using a sterile loop. The plates were incubated overnight at 37 °C. The same procedure was done for the control reaction, excluding the addition of 1μL of the digestion reaction.

### 1.3 DNA Plasmid Preparation/Purification using QIAprep Spin Miniprep Kit

Colonies were collected from the incubated plates (Fig. 2) and a 7 mL LB + 7μL Ampicillin<sub>100mg/mL</sub> overnight culture was grown in the shaker at 37°C. The next day, a 1:1 1 mL 50% glycerol stock of the cells was made from the overnight culture.

![Figure 2: Plate containing colonies collected for DNA plasmid preparation and purification using the QIA prep spin Miniprep kit.](image)

The cells were harvested by centrifuging the overnight culture in three, 1.5 mL micro-centrifuge tubes at 13,000 rpm on a tabletop centrifuge (fig.3). The supernatant was poured out and an additional 1.5mL of the overnight culture was added to the tubes and centrifuged at the same settings.
Figure 3: Eppendorf tabletop centrifuge, model 5418, that was used in the purification of DNA plasmid colonies using the QIA prep spin Miniprep kit collected from transformation LB agar plates containing 100mg/ml ampicillin.

Using a QIAprep Miniprep kit protocol\textsuperscript{24}, the pellets in the three 1.5mL microcentrifuge tubes were separately suspended in 250\(\mu\)L Buffer P1 (containing RNAse) 250 \(\mu\)L Buffer P2, 350 \(\mu\)L Buffer N3 and centrifuged for 10min at 13,000 rpm. The supernatant from all three tubes were added gradually to the spin column and centrifuged for 30s at 13,000 rpm until all the supernatant was added to the column. The column was washed with 0.5mL Buffer PB and 0.75mL Buffer PE (containing 20mL 100% Ethanol) each time centrifuging for 30s at 13,000rpm. The column was centrifuged for 2min at 13,000rpm to remove the excess ethanol from Buffer PE. Finally, 30\(\mu\)L diH\(_2\)O was added to the column, which was incubated for 5min and centrifuged for 1 min at 13,000rpm.
To determine the concentration of the eluted DNA, 2μL of the protein was placed directly onto the optical measurement surface of a Thermo Scientific™ NanoDrop 2000c UV-Vis spectrophotometer using diH2O as a blank at the DNA module setting at 260nm wavelength.

Once the concentration was determined, to confirm the mutation, 10μL of the purified DNA plasmids were sent for sequencing (done by Adam Parker of the Molecular Biology department) and the amino acid sequence alignments were done using Biology Workbench. (Refer to results section 2)

1.4 Transformation of 186A construct into BL21 Escherichia coli (Ec)

Expression Cells

The XLBlue cells transformation protocol, described in materials and methods section 1.2 was used. To 100μL of competent BL21 Ec. cells, 1μL of purified I86A DHFR plasmid DNA was directly added, to the transformation reaction. No plasmid was added to the Ec. cells for the control reaction.

2. Protein Expression, Purification and Preparation of MDCC Labeled DHFR

2.1 Protein Expression DHFR in BL21 Ec. Cells

BL21 C73A/S131C DHFR from a glycerol stock was added to two culture tubes containing 5mL LB broth+ 5uL Amp100mg/ml. The cells were grown overnight and 2.5mL were added to 4 flasks containing 250mL LB + 250μL Amp100mg/ml at 37°C. At an OD_{600} of 0.6, the cells were induced with 50μL of 1M isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.2mM and grown for 18h at 30°C and 225rpm. The cells were centrifuged at 5000rpm for 15min at 4°C in a Beckman Coulter Avanti J-
265 XP centrifuge using the JA-17 rotor and washed with 200ml 0.9% Sodium Chloride (NaCl) and centrifuged again as above. The pellets were stored at -80°C. A similar protocol was used for the purification of I86A and Y127A (50% glycerol stock of Y127A was given by Sarah Cho).

2.2 Protein Purification of DHFR

The pellets were suspended in 40mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer at pH6.8 and lysed by sonication for 5min at setting 5, 10% duty cycle on ice and centrifuged at 17,000rpm for 20min. The supernatant was loaded onto an equilibrated SP-Sepharose C-25 column; saving 80μL and collecting 80μl of the flow through, The column was washed with 25 ml of HEPES and the protein was eluted with 0.2M NaCl, 40mM HEPES, 1mM DTT, 10% glycerol (pH 6.8). Presence of protein in each fraction was confirmed using 5X Biorad Bradford assay (80μL Bradford solution +20μL sample)(fig 4).

Figure 4:A. Columns containing SP-Sepharose C-25 resin that the lysate was loaded onto. B. Bradford protein assay (80μL Bradford solution +20μL sample) done in a 96 well plate. Blue indicates presence of protein while brown shows no protein present.
The protein was concentrated and washed with 40mM HEPES, 1mM DTT and 10% glycerol in a swing bucket rotor in an Eppendorf tabletop Centrifuge, model 5810R at 3x20min at 4000rpm. The concentration of the protein was calculated using beer law equation (eq. 1) by determining the absorbance at 280nm from the Thermo Scientific™ NanoDrop 2000c on UV-Vis setting and an extinction coefficient of 25565 M$^{-1}$cm$^{-1}$, 1.386mg$^{-1}$cm$^{-1}$. Typical final concentration and volume of protein obtained from a 500mL pellet of protein is 1.5mL of 31μM and 0.58 mg/mL at an absorbance of ≈ 0.08 at 280nm wavelength. Giving a typical yield 0.88mg per 500mL.

\[
C = \frac{A}{\varepsilon l}
\]

C in mg/ml: \( \varepsilon = 1.368\text{mLmg}^{-1}\text{cm}^{-1} \)

C in M: \( \varepsilon = 22565\text{M}^{-1}\text{cm}^{-1} \)

\( l = 0.1\text{cm} \)

**Equation 1: Beers law equation used to calculate protein concentration of purified C73A/S131C DHFR.**

An SDS-PAGE electrophoresis was done at 145V for 45min (10μL of 4X SDS loading dye +30μL sample) to view the size of the protein and its % purity (Fig 5). The same protocol was used for I86A and Y127A constructs.
2.3 Preparation of C73A/S131C<sub>MDCC</sub>, I86A<sub>MDCC</sub> and Y127A<sub>MDCC</sub> DHFR

To covalently attach the fluorescent label to C73A/S131C DHFR, the enzyme was washed in pH 7.2 40mM HEPES to remove excess DTT and exchange the buffer. A 4-fold molar excess of 0.22mM stock solution of MDCC fluorophore (MW 383.4 g/mol), dissolved in dimethyl sulfoxide (DMSO), was added drop-wise to the purified C73A/S131C DHFR in pH 7.2 HEPES buffer. The final concentrations were 128μM of MDCC and 32μM and enzyme respectively.

The mixture was covered with aluminum foil to protect the sample from light and incubated at room temperature for 2 h. The sample was dialyzed twice, once overnight and 4h the next day at 4 °C in at least 200X 40mMHEPES, pH 6.8 to remove excess dye and to exchange buffer. A similar procedure was used to attach MDCC to I86A and Y127A with varying incubation times, I86A (3h), Y127A (5 h shaking).
2.4 Calculation of %Labeling efficiency of MDCC attachment to DHFR

The extinction coefficient of DHFR (25,565 M⁻¹cm⁻¹ at 280 nm) and of MDCC (10,000 M⁻¹cm⁻¹ at 280 nm and 50,000 M⁻¹cm⁻¹ at 419 nm) were used to calculate labeling efficiency. Labeling efficiency was found to be ~87 % from absorbance measurements at 280 and 419 nm using a Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer for C73A/S131C<sub>MDCC</sub> and 186A<sub>MDCC</sub>. A labeling efficiency of 40% was obtained for Y127A<sub>MDCC</sub>.

**STEP 1:** Determining the concentration of MDCC present at 419nm

MDCC concentration is calculated using beer's law and absorbance obtained at 419nm

$$\text{Labeling Efficiency}$$

$$A_{419nm} = 0.017$$

$$\varepsilon = 50,000M^{-1}cm^{-1}, l = 0.1cm$$

$$C = \frac{0.017\times 10^6 \mu M}{50,000M^{-1}cm^{-1}\times 0.1cm} = 3.4 \mu M$$

**STEP 2:** Calculating absorbance contributed by MDCC at 280nm

$$A_{280nm} \text{ contributed by MDCC}$$

$$A = 3.4 \mu M \times 0.1cm \times 10,000M^{-1}cm^{-1} \times \frac{1M}{10^6 \mu M} = 0.0034$$

**STEP 3:** Determining the concentration of Enzyme present in solution

The enzyme absorbance at 280nm is calculated by subtracting the MDCC absorbance at 280nm from the overall 280nm absorbance.

**Enzyme** $$A_{280nm} = \text{Overall A}_{280nm} - \text{MDCC A}_{280nm}$$

$$0.075 - 0.0034 = 0.0716$$

**Enzyme Concentration**

$$C = \frac{0.0716\times 10^6 \mu M}{25565cm^{-1}\times 0.1cm} = 28.01 \mu M$$

**STEP 4:** Calculating % Labeling Efficiency
To obtain the % labeling efficiency, the ratio of the concentration of the label to enzyme is calculated.

\[
\text{Labeling efficiency} = \frac{\text{MDCC Concentration}}{\text{Enzyme Concentration}} \times 100\%
\]

\[
\frac{3.4\mu M}{28.01\mu M} \times 100\% = 12.1\%
\]

3. **Kinetic Analysis of Inhibitor binding to DHFR enzymes using Stopped Flow**

**Instrumentation**

3.1 **Determination of Inhibitor Binding Kinetics to C73A/S131C DHFR**

To determine the conformational motions associated with binding and after binding using stopped-flow instrumentation together with intrinsic fluorescence, 2µM of C73A/S131C DHFR was mixed with 2-200µM of an inhibitor\(^b\) in MTEN buffer (50mM 2-(N-morpholino) ethanesulfonic acid, 25mM tris (hydroxymethyl)-aminomethane, 25mM ethanolamine and 100mM NaCl) at pH 7, 25\(^\circ\)C in an applied photo physics SX20 Stopped-flow kinetics instrument with 1mm slit width. (Figure 6) giving a final concentration of, 1µM of C73A/S131C DHFR was mixed with 1-100µM of an inhibitor. The technical specification settings for the stopped flow instrumentation can be found in Appendix II \(^{25}\).

\(^b\) Inhibitor refers to MTX and PYR
Figure 6: A picture of the sampling handling unit taken from the AppliedPhotophysics SX 20, Hardware Guide. Syringe A and B indicate where sample solutions are added to unit before mixing $^{25}$.

The change in intrinsic tryptophan florescence over time (excitation at 290nm, emission with a 320nm cutoff filter) at 25°C was recorded. For each reaction at a particular concentration, five traces of the signal were collected and averaged. The experiments run are listed in table 3.
Table 3: Kinetic experiments that were done using the stopped-flow instrument. The final range of concentration of inhibitor obtained after mixing, amount of time the data was recorded, and the P.M. volts used for each inhibitor study.

Experimental controls were done before the mixing of enzyme and inhibitor (Table 4). This was done to allow for a better approximation of the concentration of enzyme inhibitor binds and the percent amplitude of the signal.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final MTX Concentration (µM)</th>
<th>Time</th>
<th>PM Volt</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX and 2µM C73A/S131C DHFR</td>
<td>1-10</td>
<td>0.1s</td>
<td>480</td>
</tr>
<tr>
<td>MTX and 2µM C73A/S131C DHFR</td>
<td>25-100</td>
<td>2s</td>
<td>480</td>
</tr>
<tr>
<td>PYR and 2µM C73A/S131C DHFR</td>
<td>1.25-12.5</td>
<td>0.1s</td>
<td>470</td>
</tr>
<tr>
<td>PYR and 2µM C73A/S131C DHFR</td>
<td>25-100</td>
<td>10s</td>
<td>470</td>
</tr>
</tbody>
</table>

Table 4: Experimental controls of stopped flow experiments to determine kinetics of binding of inhibitor to C73A/S131C DHFR. Refer to Figure 1 for position of syringe A and B.

To determine the dissociation rates of the inhibitor, 2µM of C73A/S131C was pre-incubated with 4µM of an inhibitor and mixed with 350µM (and 700µM) TMP in the stopped flow instrument at 600s (MTX) and 100s (PYR). The traces were fitted using DYNAFIT4 program. To study the conformational motions of Y127A and I86A DHFR, the same protocol was used, keeping the controls, technical settings constant and enzyme and inhibitor concentration similar to C73A/S131C.
3.2 Determination of Inhibitor Binding Kinetics to C73A/S131C$_{MDCC}$ DHFR

To determine the conformational motions associated with inhibitor binding to C73A/S131C$_{MDCC}$, stopped-flow instrumentation together with MDCC fluorescence. Equal volumes of 2μM of C73A/S131C DHFR was mixed with 2-200μM of an inhibitor in MTEN buffer at pH 7, 25$^o$C in an applied photo physics SX20 stopped flow kinetics instrument with 1mm slit.

The change in MDCC fluorescence over time (excitation at 419nm, emission with a 450nm cutoff filter) at 25$^o$C was recorded. A summary of the experiments, inhibitors and time scale used are listed in (Table 5). Controls were done using the same parameters and procedure as the C73A/S131C experiment; refer to Table 4 in material and methods chapter 3, section 3.1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Final MTX Concentration (μM)</th>
<th>Time</th>
<th>PM Volt</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX and C73A/S131C$_{MDCC}$ DHFR</td>
<td>1-10</td>
<td>0.1s</td>
<td>360</td>
</tr>
<tr>
<td>MTX and C73A/S131C$_{MDCC}$ DHFR</td>
<td>1-100</td>
<td>10s</td>
<td>360</td>
</tr>
<tr>
<td>PYR and C73A/S131C$_{MDCC}$ DHFR</td>
<td>1.25-12.5</td>
<td>0.2s</td>
<td>370</td>
</tr>
<tr>
<td>PYR and C73A/S131C$_{MDCC}$ DHFR</td>
<td>1.25-100</td>
<td>10s</td>
<td>370</td>
</tr>
</tbody>
</table>

Table 5: Kinetic experiments for 2μM C73A/S131C$_{MDCC}$ DHFR that were carried out in the stopped-flow instrument. The final range of concentration of inhibitor obtained after mixing, amount of time the data was recorded, and the P.M. volts used for each inhibitor study.

To determine the dissociation rates of the inhibitor, 2μM of C73A/S131C$_{MDCC}$ was pre-incubated with 4μM of an inhibitor and mixed with 350μM(and 700μM) TMP in the stopped flow instrument at 600s (MTX) and 100s (PYR). The traces were fitted using DYNAFIT4 program. A similar protocol was followed to study the conformational motions associated with binding for I86A$_{MDCC}$ and Y127A$_{MDCC}$ DHFR.
3.3 Analysis of Data using DYNAFIT 4 program

DynaFit4 is a program used to perform nonlinear least squares regression fitting of enzyme kinetic data to a set of chemical equations. In this context the experimental data analyzed are reaction regression curves of time (x-axis) versus fluorescence (y-axis).

The averaged individual progression curve data obtained from the reaction of 2μM of DHFR with a particular concentration of inhibitor in stopped-flow instrumentation are analyzed together; this is a “global fit”. Script files are used to fit the data to a set of chemical reactions (fig7).

The same scripts are used for I86A and Y127A. Refer to Appendix III to view the scripts used for low concentrations and high concentration fits of the progression curves from the reaction of enzyme with inhibitor for the unlabeled enzyme and the global fit scripts for all the concentrations from the labeled enzyme reactions.

Global Fit the progress of C73A/S131C DHFR vs MTX at 0.1s

[task]
data = progress
  task = fit

[mechanism]
E + MTX ----- EMTX : k1 k-1
[constants]
  ; units: uH, sec
  k1 = 50
  k-1 = 0.01
  k2 = 10

[concentrations]
  ; units: uM
  E = 1.5
  EMTX = 0.15

[responses]
  ; percent product/nM substrate
  E = 0.686
  MTX = -0.0209
  EMTX = 0.238

[output]

directory/Users/Marie/Desktop/output/WT.715/0.1

Chemical Equation for data fitting

Experimental control predictions

Individual reaction traces at varying concentrations in a global fit

**Figure 7:** Sample script used to globally fit individual progress curves obtained from the reaction of 2μM of C73A/S131C DHFR with varying MTX concentrations.

---

c Inhibitor refers to methotrexate (MTX) and Pyrimethamine (PYR)
Results

1. Characterization of DHFR

1.1 Determining Concentration of Purified I86A DHFR DNA Plasmid

Concentrations of the two samples of plasmid purified using QIA miniprep kit were high enough to send for sequencing (Table 6).

<table>
<thead>
<tr>
<th>Purified Plasmid Label</th>
<th>Expected Mutations</th>
<th>Concentration (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG1</td>
<td>I86A</td>
<td>49.9</td>
</tr>
<tr>
<td>NG2</td>
<td>I86A</td>
<td>19.9</td>
</tr>
</tbody>
</table>

Table 6: Concentration in ng/µl of I86A DHFR DNA plasmid, purified using the QIA DNA mini prep- kit.

1.2 Sequencing Results of I86A Mutant Construct

To confirm the introduction of the I86A mutation into C73A/S131C DHFR, the purified plasmid amino acid sequence was aligned with the C73A/S131C DHFR sequence. From Table 1, both NG1 and NG2 (NG2 data not shown) plasmids had the mutation and maintained the fluorescent tag site already present (Fig 8).

Figure 8:Amino acid alignment sequencing results showing the introduction of I86A mutation in the C73A/S131C DHFR purified DNA plasmid introduced by mutagenesis. Green amino acids are the fluorescent tag site C73A/S131C and the mutation of interest I86A is shown in red. Biology Bench work was used for the alignment The nucleic acid sequence is in the Appendix fig 30.
1.3 SDS-PAGE characterization of WT and I86A constructs

The molecular weight (MW) of DHFR is ≈ 18 kDa. The SDS-PAGE (Fig9) shows two bands on the gel for C73A/S131C and I86A and Y127A constructs. The lowest band was at about 18kDa MW and the highest band at 36kDa indicates a dimer. The EZ protein-ladder is shown next to the gel (Refer to Appendix III for SDS-PAGE gels showing labeled C73A/S131C, I86A and Y127A protein).

Figure 9: SDS-PAGE gel of C73A/S131C, I86A and Y127A proteins, using 4X-loading dye (10ul Dye and 30ul sample). EZ protein ladder shows the MW sizes and position

2. Kinetic Analysis of Conformational Motions Associated with Inhibitor Binding to DHFR

In previous findings27, I86A and Y127A mutations were introduced to two distinct regions distant from the DHFR active site. The mutant constructs retained catalytic activity ($k_{cat}$) compared to the wild type (WT) but showed a change in the inhibitor constant ($K_i$); the potency of the inhibitor, that was ligand specific to methotrexate (MTX) and pyrimethamine (PYR) (Table 7).
Table 7: $k_{\text{cat}}$ (s$^{-1}$) and $K_i$ (nM) of WT, I86A and Y127A for methotrexate (MTX) and pyrimethamine (PYR)\textsuperscript{21}

This validated the hypothesis that residues distal from the enzyme active site played a role in ligand specificity and binding. However, how these allosteric mutations affect inhibitor binding and specificity is not known. One hypothesis is that the mutations may have an effect on the conformational changes and rates associated with inhibitor binding and therefore affect inhibitor potency and specificity when compared to the WT.

To study the effects of the mutations on conformational changes associated with binding, intrinsic tryptophan and extrinsic (MDCC) fluorescence in combination with stopped-flow instrumentation is used. Bs. DHFR has a total of three tryptophans (Trp 22, Trp 85 and Trp 135)\textsuperscript{22,27}. Trp 85 and Trp 135 are located distant from the ligand-binding site, while Trp 22 is located on a loop that aligns to the active site Met 20 loop in Ec. DHFR. It is not clear which tryptophan responds to inhibitor binding, however studies done, show that the Ec. DHFR exhibits three conformational states (closed, occluded and open)\textsuperscript{1,28} and when a extrinsic fluorophore was attached to the Met 20 loop on the Ec. DHFR two native conformations were observed during binding of MTX to DHFR\textsuperscript{29}. It is very likely that Trp 22 mostly responds to inhibitor binding, as was seen for the Met 20 loop and the combination of the three tryptophans is responsible for the overall signal detected.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_i(\text{MTX})$ (nM)</th>
<th>$K_i(\text{PYR})$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>11</td>
<td>0.010</td>
<td>0.018</td>
</tr>
<tr>
<td>I86A</td>
<td>2.4</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>Y127A</td>
<td>10</td>
<td>0.17</td>
<td>0.084</td>
</tr>
</tbody>
</table>
Previously, the development of a fluorescently labeled DHFR allowed for the study of conformational changes associated with MTX binding\textsuperscript{22}. A minimal model was determined for MTX binding to two different conformations (E and E') of DHFR. It included an induced fit model as well as a conformational selection pathway. The induced fit model involves the inhibitor binding to one conformer (E or E') and induces a conformational change to a more energetically favored conformation\textsuperscript{30}. The conformational selection pathway is where a particular conformation (E' or E) is selected for binding \textsuperscript{31} to form the enzyme-inhibitor complex. The two conformations are thought to be in equilibrium\textsuperscript{32 33}. (Fig 10)

\[ E + \text{MTX} \rightleftharpoons \text{EMTX} \]
\[ k_1 = 23 \mu M^{-1} s^{-1} \]
\[ k_{-1} = 0.01 s^{-1} \]
\[ k_2 = 9 s^{-1} \]
\[ k_3 = 0.0067 \mu M^{-1} s^{-1} \]
\[ k_{-3} = 0.01 s^{-1} \]

Figure 10: Minimal model for the binding of MTX to E and E'. MTX binding to E (k1) or E' (k4) is the binding event step. The induced fit model relates to (k1 and k2) or (k4 and k-2). Conformational selective model fits for (k3 and k4) or (k-3 and k1). The conformational equilibrium is indicated by (k3 and k-3). The binding affinity rate constants and conformational change rate constants are from the previous study of the fluorescently labeled DHFR. \textsuperscript{22}

A fluorescent label (MDCC) was covalently attached to the modified C73A/S131C DHFR (Fig 11) and conformational changes associated with MTX binding that were different from those observed from intrinsic tryptophan fluorescence were confirmed. The conformational changes recorded from changes in MDCC extrinsic
fluorescence were associated with MTX binding to E’ and the changes in intrinsic tryptophan fluorescence overtime, to MTX binding to E.

Figure 11: A cartoon (left) and surface (right) representation of the structure of Bs.DHFR (PDB ID: 1ZDR). Residue 131, the site of labeling, is shown as a magenta sphere. The MTX binding site is labeled in the surface representation. Data shown is from previously published work from Goodey et al.22

3. Methotrexate (MTX) binding to DHFR

3.1 MTX Binding to C73A/S131C, I86A and Y127A DHFR

To study the events occurring during methotrexate (MTX) binding to C73A/S131C, I86A and Y127A DHFR (E), 2µM of enzyme (C73A/S131C, I86A and Y127A separately) was mixed with 2-200µM of MTX in stopped-flow instrumentation in equal volumes and the change in intrinsic fluorescence intensity over time was recorded (excitation 290nm, emission 320nm cutoff filter). For all MTX concentrations, a decrease in fluorescence over time was observed, similar to previous research22. The individual

22
traces from the different concentrations were fit to an induced fit two-step model, similar to that of the previous study (fig 12), on a "global fit" in DYNAFIT4 program.

$$E + MTX \xrightleftharpoons[k_1][k_{-1}] EMTX$$

$$k_2 \quad \downarrow \quad k_2$$

$$E'MTX$$

Figure 12: Minimal model used for the binding of PYR to E detected by intrinsic tryptophan fluorescence. The induced fit model relates to ($k_1$ and $k_2$).

A large concentration dependent decrease in tryptophan fluorescence emission over time associated with MTX binding was observed during binding of low concentrations (2-20μM) of MTX to C73A/S131C, I86A and Y127A (Fig 13). It is concentration dependent because the rate of binding increases along with MTX concentration. These binding event traces were fit using Dynafit4 program.
Figure 13: The binding event step (D) with a large MTX concentration dependent decrease in fluorescence emission over time is observed with different association rates \( k_i \) for A. C73A/S131C (60 \( \mu M^{-1} s^{-1} \)), B. I86A (71 \( \mu M^{-1} s^{-1} \)), and C. Y127A (32 \( \mu M^{-1} s^{-1} \)). Low concentrations (2-20\( \mu M \)) of MTX are rapidly mixed with 2\( \mu M \) of enzyme in equal volumes and the traces were “globally fit” using DYNAFIT 4 program.

A second small MTX concentration independent decrease in fluorescence emission over time was observed for large concentrations (50-200\( \mu M \)) of MTX binding to all three enzymes (C73A/S131C, I86A and Y127A). It is concentration independent because the rate does not change with increasing concentration of MTX. The first large decrease happened too fast for the higher concentrations of MTX and was over within the

\[ k_1 = 60 \mu M^{-1} s^{-1} \]

\[ k_1 = 71 \mu M^{-1} s^{-1} \]

\[ k_1 = 32 \mu M^{-1} s^{-1} \]
dead time of the instrument while the second decrease was too small to be detected for lower MTX concentrations. (Fig 14).

Figure 14: A small MTX concentration independent decrease in fluoresce emission over time for is observed with different rates ($k_2$) for A. C73A/S131C ($9 \text{s}^{-1}$), I86A ($23 \text{s}^{-1}$) and Y127A ($10 \text{s}^{-1}$). High concentrations of MTX (50-200µM) are rapidly mixed with 2µM of enzyme in equal volumes and the Traces were “globally fit” using a one directional step model (D) on DYNAFIT 4 program.

The dissociation rates were determined by using Trimethoprim (TMP) to displace the enzyme MTX complex for C73A/S131C, I86A and Y127A separately in stopped-flow instrumentation as was done previously by Goodey study. Enzyme (2µM) was pre-incubated with MTX (4µM) and mixed in the stopped-flow instrumentation with 350µM and 700µM of TMP. Traces (600s) showed an increase in tryptophan fluorescence emission indicating displacement of MTX by TMP and were sufficiently fit
using a single exponential equation (not shown) and confirmed using Dynafit4 program (Fig 15).

**Figure 15:** An increase in fluorescence emission over time is observed for the dissociation of MTX from the enzyme complex. C73A/S131C (A), I86A (B) and Y127A (C) all have different rates with different amplitudes. Y127A having the highest %change amplitude ($k_{-1}$). The increase shows the displacement of MTX by varying concentrations (350pM (not shown) and 700pM) of TMP from the EMTX complex and was obtained by mixing pre-incubated enzyme (2pM) with MTX (4pM) with (350μM and 700μM) TMP in the stopped flow instrumentation.

The first decrease is attributed to MTX binding to E for all enzymes with an association rate ($k_{1}$) of 60μM⁻¹s⁻¹ (C73A/S131C), 71μM⁻¹s⁻¹ (I86A) and 32μM⁻¹s⁻¹ (Y127A). The 60μM⁻¹s⁻¹ $k_{1}$ rate constant obtained for C73A/S131C differed from the Goodey Paper at 23μM⁻¹s⁻¹. This difference could be as a result of inclusion of the higher concentrations in the single exponential fittings, something we did not do in this work.
When comparing the mutants to C73A/S131C, the I86A association rate varied slightly to C73A/S131C; it was showed an increase with no significant change in the % amplitude. The $k_1$ of Y127A differed significantly to both C73A/S131C and I86A with a change in the % amplitude. The fitting provided an approximation of the percentage of conformer E present in the total amount of enzyme, prior to binding of MTX.

The second decrease is associated with a conformational change from the E'MTX complex to E'MTX it was however not clear what the best value for the $k_2$ is, given the small amplitude of the signal. The conformational change step for I86A was faster at $23s^{-1}$ compared to $9s^{-1}$ for C73A/S131C and $10s^{-1}$ for Y127A. (Table8). The % amplitude was lower for I86A compared to Y127A, accounting for the faster conformational change rate constant

<table>
<thead>
<tr>
<th>Dynafit Data for MTX</th>
<th>C73A/S131C</th>
<th>I86A</th>
<th>Y127A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ ($\mu M^{-1}s^{-1}$)</td>
<td>60±3.0</td>
<td>71±2.0</td>
<td>32±1.0</td>
</tr>
<tr>
<td>$k_2$ ($s^{-1}$)</td>
<td>9±1.0</td>
<td>23±3.0</td>
<td>10±0.6</td>
</tr>
<tr>
<td>%E concentration</td>
<td>71±16.0</td>
<td>78±3.0</td>
<td>83±10.0</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal of $k_1$</td>
<td>3.40</td>
<td>2.60</td>
<td>1.10</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal of $k_2$</td>
<td>1.10</td>
<td>0.03</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Table 8: Parameters for C73A/S131C, I86A and Y127A obtained from DYNAFIT4 program. Association rates, $k_1$ ($\mu M^{-1}s^{-1}$), conformational change, $k_2$ ($s^{-1}$) concentration of E prior to binding in the total amount of enzyme used in the experiment. The % amplitude of the signal was calculated by the change in fluorescence divided by the initial fluorescence at time zero. The standard error shown is a regression error from fitting.

Dissociation rates ($k_{-1}$) of 0.01s$^{-1}$(C73A/S131C), 0.016s$^{-1}$(I86A) and 0.02s$^{-1}$ (Y127A) were obtained from fitting the traces to a single exponential equation (data not shown) and confirmed using Dynafit4 program (Table 9). The % amplitude in the
fluorescent signal was higher for Y127A indicated a larger change in the dissociation rate compared I86A.

<table>
<thead>
<tr>
<th>k_{-1} (s^{-1}) (MTX)</th>
<th>C73A/S131C</th>
<th>I86A</th>
<th>Y127A</th>
</tr>
</thead>
<tbody>
<tr>
<td>350\mu M</td>
<td>0.011</td>
<td>0.017</td>
<td>0.024</td>
</tr>
<tr>
<td>700\mu M</td>
<td>0.010</td>
<td>0.017</td>
<td>0.025</td>
</tr>
<tr>
<td>Average</td>
<td>0.010±0.007</td>
<td>0.017±0.004</td>
<td>0.024±0.005</td>
</tr>
<tr>
<td>%Amplitude of fluorescent signal of k_{-1}</td>
<td>-0.15</td>
<td>-0.15</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

Table 9: Dissociation rates (k_{-1}) for C73A/S131C, I86A, and Y127A obtained from DYNAFIT4 program, for the varying concentrations of TMP after mixing with pre-incubated E (2\mu M) and MTX (4\mu M). Final k_{-1} values are calculated by taking the average of values from 350\mu M and 700\mu M TMP. The % amplitude of the fluorescent signal for Y127A was higher compared to I86A and C73A/S131C. Errors indicate standard deviations from three sets of actual data.

For binding of MTX to conformer E, the mutants did affect the binding rate constants (k_1 and k_{-1}) and conformational change step (k_2) compared to C73A/S131C (modified wild type). Although I86A had a similar k_1 to C73A/S13C, this mutation did affect the conformational change step. In the case of Y127A, the binding rate constant k_1 was significantly slower compared to C73A/S131C and I86A and a much faster k_{-1}.

In the case of the I86A mutation, an increase in both the forward and reverse rate constant is seen. For Y127A we see a decreases in the forward rate and an increases the reverse. This may answer the question of how the mutations affect MTX binding but it does not answer what role they play in the allostERIC regulation of the global protein dynamics.

### 3.2 MTX Binding to MDCC Labeled DHFR

Varying concentrations of MTX (2-200\mu M) was mixed with 2\mu M of MDCC labeled DHFR(E'), (C73A/S131C_{MDCC}, I86A_{MDCC} and Y127A_{MDCC}) in stopped-flow
instrumentation in equal volumes and the change in MDCC fluorescence emission over time was recorded (excitation 419nm, emission 450nm cutoff filter). Similar to MTX binding to E and to the previous fluorescent labeled DHFR studies\textsuperscript{22}, a large concentration dependent decrease in fluorescence over time was observed during MTX binding. This decrease is not the same conformational change associated with MTX binding to E, instead it is associated with MTX binding to E'. The binding affinity rate constants ($k_4$ and $k_{-4}$) were very different from those recorded for changes in intrinsic tryptophan fluorescence, attributing this particular change to MTX binding to conformer E'.

The individual traces from the different concentrations were fit to a second order rate constant in a global fit on DYNAFIT\textsuperscript{4}. The conformational selection step from the minimal model (fig10), that includes an association rate ($k_4$) and dissociation rate constant ($k_{-4}$), is the event represented here (Fig 16). There is no conformational change step observed for E'MTX to EMTX as was seen for E from changes of intrinsic tryptophan fluorescence.
Figure 16: The binding event step for binding of MTX to E' (D) with a large MTX concentration dependent decrease in fluorescence emission over time is observed with different association rates (k4) for A. C73A/S131C (0.069 µM$^{-1}$ s$^{-1}$), B. I86A (0.074 µM$^{-1}$ s$^{-1}$), and C. Y127A (0.070 µM$^{-1}$ s$^{-1}$). Varying concentrations of MTX (2-200 µM) are rapidly mixed with 2 µM of enzyme.

Dissociation rates were determined by using Trimethoprim (TMP) to displace MTX from the E'MTX complex for C73A/S131C$_{MDCC}$, I86A$_{MDCC}$ and Y127A$_{MDCC}$ separately in stopped-flow instrumentation as was done for conformer E. Enzyme (2 µM) was pre-incubated with MTX (4 µM) and mixed in the stopped-flow instrumentation with 350 µM and 700 µM of TMP. Traces (600 s) showed an increase in tryptophan fluorescence emission indicating displacement of MTX from conformer E' by TMP and were sufficiently fit using a single exponential equation (not shown) and confirmed using Dynafit4 program (Fig 17).
Figure 17: An increase in fluorescence emission over time is observed for the dissociation of MTX from E’MTX. C73A/S131C_{MDCC} (A), 186A_{MDCC} (B) and Y127A_{MDCC} (C) all have different rates ($k_4$). The increase shows the displacement of MTX by varying concentrations (350µM and 700µM) of TMP from the E’MTX complex and was obtained by mixing pre-incubated enzyme (2µM) and MTX (4µM) with (350µM and 700µM) TMP in the stopped flow instrumentation.
Dynafit Data for MTX

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C73A/S131CMDCC</th>
<th>I86AMDCC</th>
<th>Y127AMDCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_4$ (μM$^{-1}$s$^{-1}$)</td>
<td>0.069±0.0005</td>
<td>0.074±0.0004</td>
<td>0.070±1</td>
</tr>
<tr>
<td>$k_4$ (s$^{-1}$)</td>
<td>0.010±0.0001</td>
<td>0.023±0.0001</td>
<td>0.026±0.0002</td>
</tr>
<tr>
<td>%E' concentration</td>
<td>38±0.1</td>
<td>25±0.1</td>
<td>23±0.1</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal</td>
<td>15</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal</td>
<td>-5.9</td>
<td>-11.5</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

Table 10: Parameters for C73A/S131CMDCC, I86AMDCC and Y127AMDCC obtained from DYNAFIT4 program. Association rates, $k_4$ (μM$^{-1}$s$^{-1}$), dissociation rates, $k_4$ (s$^{-1}$), concentration of E' prior to binding in the total amount of enzyme used in the experiment is obtained from DYNAFIT fitting, and the % amplitude of the signal calculated from the change in fluorescence divided by the initial fluorescence at time zero. The standard errors shown are regression errors from fitting.

This data confirms the previous Goodey study that *B. DHFR* exists in two different conformers (E and E') that have different binding affinity rate constants. For all the enzymes (C73A/S131C, I86A, Y127A) MTX binding to E has a faster association rate constant than E'. There are two conformational change events happening in the case for E; a conformational change associated with binding of MTX followed by a conformational change from EMTX to the more stable complex E'MTX. The $k_2$ was not easily resolved for the case of E' where we only see a conformational change associated with binding to form the E'MTX complex.

During binding of MTX to both E and E' we see similar conformational motions for the mutant (I86A, Y127A) compared to C73A/S131C (the modified wild type). The effects of the mutations are visible from changes in binding and conformational rate constants compared to C73A/S131C (Fig 18).
Figure 18: Binding Mechanism of MTX to E and E' for A. C73A/S131C, B. I86A and C. Y127A showing the Binding rates and conformational change rates.

The $k_2$ and $k_4$ differ the most for I86A with a large changes in % amplitude to C73A/S131C, indicating they are different rates from those of C73A/S131C. The biggest change we see for Y127A is in the % amplitudes of $k_4$ and $k_4$. The $k_4$ is within the same range as C73A/S131C however they are different because the % amplitudes are different.

4. Pyrimethamine (PYR) binding to DHFR

4.1 PYR Binding to C73A/S131C, I86A and Y127A DHFR

A similar method to section (2.1) in results Chapter 2 for the binding of MTX to DHFR was applied to study conformational changes associated with pyrimethamine (PYR) binding to C73A/S131C, I86A and Y127A.

Upon binding of low concentrations (2-25µM) of PYR, a large concentration-dependent increase in intrinsic tryptophan fluorescence emission overtime was observed
for C73A/S131C. Yet, when compared to C73A/S131C and I86A, Y127A revealed a small increase with a very small amplitude signal (Fig 19). From the MTX studies, we know that conformational motions of E are detected by intrinsic tryptophan fluorescence; it is safe to assume that this conformational change is attributed to PYR binding to conformer E.

**Figure 19:** An increase in intrinsic tryptophan fluorescence emission over time is observed for the binding of PYR to E (D) when low concentrations (2-20μM) of PYR are rapidly mixed with 2μM of enzyme in equal volumes and the traces “globally fit” using DYNAFIT 4 program. The association rate constants (k_1) were different for A. C73A/S131C (35μM⁻¹ s⁻¹), B. I86A (47μM⁻¹ s⁻¹), and C. Y127A (87μM⁻¹ s⁻¹).

The increase in intrinsic tryptophan fluorescence emission over time is a concentration dependent increase because the rate of binding increases with increasing concentrations of PYR. This is the binding event step.
After the binding event was over, a second conformational change that showed a decrease in intrinsic tryptophan fluorescence over time was observed for higher PYR concentrations (50-200μM). For all three enzymes, this decrease was concentration independent because the rate of the event did not change with increasing concentrations of PYR and is therefore the conformational change of the complex EPYR to E'PYR.

When the conformational change rates \( (k_2) \) for the mutants are compared to C73A/S131C (the modified wild type); the rates for the mutants are much slower and at a slower time scale than C73A/S131C. Y127A shows the slowest conformational change at a 100s range to I86A at 20s and C73A/S131C at 10s. (Fig20).

![Fluorescence Signal vs Time for C73A/S131C, I86A, and Y127A](image)

**Figure 20:** A small PYR concentration independent decrease in tryptophan fluorescence emission over time is attributed to the conformational change of complex EPYR to E'PYR (D) with different rates \( (k_2) \) for A. C73A/S131C (2.80 s\(^{-1}\)), I86A (0.30 s\(^{-1}\)) and Y127A (0.08 s\(^{-1}\)). High concentrations of MTX (50-200μM) are rapidly mixed with 2μM of enzyme in equal volumes and the Traces were “globally fit” using a one step model (D) on DYNAFIT 4 program.
The binding event observed with an increase in tryptophan fluorescence happens very fast that it is over within the dead time of the instrument for high concentrations. Similar to the MTX experiment, the optimal value for $k_2$ was difficult to obtain for C73A/S131C and I86A because of the small change in the tryptophan fluorescence signal.

To displace PYR from the enzyme complex (EPYR), a competition experiment was done, by mixing pre-incubated enzyme (2μM) and PYR (4μM) with varying concentrations of TMP (350μM and 700μM) in the stopped flow instrumentation. A large decrease in tryptophan fluorescence emission over time (100s) was recorded. The decrease indicates the displacement of PYR by TMP, since the change in the overall tryptophan fluorescence signal from the binding event was a large increase. The values of $k_{-1}$ were obtained by fitting the traces to a single exponential (not shown) and confirmed on Dynafit 4. (Fig21)
Figure 21: A decrease in fluorescence emission over time is observed for the dissociation of PYR from the enzyme complex EPYR (D). C73A/S131C (A), I86A (B) and Y127A (C) all have different dissociation rates (k−1). This was obtained by mixing pre-incubated enzyme (2µM) with MTX (4µM) with (350µM and 700µM) TMP in the stopped flow instrumentation.

The first phase increase is attributed to PYR binding to E for all enzymes with an association rate (k+) of 35µM⁻¹s⁻¹ (C73A/S131C), 47µM⁻¹s⁻¹ (I86A) and 87µM⁻¹s⁻¹ (Y127A). The association rates were obtained from a “global fit “ of the traces on DYNAFIT4. Y127A has a significant effect on the forward binding affinity rate constant when compared to C73A/S131C and I86A, with a decrease in the % amplitude signal. The small change in tryptophan signal along with a very fast association rate, may suggest that Y127A has a different conformational change associated with PYR binding, however the nature of this conformational change is not known. (Table 11)
The second phase small decrease in intrinsic tryptophan fluorescence is associated with a conformational change step from the EPYR complex to the E'PYR with a conformational rate of 2.80 s⁻¹ (C73A/S131C), 0.30 s⁻¹ (I86A) and 0.16 s⁻¹ (Y127A). Similar to the conformational change rate for EMTX to E'MTX, it was not clear what the optimal k₂ value was for C73A/S131C and I86A, since the amplitude of the signal was so small (Table 11). However the k₂ for the mutants were significantly slower than C73A/S131C and they showed a decrease in the % amplitude signal.

<table>
<thead>
<tr>
<th>Dynafit Data for PYR</th>
<th>C73A/S131C</th>
<th>I86A</th>
<th>Y127A</th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁ (µM⁻¹s⁻¹)</td>
<td>35±1.1</td>
<td>47±1.2</td>
<td>87±9.1</td>
</tr>
<tr>
<td>k₂ (s⁻¹)</td>
<td>2.80±0.39</td>
<td>0.30±0.03</td>
<td>0.08±0.01</td>
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<tr>
<td>%E concentration</td>
<td>74±0.12</td>
<td>86±0.30</td>
<td>85±0.14</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal k₁</td>
<td>-3.1</td>
<td>-4.4</td>
<td>-1.5</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal k₂</td>
<td>0.07</td>
<td>0.10</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 11: Kinetic parameters for PYR binding to C73A/S131C, I86A and Y127A obtained from DYNAFIT4 program. Association rates, k₁ (µM⁻¹s⁻¹), conformational change, k₂ (s⁻¹) concentration of E prior to binding in the total amount of enzyme used in the experiment, and the % amplitude of the signal. The standard error shown is a regression error from fitting.

Dissociation rates (k⁻¹) of 0.21 s⁻¹ (C73A/S131C), 0.17 s⁻¹ (I86A) and 0.14 s⁻¹ (Y127A) were obtained from fitting the traces to a single exponential equation and confirmed using Dynafit4. Y127A showed a decrease in the % amplitude signal, indicating the biggest difference in k⁻¹ when compared to C73A/S131C and I86A. This rate as well as the conformational change observes is different from the other two enzymes. (Table 12)
Table 12: Dissociation rates ($k_{-1}$) for C73A/S131C, I86A and Y127A obtained from DYNAFIT4 program, for the varying concentrations of TMP after mixing with pre-incubated E (2pM) and PYR (4pM). Final $k_{-1}$ values are calculated by taking the average of values from 350μM and 700μM TMP. The errors shown are calculated from the actual data.

<table>
<thead>
<tr>
<th>$k_{-1}$ (s$^{-1}$) (MTX)</th>
<th>C73A/S131C</th>
<th>I86A</th>
<th>Y127A</th>
</tr>
</thead>
<tbody>
<tr>
<td>350μM</td>
<td>0.21</td>
<td>0.017</td>
<td>0.15</td>
</tr>
<tr>
<td>700μM</td>
<td>0.20</td>
<td>0.016</td>
<td>0.14</td>
</tr>
<tr>
<td>Average</td>
<td>0.21±0.01</td>
<td>0.17±0.01</td>
<td>0.15±0.01</td>
</tr>
</tbody>
</table>

The conformational motions of DHFR (C73A/S131C, I86A and Y127A) associated with PYR binding, detected from changes intrinsic tryptophan fluorescence, revealed the presence of two conformations of DHFR. This aligned with the data obtained from MTX binding to DHFR and a similar model can be used for PYR binding (fig. 22).

\[
E + PYR \xrightleftharpoons[k_{-1}]{k_1} EPYR
\]

Figure 22: Minimal model used for the binding of PYR to E detected by intrinsic tryptophan fluorescence. The induced fit model relates to ($k_1$ and $k_2$).

4.2 PYR Binding to C73A/S131CMDCC, I86AMDCC and Y127AMDCC DHFR

To study the conformational motions of DHFR from changes in MDCC fluorescence over time, PYR (2-200μM) was mixed with 2μM of MDCC labeled DHFR.
(C73A/S131C<sub>MDCC</sub>, I86A<sub>MDCC</sub> and Y127A<sub>MDCC</sub>) in the stopped-flow instrumentation at equal volumes and the change in MDCC fluorescence over time was recorded (excitation 419nm, emission 450nm cutoff filter). A large concentration dependent decrease in fluorescence over time was observed during PYR binding to C73A/S131C<sub>MDCC</sub> and Y127A<sub>MDCC</sub>. A large concentration dependent increase is observed for PYR binding to I86A<sub>MDCC</sub>, this is a very different conformational change and may give an explanation for the large increase in in the inhibitor constant (K<sub>i</sub>) seen in the ligand specificity study<sup>21</sup>. (Fig23)

Figure 23: D shows the binding event of PYR to E'. A large PYR concentration dependent decrease in MDCC fluorescence emission over time is observed with association rates (k<sub>a</sub>) for A. C73A/S131C<sub>MDCC</sub> (0.55μM<sup>-1</sup>s<sup>-1</sup>) and C. Y127A<sub>MDCC</sub> (1.15μM<sup>-1</sup>s<sup>-1</sup>). A large PYR concentration increase in MDCC fluorescence is observed for B. I86A<sub>MDCC</sub> (0.83μM<sup>-1</sup>s<sup>-1</sup>). Varying concentrations of PYR (2-200μM) are rapidly mixed with 2μM of enzyme.
The decrease in MDCC fluorescence was not the same conformational change associated with PYR binding to conformer E for C73A/S131C_{MDCC} and Y127A_{MDCC}, where we see an increase in intrinsic tryptophan fluorescence. It is a conformational change associated with PYR binding to E’ and both C73A/S131C_{MDCC} and Y127A_{MDCC} have a different association rate constant ($k_4$) compared to the $k_1$ for PYR binding to conformer E.

In the case of I86A_{MDCC}, we see an increase in both MDCC fluorescence and intrinsic fluorescence emission over time, a conformational change associated with PYR binding to E’ and E respectively. We attribute the increase in MDCC fluorescence to the binding event associated with E’ because a different association rate constant ($k_4$) is obtained compared to the $k_1$ of PYR binding to E.

For higher PYR concentrations (50-200μM), after the binding event was over we observe a small decrease in MDCC fluorescence over time that was concentration independent. The rate of the event does not increase with increasing PYR concentrations and is attributed to the conformational change of the E’PYR complex to EPYR, a step not seen during MTX binding to E’. The % amplitude of MDCC signal for I86A differs significantly from C73A/S131C and Y127A suggesting a different conformational change observed. (Fig 24)
Figure 24: D shows the conformational change step for E’PYR to EPYR. A PYR concentration independent decrease in MDCC fluorescence emission over time is observed with rates (k-2) for A. C73A/S131C_{MDCC} (0.03 s⁻¹), C. Y127A_{MDCC} (0.11 s⁻¹) and B. I86A_{MDCC} (0.05 μM⁻¹ s⁻¹). High concentrations of PYR (50-200 μM) are rapidly mixed with 2 μM of enzyme.

A similar competition experiment for MTX in section 3.2 was used to determine the dissociation rates of PYR from the E’PYR complex. Trimethoprim (TMP) to displace MTX from the E’MTX complex for C73A/S131C_{MDCC}, I86A_{MDCC} and Y127A_{MDCC} separately in stopped-flow instrumentation; Enzyme (2 μM) was pre-incubated with MTX (4 μM) and mixed in the stopped-flow instrumentation with 350 μM and 700 μM of TMP.
Traces (100s) showed an increase in tryptophan fluorescence emission indicating displacement of PYR from conformer E’ by TMP and were sufficiently fit using a single exponential equation (not shown) and confirmed using Dynafit4 program (Fig25). We see an increase in MDCC fluorescence for the dissociation event for I86A\textsubscript{MDCC} because the overall signal was a decrease. Y127A\textsubscript{MDCC} showed the largest decrease in % amplitude when compared to I86A and C73A/S131C.

![Figure 25](image_url)

Figure 25: An increase in fluorescence emission over time is observed for the dissociation of PYR from E’MTX. C73A/S131C\textsubscript{MDCC} (A), I86A\textsubscript{MDCC} (B) and Y127A\textsubscript{MDCC} (C) all have different rates (k\textsubscript{-4}). The increase shows the displacement of MTX by varying concentrations (350\textmu M and 700\textmu M) of TMP from the E’PYR complex and was obtained by mixing pre-incubated enzyme (2\mu M) and PYR (4\mu M) with (350\mu M and 700\mu M) TMP in the stopped flow instrumentation.
The binding affinity rate constants were similar for the mutants (Y127AMDCC and I86AMDCC) compared to C73A/S131C_MDCC and the conformational change step rate was within the same range for all three enzymes (C73A/S131C_MDCC, I86AMDCC, Y127AMDCC). We observe a different conformational change associated with PYR binding to E’ for I86AMDCC as well as different % amplitude in MDCC fluorescence signal; it is interesting that the binding affinity rate constants and the conformational change rate do not differ significantly for I86AMDCC C73A/S13C_MDCC and Y127AMDCC. The major difference is observed in the % amplitude of MDCC signal. (Table 13).

<table>
<thead>
<tr>
<th>Dynafit Data for PYR</th>
<th>C73A/S131C_MDCC</th>
<th>I86AMDCC</th>
<th>Y127AMDCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{4}$ (µM$^{-1}$s$^{-1}$)</td>
<td>0.55±1.1</td>
<td>0.83±0.01</td>
<td>1.15±0.03</td>
</tr>
<tr>
<td>$k_{-4}$ (s$^{-1}$)</td>
<td>0.16±0.001</td>
<td>0.14±0.001</td>
<td>0.27±0.002</td>
</tr>
<tr>
<td>$k_{-2}$ (s$^{-1}$)</td>
<td>0.03±0.002</td>
<td>0.05±0.003</td>
<td>0.11±0.001</td>
</tr>
<tr>
<td>%E’ concentration</td>
<td>39±0.18</td>
<td>25±0.30</td>
<td>30±0.14</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal for $k_{4}$</td>
<td>-4.09</td>
<td>-1.40</td>
<td>1.10</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal for $k_{-4}$</td>
<td>-4.09</td>
<td>-3.04</td>
<td>1.06</td>
</tr>
<tr>
<td>% Amplitude of fluorescent signal for $k_{-2}$</td>
<td>-0.54</td>
<td>-0.11</td>
<td>-0.69</td>
</tr>
</tbody>
</table>

Table 13: Kinetic parameters for PYR binding to C73A/S131C_MDCC, I86AMDCC and Y127AMDCC obtained from DYNAFIT4 program. Association rates, $k_{4}$ (µM$^{-1}$s$^{-1}$), dissociation rates, $k_{-4}$ (s$^{-1}$), conformational change rates $k_{-2}$ (s$^{-1}$), concentration of E prior to binding in the total amount of enzyme used in the experiment, and the % amplitude of the signal are shown. The standard error shown is a regression error from fitting.

The data obtained from the MDCC fluorescent experiment of PYR binding to E’, confirms the existence of the two conformers observed in the MTX experiments. It is therefore suitable to use a similar overall minimal model for the events associated with binding of PYR to the two conformers (E and E’) (Fig 26) as was used for MTX (refer to figure10).
Figure 26: Minimal model for the binding of PYR to E and E’ similar to MTX model. PYR binding to E (k₁) or E’ (k₄) is the binding event step. The induced fit model relates to (k₁ and k₂) or (k₄ and k₂). Conformational selective model fits for (k₃ and k₄) or (k₃ and k₁). The conformational equilibrium is indicated by (k₃ and k₄).

The nature of the conformational changes observed is not known however the different % amplitude in MDCC fluorescence for k₄, k₃ and k₂, indicate a different conformational change for I86A and Y127A when compared to C73A/S131C with different rate constants. (Fig 27)

Figure 27: Binding Mechanism of PYR to E and E’ for A. C73A/S131C, B. I86A and C. Y127A
Summary

1. Conformational Motions Associated with Inhibitor Binding

In this work, we reveal the conformational changes associated with inhibitor binding are inhibitor specific. Upon binding of MTX to conformer E of all the DHFR (C73A/S131C, I86A and Y127A) we observe a large concentration dependent decrease in tryptophan fluorescence over time, while binding of PYR to E shows an increase in tryptophan fluorescence. (Fig 28)

![Figure 28: Different conformational changes associated with MTX and PYR binding to C73A/S131C DHFR detected by intrinsic tryptophan fluorescence. A. MTX binding to Conformer E revealed a concentration dependent decrease in tryptophan fluorescence emission. B. PYR binding to E shows an increase in tryptophan fluorescence.](image)

Inhibitor binding to the other conformer of DHFR (E’) reveals a different conformational change compared to E, which was also inhibitor specific. We observe a large concentration dependent decrease in MDCC fluorescence for both MTX and PYR binding to E’ of C73A/S131C<sub>MDCC</sub> and Y127A<sub>MDCC</sub>. This decrease is different from the
decrease observed during binding of MTX to E (Fig 29). Although I86A MDCC had a similar conformational change associated with MTX binding, it showed a completely different motion (an increase in MDCC fluorescence) during PYR binding (refer to Fig 23).

**Figure 29:** Different conformational changes associated with MTX and PYR binding to Y127A DHFR detected by intrinsic tryptophan fluorescence and MDCC fluorescence. A. MTX binding to Conformer E. B. PYR binding to E. C. MTX binding to Conformer E'. D. PYR binding to E'.

MTX and PYR are competitive inhibitors of DHFR that bind in the active site of the enzyme and therefore interact with the active site residues. One might assume to assume that the conformational change associated with binding of any competitive inhibitor of DHFR would not be different across inhibitors. This is not the case as we see
for MTX and PYR. Although the nature of the conformational change is not known, each inhibitor induces a different conformational motion of DHFR upon binding. A structural understanding of these ligand induced conformational motions may be potential targets in drug design and therefore have implications for structure-based and molecular dynamics drug design.

2. Effects of Allosteric Motions on DHFR

2.1 Effects of Allosteric Mutations on association rate constants

The allosteric mutations affected the binding affinity rate constants of MTX and PYR binding to conformer E when compared to C73A/S131C (the modified wild type). There was no significant change in the binding affinity rate constants for binding of MTX and PYR to E' for all three enzymes (C73A/S131C, Y127A and I86A). The effects were also inhibitor specific, with an increase in the association rates ($k_1$) of MTX and PYR to I86A and a decrease for MTX to Y127A and an increase for PYR to Y127A.

The most significant difference for Y127A was in the $k_1$, $k_{-1}$ and $k_4$ for MTX and $k_1$ and $k_2$ during PYR binding. I86A showed the biggest change in $k_2$ and $k_4$ for MTX and $k_2$ for PYR. When we compare both mutants to each other Y127A seems to have a larger effect on the association and dissociation rates while I86A affects the conformational changes.

The $k_{off}/k_{on}$ values are calculated from the experimental values of $k_{-1}$ and $k_1$ for conformer E and $k_4$ and $k_4$ for conformer E' (Table 13).
Table 13: \( k_{\text{off}}/k_{\text{on}} \) parameters calculated from binding affinity rate constant resolved from kinetic data from stopped-flow experiments.

These values give an estimate of the equilibrium dissociation constants, \( k_D \) for MTX and PYR to E and E'. Based on these estimates the E conformer of C73A/S131C has a higher affinity for MTX than E'. This trend is the same for the mutants, Y127A, and I86A although when compared to C73A/S131C both mutants show a decrease for affinity of MTX.

PYR binds better to DHFR of protozoan species, so it makes sense that DHFR from \( B.s. \) would have a lower affinity for PYR than MTX. Similar to MTX, the E for all three enzymes has a higher affinity for PYR compared to E'.

The data aligns with the inhibitor specificity study\(^{21}\) where we see the allosteric mutations affecting inhibitor binding. A small perturbation such as a distal allosteric mutation is able to affect the binding affinity rate constants of MTX and PYR to DHFR far from the active site. These minimal changes in the rate constants and the conformational change rates more information for the effects of the allosteric mutations on inhibitor binding.

2.2 Effects of Allosteric Mutations on the Conformational Equilibrium of DHFR Prior to Inhibitor Binding

The advantage of using DYNAFIT4 program for enzyme kinetic fitting is that, it can give a calculation for the concentration of enzyme involved in inhibitor binding. One hypothesis of how allosteric mutations affect inhibitor binding and specificity is by
altering the conformational equilibrium of DHFR. This can be significant if the low energy, binding event involves the induced fit pathway as opposed to the conformational selection pathway and a shift in equilibrium may alter the presence of the preferred conformer for initial inhibitor binding. Kinetic data retrieved from the stopped-flow instrumentation experiments, reveal approximations of the % concentration of E and E’ prior to inhibitor binding. (Table 14)

<table>
<thead>
<tr>
<th></th>
<th>%E (MTX)</th>
<th>%E (PYR)</th>
<th>Overall %E</th>
<th>%E’ (MTX)</th>
<th>%E’ (PYR)</th>
<th>Overall %E’</th>
</tr>
</thead>
<tbody>
<tr>
<td>C73A/S131C</td>
<td>71</td>
<td>74</td>
<td>≈70</td>
<td>38</td>
<td>39</td>
<td>≈30</td>
</tr>
<tr>
<td>I86A</td>
<td>73</td>
<td>86</td>
<td>≈70</td>
<td>25</td>
<td>25</td>
<td>≈30</td>
</tr>
<tr>
<td>Y127A</td>
<td>86</td>
<td>85</td>
<td>≈80</td>
<td>23</td>
<td>30</td>
<td>≈20</td>
</tr>
</tbody>
</table>

Table 14: An approximation of the conformational equilibrium of E and E’ prior to binding, obtained from DYNAFIT4 kinetic data fitting. The % concentration of conformer E available to bind MTX and PYR and % concentration of E’ for MTX and PYR binding are shown in the table.

From the data we can estimate that for all DHFR enzymes (C73A/S131C, Y127A and I86A) ≈70 % of E and ≈30 % of E’ exist in solution. The conformational equilibrium prior to inhibitor binding is not affected very significantly by the introduction of allostERIC mutations. This is just an approximation and cannot rule out fully, a slight shift in conformational equilibrium as a factor involved in the role of allostERIC residues in the regulation of protein motions necessary for ligand specificity and binding.
**Conclusion**

We wanted to investigate the effects of distal allosteric mutations on the conformational motions of DHFR upon inhibitor binding and validate the previous study done to support the effects of allosteric mutations of inhibitor specificity and binding \(^{21}\). It is clear, that the allosteric mutations do affect ligand binding and the conformational motions associated with inhibitor binding without altering the conformational equilibrium of DHFR prior to binding.

I86A had very similar binding affinity rate constants and conformational change rates to C73A/S131C, but affected the conformational motions associated with PYR binding to conformer E'; It showed an increase in MDCC fluorescence emission overtime compared to the decrease seen for C73A/S131C.

Y127A exhibited very different binding affinity rate constants to C73A/S131C and I86A. The association rate constant decreased for binding to MTX while it increased for PYR binding. For the conformational change rates, we see a very slow rate, \(0.08s^{-1}\) in the millisecond time scale compared to \(2.80s^{-1}\) for C73A/S131C. Slowing down the conformational change step may affect the binding kinetics of the enzyme. The conformational change associated with PYR binding to E, revealed a small concentration dependent increase in tryptophan fluorescence over time, which can be attributed to a different conformation change compared to that seen for C73A/S131C and I86A.

These mutations are located at sites distal from the active site and do not interact with the inhibitor in the binding pocket. Yet, they are able to affect the conformational motions associated with inhibitor binding along with the binding and conformational
change rates. The mutations introduced are also located at different distal sites on the enzyme and the effects on DHFR are very different between the two mutations.

It is most likely that allosteric mutations may exert their effects by affecting the conformational motions, particularly by slowing them down, speeding up or changing them completely as in the case of PYR binding to E' of I86A. The residues located in the mutation regions may exhibit the same effects as the individual mutations and further studies on cluster mutations as well as double cluster mutations might reveal more information on which conformational motions are severely affected.

Another important factor revealed from this work, was that the conformational changes associated with inhibitor binding were different for MTX compared to PYR. It is very easy to assume that inhibitors that bind to the same site would induce the same conformational change even though they may interact with different active site residues. This is not the case, as the conformational changes are inhibitor specific, and also induce different conformations when binding to the different conformers. This is relevant for structure based drug design, molecular docking and molecular dynamics computational studies for drug design, as these motions may be potential drug targets or reveal more for design of better and selective inhibitors.
References


## APPENDIX

<table>
<thead>
<tr>
<th>Sx 20 Stopped Flow Instrumentation</th>
<th>Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light source</td>
<td>150W air cooled Xe arc Lamp</td>
</tr>
<tr>
<td>Lamp Stability</td>
<td>&lt;0.001 AU peak to peak</td>
</tr>
<tr>
<td>SF cell Volume</td>
<td>20µL</td>
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<tr>
<td>Pathlength (Fluorescence)</td>
<td>3mm &amp; 1mm</td>
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<tr>
<td>Dead time</td>
<td>1.1ms for 1:1 mixing</td>
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<tr>
<td>Drive volume</td>
<td>40 µL per syringe for 1:1 mixing</td>
</tr>
<tr>
<td>Dead Volume</td>
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<tr>
<td>Mixing options</td>
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<td>Windows XP OS</td>
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</table>

Table 15: SX 20 Stopped flow instrumentation technical settings held constant for stopped-flow kinetic experiments
Figure 30: C73A/S131C (Bs.DHFR) and I86A (NG1) Nucleic acid sequence.
Global Fit the progress of WT vs MTX 0.1s

[task]
  data = progress
  task = fit

[mechanism]
  E + MTX <=> E'MTX : k1 k-1
  EMTX ----> E'MTX : k2 ;k-2

[constants]
  ; units: uM, sec
  k1 = 67
  k-1 = 0.01
  k2 = 9
  ;k-2 = 0.1

[concentrations]
  ; units: uM
  E = 1.5
  EMTX = 0.000000000000000001

[responses]
  ; percent product/nM substrate
  E = 0.686?
  MTX = -0.0209?
  EMTX = 0.238?
  E'MTX= 0.79?

[data]

directory /Users/Marie/Desktop/Data/Low
file 10.1s.txt | offset 7.47| concentration MTX =1

directory /Users/Marie/Desktop/Data/Low
file 1.50.07s.txt | offset 7.37| concentration MTX = 1.5

directory /Users/Marie/Desktop/Data/Low
file 20.06s.txt | offset 7.32| concentration MTX = 2

directory /Users/Marie/Desktop/Data/Low
;file 2.50.1s.txt | concentration MTX =2.5 | offset 6.8

directory /Users/Marie/Desktop/Data/Low
file 40.05s.txt | concentration MTX =4 | offset 7.29

directory /Users/Marie/Desktop/Data/Low
file 50.04s.txt | concentration MTX =5 | offset 7.26

directory /Users/Marie/Desktop/Data/Low
;file 80.03s.txt | concentration MTX =8 | offset 7.4?

directory /Users/Marie/Desktop/Data/Low
;file 100.1s.txt | concentration MTX =10| offset 7.4?

[output]

directory /Users/Marie/Desktop/output/WT.7.15/0.1

Figure 31: C73A/S131C Script file for DYNAFIT4 fitting of low MTX concentrations to give a value for the association rate k1. Similar script was used for I86A and Y127A as well as data from PYR binding.
Global Fit the progress of WT vs PYR 10s
;-------------------------------------------------------------------------

[task]
data = progress
    task = fit

[mechanism]
;E + PYR        = EPYR ; k1 k-1
    EPYR ----> E'PYR ; k2 ; k-2

[constants] ; units: uM, sec
; k1 = 78
; k-1 = 0.01
    k2 = 3?
; k-2 = 0.1?

[concentrations] ; units: uM
; E = 1.5?
    EPYR = 1.5?

[responses] ; percent product/nM substrate
; E = 0.686
; PYR = -0.0209
    EPYR = 0.675?
    E'PYR = 0.6?

[data]
directory /Users/Marie/Desktop/Data/High
    file 2510s.txt | offset 6.14?|; concentration MTX = 25
    directory /Users/Marie/Desktop/Data/High
; file 5010s.txt | offset 6.28?|; concentration MTX = 50
    directory /Users/Marie/Desktop/Data/High
    file 10010s.txt | offset 6.57?|; concentration MTX = 100

[output]
directory /Users/Marie/Desktop/output/WT.715/10

Figure 32: C73A/S131C Script file for DYNAFIT fitting analysis of high PYR concentrations to give a value for the conformational change rate k2. Similar script was used for I86A and Y127A as well as data from MTX binding.
Global Fit the progress of Y127A vs MTX 600s

[task]
data = progress
task = fit

[mechanism]
EMTX ----> E + MTX : k-1

[constants]
; units: uM, sec
k-1 = 0.01?

[concentrations]
; units: uM
EMTX = 1?

[responses]
; percent product/nM substrate
E = 3.4?
MTX = 3.4?
EMTX = 0.1?

[data]
directory /Users/Marie/Desktop/Data/358
file 600s.txt | offset 4.29?
directory /Users/Marie/Desktop/Data/700
file 700s.txt | offset 4.93?

[output]
directory /Users/Marie/Desktop/output/offrate.15/Y127AMTX.700.350

Figure 33: Y127A Script file for DYNAFIT fitting of competitive experiment data
with TMP give a value for the dissociation rate k-1. Similar script was used for I86A
and Y127A as well as data from MTX binding and MDCC labeled enzyme
dissociation.
Global Fit the progress of I86AMDCC vs MTX 10s

[:task]
  data = progress
  task = fit

[:mechanism]
  E' + MTX $\Leftrightarrow$ E'MTX : k4 k-4
  ;E + MTX $\Leftrightarrow$ EMTX : k1 k-1
  ;EMTX $\rightarrow$ E'MTX : k-2
  ;E $\rightarrow$ E' : k3

[:constants] ; units: uM, sec
  ;k1 = 74
  ;k-1 = 0.02
  k4 = 0.092
  k-4 = 0.024
  ;k-2 = 9
  ;k3 = 28

[:concentrations] ; units: uM
  ;E' = 0.9
  ;E = 0.5
  ;EMTX = 0.000001

[:responses] ; percent product/nM substrate
  ;E = 3.1
  E' = 3.01
  MTX = 0
  E'MTX = 1.95
  ;EMTX= 1

[:data]
  directory /Users/Marie/Desktop/Data/10
  ;file 1.5.txt | offset 5.92|concentration MTX = 1.5
  directory /Users/Marie/Desktop/Data/10
  ;file 2.5.txt| offset 5.98|concentration MTX = 2.5
  directory /Users/Marie/Desktop/Data/10
  file 5.txt | offset 5.95|concentration MTX = 5.04
  directory /Users/Marie/Desktop/Data/10
  file 10.txt | offset 5.90|concentration MTX = 10.77
  directory /Users/Marie/Desktop/Data/10
  file 25.txt | offset 5.8|concentration MTX = 26.88
  directory /Users/Marie/Desktop/Data/10
  file 50.txt | offset 5.75|concentration MTX = 54.83
  directory /Users/Marie/Desktop/Data/10
  file 100.txt | offset 5.6|concentration MTX = 93.97

[:output]
  directory /Users/Marie/Desktop/output/186A.2/MTX/10

Figure 34: I86AMDCC Script file for DYNAFIT fitting of MTX vs MDCC labeled enzyme to give a value for the association rate k4. Similar script was used for C73A/S131CMDCC and Y127AMDCC as well as data from PYR binding.
Figure 35: SDS-PAGE gel of I86A and I86AMDCC proteins, using 4X loading dye (10ul Dye and 30ul sample). EZ protein ladder shows the MW sizes and position.

Figure 36: SDS-PAGE gel of Y127A proteins, using 4X loading dye (10ul Dye and 30ul sample). EZ protein ladder shows the MW sizes and position. Higher band is dimer.