Supplementary materials for: MULTIPLE CONFORMERS IN ACTIVE SITE OF HUMAN DIHYDROFOLATE REDUCTASE F31R/Q35E DOUBLE MUTANT SUGGEST STRUCTURAL BASIS FOR METHOTREXATE RESISTANCE

Jordan Volpato, Brahm J. Yachnin, Jonathan Blanchet, Vanessa Guerrero, Lucie Poulin, Elena Fossati, Albert M. Berghuis and Joelle N. Pelletier

Experimental procedures

Expression and purification of His_6-hDHFR F31R/Q35E – An overnight preculture of SK037/His_6-hDHFR F31R/Q35E-pQE32 was used to inoculate 1 L of LB medium. The culture was grown at 37°C until the $A_{600\ nm} \approx 0.7$. Protein expression was induced with the addition of 1 mM of isopropyl 1-thio-β-D-galactopyranoside (IPTG), after which the cells were grown for 3 h at 37°C. Induced cells were harvested by centrifugation (4000*g* for 30 min at 4°C). The cell pellet was resuspended in 0.1 M phosphate buffer, pH 8.0, at 4°C. The cells were lysed on ice using a Branson sonicator (four pulses at 200 W for 30 s with a tapered micro-tip). The cellular debris was pelleted by centrifugation (4000*g* for 30 min at 4°C) and the supernatant was filtered through a 0.2μm filter before purification.

Purification was performed following a 2-step purification protocol on an AKTA FPLC (GE Healthcare, Piscataway, NJ) at 5°C. First, the supernatant was applied to a HisTrap FF 1 mL prepacked cartridge (GE Healthcare) followed by a 10 column volumes (CV) wash with 0.1 M phosphate buffer, pH 8.0, at 1 mL/min. A step-wise gradient of imidazole (10, 20, 50 and 200 mM; 5 CV for each step) in 0.1 M phosphate buffer, pH 8.0, was used to elute the F31R/Q35E mutant. hDHFR activity was monitored in MATS buffer, pH 7.6, in the presence of 100 μM each NADPH and DHF. Activity was measured in flat-bottom plates (Costar #3595) by monitoring concurrent depletion of NADPH and DHF ($\Delta\varepsilon_{340nm}$ = 12 800 $M^{-1}cm^{-1}$) on a FLUOstar OPTIMA UV-vis plate reader (BMG Laboratories, Offenburg, Germany). Active fractions were pooled and dialysed overnight at 4°C against 50 mM phosphate buffer, pH 7.5. Following dialysis, the sample (15 mL) was concentrated to 1.5 mL using an Amicon concentrator (MCWO 10000, Millipore), for injection on a Superose12 column (1.6 × 55 cm). The sample was eluted with 50 mM phosphate buffer, pH 7.5, at a flowrate of 1.5 mL/min. hDHFR

activity was monitored as described above. Enzyme purity was evaluated following separation by SDS-PAGE (15% (w/v) polyacrylamide gel) stained by the zinc-imidazole method (1) and quantified using the public domain image analysis software Scion Image (NIH, rsb.info.nih.gov/ nih-image). Protein concentration was quantified using the Bradford assay (Biorad, Hercules, CA).

Crystallization and X-ray data collection of His_6-hDHFR F31R/Q35E – Purified His_6-hDHFR F31R/Q35E enzyme was buffer-exchanged into 50 mM Imidazole pH 7.5 and concentrated to 20 mg/mL using an Amicon concentrator (MCWO 10000). MTX and NADPH were prepared as described previously (2) and were added at a final concentration of 4 mM each (5-fold molar excess) to the protein sample. Crystallization experiments were set up using hanging drop vapour diffusion experiments, with a reservoir volume of 1 mL and a drop size of 4 μL of equal volumes of protein and reservoir solutions. A reservoir solution containing 0.1 M Tris pH 8.5, 0.01 M $NiCl_2$ and 21% PEG 2000 MME yielded crystals that diffracted at 2.2 Å. The crystals were soaked in the mother liquor supplemented with 30% PEG 400 as a cryoprotectant, frozen in a nitrogen cryostream (model X-stream 2000), and stored in liquid nitrogen. Data collection was performed with a X8C beam at the National Synchrotron Light Source, Brookhaven National Laboratory, and processed using HKL2000 (3). (Table S1).

Structure Determination and Refinement – The structure was determined by molecular replacement using Phaser (4), which found six protein molecules in the asymmetric unit (Resolution Range Used: 2.21-40.89; Log Likelihood Gain (Refined): 1932.291). PDB ID 1U72 was used as a molecular replacement model. Reciprocal-space refinement was performed using Refmac (5), and included individual isotropic B-factor refinement. Manual model building was performed periodically using Coot (6) (Table S1). The full length of the hDHFR backbone could be modelled from the electron density map in all six molecules in the asymmetric unit, as could some portion of the N-terminal His_6-tag. In all six protein molecules in the asymmetric unit, large regions of electron density were observed in the DHF- and NADPH-binding sites. MTX and NADPH were modelled into these sites, respectively.

Table S1: Crystallographic data for His_6-hDHFR F31R/Q35E

Data collection statistics	
Space group	P1
Number of Molecules per Asymmetric Unit	6
a (Å)	62.605
b (Å)	83.334
c (Å)	83.272
α (º)	62.92
β (º)	78.68
γ (º)	71.81
Wavelength (Å)	1.100
Resolution Range (Å)[†]	2.21-40.89 (2.21-2.29)
Completeness (%)[†]	92.1 (94.2)
Redundancy[†]	3.6 (3.1)
R_{merge} (%)[†]	6.2 (15.6)
Refinement statistics	
Total number of reflections (reflections in R-free set)	65,777 (3284)
R_{factor} (%)	31.420
R_{free} (5% free test set) (%)	40.028
Number of atoms	10,446
Protein	9720
Water	240
Co-factor	288
Inhibitor	198
RMSD	
Bond length (Å)	0.010
Bond angle (º)	1.847
Average atomic B-Factor (Å^2)	32.789
Protein (Å^2)	32.85
Water (Å^2)	22.74
Co-factor (Å^2)	41.01
Inhibitor (Å^2)	30.06
Wilson B-Factor (Å^2)	32.253
Ramachandran Plot (non-Gly, non-Pro residues)	1026 (100%)
Residues in Favoured Positions	822 (80.1%)
Residues in Allowed Positions	137 (13.4%)
Residues in Generously Allowed Positions	34 (3.3%)
Residues in Disallowed Positions	33 (3.2%)

[†]Items in parentheses refer to the highest resolution shell.

Supplementary figures

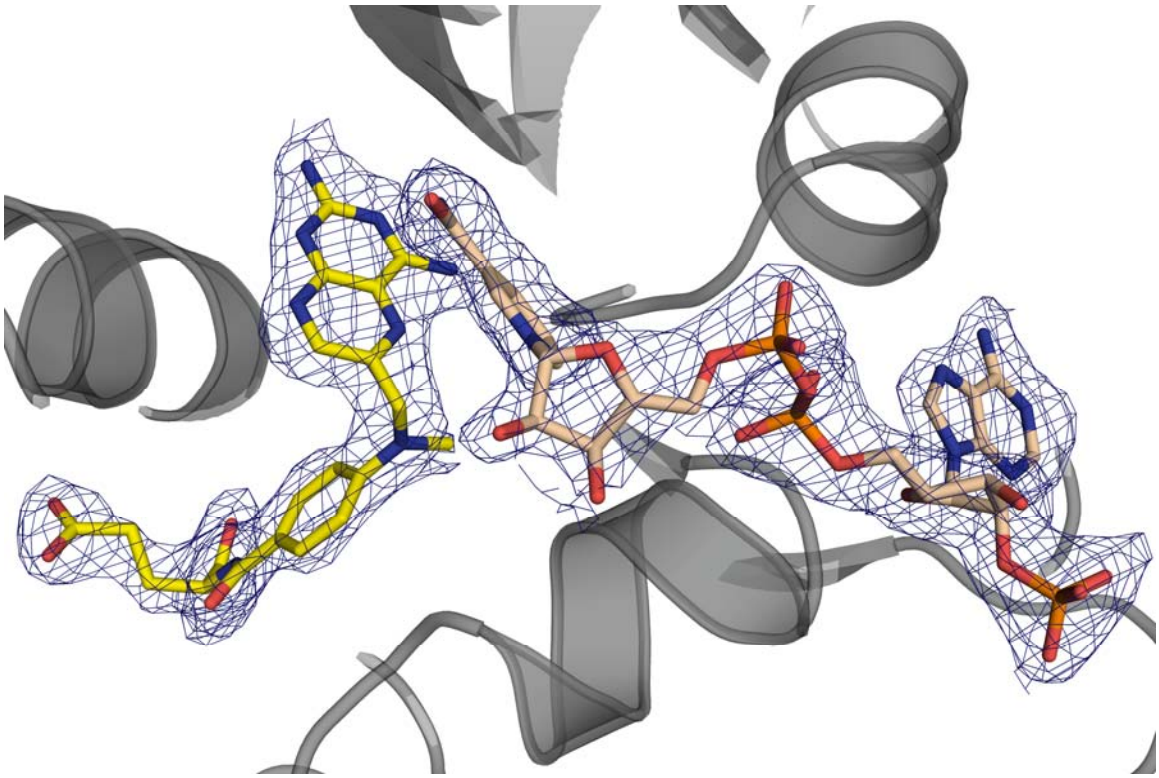

Figure S1: Electron density around MTX and NADPH from low resolution structure of His_6-hDHFR F31R/Q35E. The electron density map is a $2F_o$-F_c map contoured at 1 sigma.

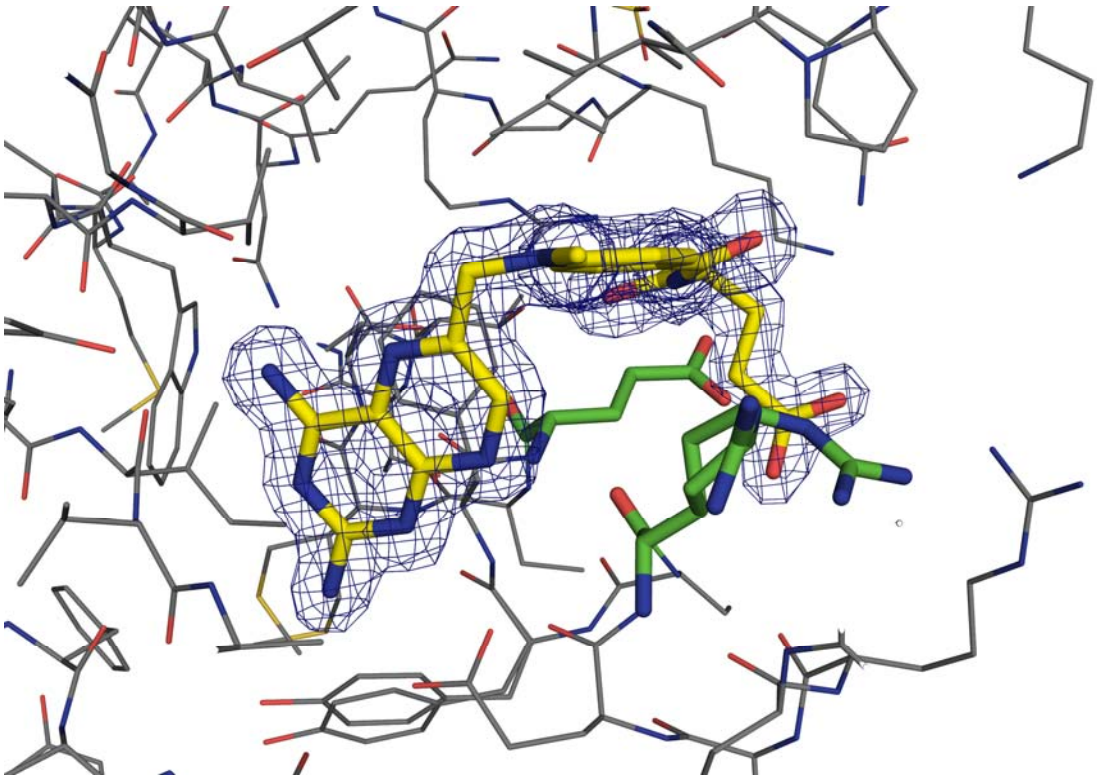

Figure S2: Electron density around MTX molecule in 3EIG. The electron density map is a $2F_o$-F_c map contoured at 1 sigma.

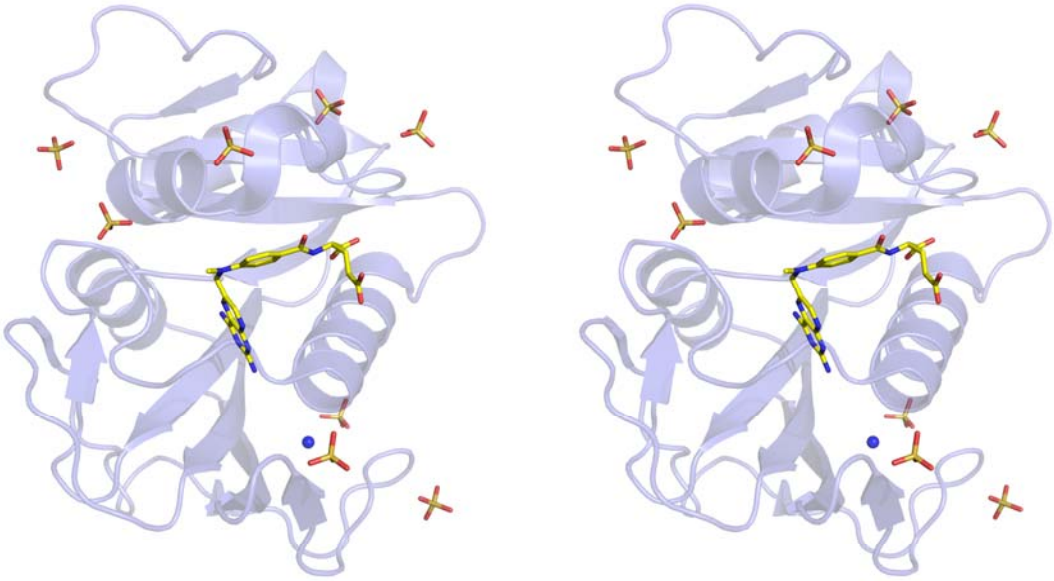

Figure S3: Stereo view of structure of hDHFR variant F31R/Q35E (3EIG). The macromolecule is shown in cartoon representation, while MTX and sulphate molecules are shown in sticks representation, colored by atom (C: yellow, N: blue, O: red and S: orange). A Cd atom is shown as a blue sphere; it is coordinated by the negatively charged groups of Glu183, Glu150 (symmetry-related), and SO_4 and by Met14.

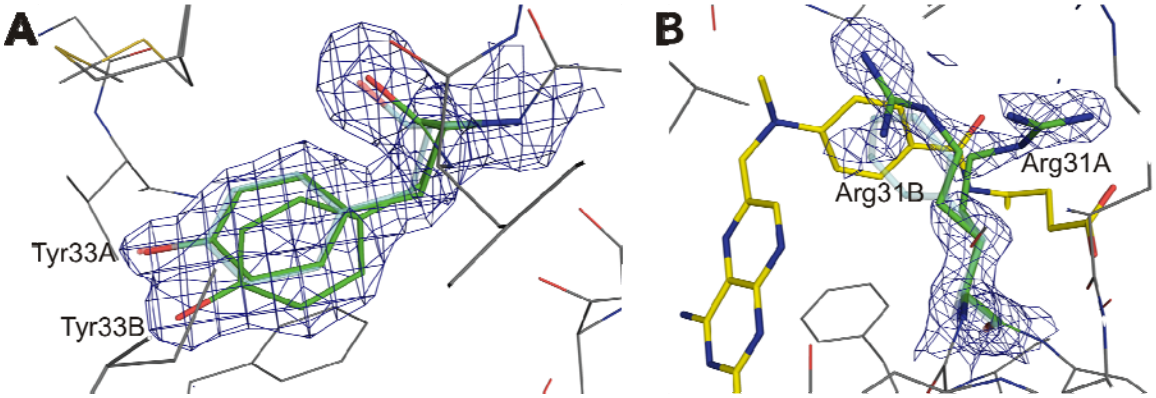

Figure S4: Alternate side chain conformers at active site residues Tyr33 (A) and Arg31 (B) in hDHFR variant F31R/Q35E (3EIG). Residues from the F31R/Q35E and superposed WT hDHFR (1U72) structure are shown in stick representation, colored by atom (C: green (F31R/Q35E), yellow (MTX), transparent cyan (WT hDHFR); O: red; N: blue). The Tyr33 residue from WT hDHFR is barely visible as it is almost perfectly superposed upon conformer Tyr33A from F31R/Q35E. Superposition was performed by C_α alignment of the crystal structures. The electron density map is a $2F_o$-F_c map contoured at 1 sigma.

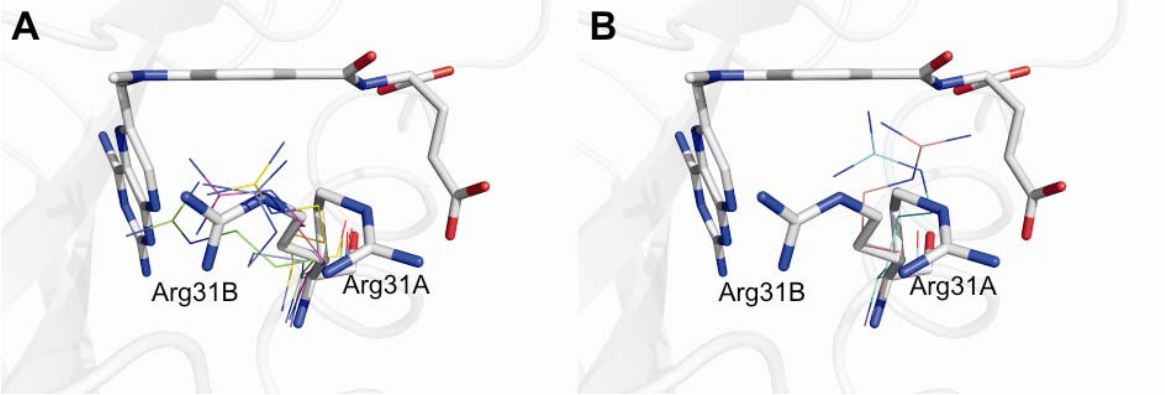

Figure S5: Multiple conformers of Arg31 in the lower resolution His_6-hDHFR F31R/Q35E (in lines) relative to 1.7 Å resolution hDHFR F31R/Q35E (in sticks). (A) Four of the six Arg31 residues found in the lower resolution structure adopt a conformation that clusters with Arg31B. (B) Two Arg31 residues found in the lower resolution structure adopt a conformation that neither resembles that of Arg31A or Arg31B.

References

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