Gene Expression, Vol. 6, pp. 231-239, 1996
Printed in the USA.

1052-2166/96 $10.00 + .00

Isolation and Characterization of a Dihydrofolate Reductase Gene Mutation in Methotrexate-Resistant *Drosophila* Cells

HONG HAO, MICHAEL G. TYSHENKO, AND VIRGINIA K. WALKER[1]

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Stepwise increases in methotrexate (MTX) concentration over a 4-year period led to the selection of a highly drug-resistant (2×10^{-4} M MTX) *Drosophila* cell line. Uptake experiments with [^{3}H]MTX showed a slightly lower level of intracellular MTX in the resistant S3Mtx cells than in the susceptible S3 parental cell line. Southern blot analysis demonstrated that the gene for the MTX target, dihydrofolate reductase (DHFR), was not significantly amplified in the resistant line. To determine the molecular basis for resistance, the DHFR cDNA sequence was amplified by polymerase chain reaction from both the resistant and susceptible cells. Sequence comparison revealed a single T to A base change at nucleotide 89, which resulted in the substitution of Gln for Leu at residue 30 in S3Mtx cells. Expression and purification of the wild-type and mutant DHFR from *E. coli* cells showed that the S3Mtx enzyme had a reduced binding affinity for the antifolates, MTX and trimethoprim, with 15-fold higher K_d and K_i values than those from the wild-type enzyme. Molecular modeling confirmed that the replacement of the hydrophobic Leu by the more polar Gln was in the substrate binding site and thus would decrease the binding of MTX. These results suggest that the high level of MTX resistance in the selected cell line can be attributed to the mutation in the DHFR gene and also provides a model for pesticide resistance in insects.

Methotrexate resistance *Drosophila* cells Dihydrofolate reductase Gene mutation

THE enzyme dihydrofolate reductase (DHFR, 5,6,7,8-tetrahydrofolate:NADP oxidoreductase, E.C.1.5.1.3.) catalyses the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. The enzyme is necessary for maintaining intracellular pools of tetrahydrofolate, which is an essential carrier of one-carbon units in the biosynthesis of thymidylate, purines, and several amino acids. DHFR is the target enzyme of antifolate drugs, such as methotrexate (MTX) and trimethoprim (TMP), which are widely used as anticancer and antimicrobial agents.

The development of acquired resistance to MTX in cultured mammalian cells has been well studied at the genetic and molecular level. Many of these studies have used cell lines in which the resistance to MTX is the result of DHFR gene (*Dhfr*) amplification, associated with the production of large quantities of DHFR (2,11,18,28, 30,38,44,46,49). Equally important, though less frequent than resistance due to gene amplification, is the decreased transport of MTX (10,17, 20,22,24,50) and alteration of DHFR structure (3,16,25,27,42) as well as other mechanisms. A single amino acid substitution can result in a reduced binding affinity to MTX (8,9,14,18,43) in addition to ligand binding and chemical catalysis (4,21,23).

In contrast to the understanding of MTX resistance in mammalian systems, little is known about the mechanism of resistance in insect cells. This is curious considering the importance of insect resistance mechanisms in the management of agricultural pest insects and vectors of human disease.

Received July 18, 1996; revision accepted January 14, 1997.
[1]Address correspondence to Dr. Virginia K. Walker. Tel: 613-545-6123; Fax: 613-545-6617; E-mail: walkervk@biology.queensu.ca

The only example that has been studied, hitherto, is a 3000-fold MTX-resistant mosquito cell line in which resistance was accompanied by the amplification of *Dhfr* to 1200 copies per nucleus (40,41). In this study, sequence information, kinetic analysis, and protein modeling indicate that an altered DHFR is associated with the high level of MTX resistance. The molecular explanation for drug resistance in this case thus parallels recent findings for some insecticide resistance phenotypes.

MATERIALS AND METHODS

Cell Line and Culture Conditions

Drosophila S3 cells were cultured at 25 ± 1°C in Schneider's *Drosophila* medium modified with L-glutamine (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 50 units/ml penicillin, 50 μg/ml streptomycin. A MTX-resistant *Drosophila* cell line, S3Mtx, was selected by stepwise increases of MTX (Sigma Chemical Co., St. Louis, MO) concentration from 1×10^{-7} to 2×10^{-4} M over a period of 4 years. This regime resulted in a 2000-fold higher level of resistance in the S3Mtx cells than in the parental wild-type S3 cells.

MTX Transport

Logarithmically growing cells (S3 and S3Mtx) were collected and resuspended in MTX uptake medium (Schneider's *Drosophila* medium with 10% FBS and 0.01 M HEPES, pH 7.4) at a concentration of 5.6×10^6 cells per ml. [^{3}H]MTX (14.6 Ci/mmol, 32.1 mCi/mg; Amersham, Oakville, Ont.) was added (4.5 μCi), together with unlabeled MTX, to final concentration of 2×10^{-7} and 2×10^{-4} M. Aliquots of cells were taken over the 90–120-min time course (see Results), washed in cold PBS, and assayed for [^{3}H]MTX as described by Flintoff et al. (13). Protein concentration was determined with the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Southern Analysis

DNA (6 μg) from S3Mtx cells and S3 cells was digested with *Kpn*I and *Sal*I restriction enzymes (BRL, Burlington, Ont.), electrophoresed through 0.7% agarose, and transferred by capillary blotting to nylon membrane (Hybond-N, Amersham). The blot was hybridized with nick translation-labeled (BRL, Burlington, Ont.) *Drosophila* DHFR cDNA (19) according to the membrane manufacturer's recommendations and finally washed under conditions of high stringency (1 × SSPE, 0.1% SDS at 65°C for 15 min, then 0.1 × SSPE, 0.1% SDS at 65°C for 15 min). The blots were stripped and subsequently hybridized with a 1.6 kb *Drosophila* α-tubulin cDNA (26). The relative signal strength in each lane was determined using a scanning densitometer (Hoeffer GS 300).

Cloning and Sequencing of DHFR cDNA

Two 17-oligonucleotide primers (CCGGATC CATGCTTCGATTCAATTTA and CCGAATT CTTATGAGTGTTTCTCCAAAA) corresponding, respectively, to the sequence at the 5′ (including the addition of a *Bam*H1 linker) and 3′ end (including the addition of a *Eco*RI linker) of the *Drosophila Dhfr* coding region [(19); from adult flies], were used to amplify cDNA from S3 and S3Mtx cells. Polymerase chain reaction (PCR) was performed using 50 pmol of each primer, 0.2 mM each deoxyribonucleotide triphosphate, 100 mM Tris, pH 9.0, 1.5 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, and 2.0 U Vent DNA polymerase (New England Biolabs, Mississauga, Ont.). Reaction conditions were: 1 min at 94°C, 1 min at 56°C, 1 min at 72°C (30 cycles), and an 8-min final extension at 72°C. Amplified DHFR cDNA products were ligated to pBluescript SK^+ vector (Stratagene, La Jolla, CA) and XL1-Blue (Stratagene) cells were electroporated and transformed. Sequencing reactions were performed at least twice on double-stranded templates using automated DNA sequencing (Applied Biosystems 373A Fluorescent Sequencer).

Expression and Purification of Recombinant DHFR

The DHFR cDNA from S3Mtx cells, as well as from S3 cells, was cloned into a pTrcHis expression vector (Invitrogen, San Diego, CA) as previously described for the wild-type DHFR cDNA from adult flies (19) and used to transform *E. coli* cells (TOP 10, Invitrogen). The expressed recombinant DHFR enzymes were purified using a protocol similar to that previously described for the isolation of *Drosophila* DHFR (36). Briefly, cell lysates of the transformed *E. coli* were prepared by three cycles of sonication followed by freeze-thawing before salting out the DHFR at 40–80% $(NH_4)_2SO_4$. The fraction containing DHFR activity was dialyzed against equilibrium buffer (36) overnight, then bound to an Affi-Gel blue column (BioRad Labs, Richmond, CA) and eluted with 1 M KCl, rather than 2 mM dihydrofolate. After

dialysis against 100 mM Tris, pH 8.5, each purified DHFR was added to a final concentration of 100 μg/ml BSA and 1 mM DTT to stabilize the enzyme for kinetic studies.

Kinetic Analysis of Purified Recombinant DHFR

Both of the purified recombinant DHFRs expressed either from S3 or from the S3Mtx DHFR cDNAs were used for kinetic studies. Enzyme activity was measured spectrophotometrically (36) in triplicate. The K_m values for NADPH and dihydrofolate (Aldrich, Milwaukee, WI) were determined by primary and secondary Hanes plots, and the K_i for trimethoprim (TMP; Sigma) was determined by a primary Hanes and secondary Dixon plot (35). The concentration of NADPH, dihydrofolate, MTX, and TMP was judged spectrophotometrically. The K_d for MTX was determined by equilibrium dialysis against different concentrations of [^{3}H]MTX as previously described (35).

Molecular Model

Drosophila DHFR (GeneBank™ accession #UO6861) was aligned to DHFRs from other organisms as previously shown (19). To obtain a homology model of the *Drosophila* S3 or wild-type DHFR, crystal structures of chicken (26) and human (7) DHFRs were obtained from the Brookhaven Protein Data Bank [(1,5); entries 8dfr and 1dhf, respectively] and the model was created using the tools available in the COMPOSER (6,47,48) module of SYBYLR (Tripos Inc., St. Louis, MO). The model of the S3Mtx DHFR was made by substituting Leu 30 of the S3 enzyme with Gln according to the S3Mtx sequence (see Results). The SYBYL MAXIMIN2 molecular mechanics minimization module (Kollman All Atom charges and force field) was used for refinement of the models. This involved 50 steps of steepest descent minimization, followed by 100 steps of Powell minimization. Simulations of the binding of MTX and NADPH to the S3 and S3Mtx DHFRs were carried out using the SYBYL DOCK module.

RESULTS

3H-MTX Transport in the Drosophila *Cell Lines*

The ability of S3Mtx cells to transport [^{3}H]MTX into the cytoplasm was examined at two different concentrations of the drug (2×10^{-7} and 2×10^{-4} M). S3 cells showed a more rapid increase in intracellular [^{3}H]MTX and had a higher level of accumulation compared to the S3Mtx cell line (Fig. 1). The intracellular MTX concentration in the resistant cell line plateaued at 77% and 63%, in 10^{-7} and 10^{-4} M MTX, respectively, of that found in the parental cell line. Culture in either MTX concentration for 2 h did not result in cell death, as judged by trypan blue staining of the S3MTX or the S3 cell line (data not shown).

Southern Analysis of Dhfr

Because amplification of *Dhfr* with the concomitant overproduction of DHFR frequently accompanies MTX resistance in selected cell lines, especially at high level of drug concentration ($> 10^{-4}$ M), the *Dhfr* copy number in S3Mtx cells

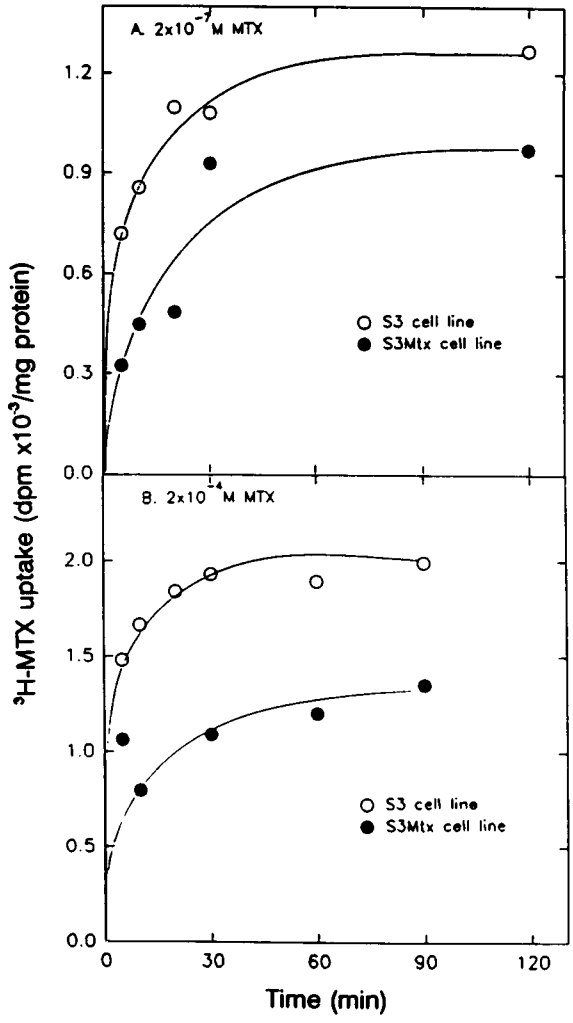

FIG. 1. Accumulation of [^{3}H]MTX in the parental S3 cell line and the MTX-resistant S3Mtx cells. The time course of [^{3}H]MTX uptake and accumulation in the S3 cell line (○) and the S3Mtx cell line (●) is shown at (A) 2×10^{-7} M MTX and (B) 2×10^{-4} M MTX. Each point represents the average value from three independent experiments.

was determined. Southern blots containing genomic DNA isolated from S3Mtx and S3 cells were hybridized with *Dhfr* cDNA (Fig. 2). There was a 1.4-fold increase in the *Dhfr* copy number in S3Mtx cell line compared to the S3 cell line.

Sequence Analysis of DHFR cDNA From S3 and S3Mtx Cells

The cloning of DHFR cDNA from *D. melanogaster* adult flies by nested PCR has been previously reported (19). Using the amplified partial cDNA as a probe, full-length *Dhfr* was obtained by screening a *Drosophila* genomic library. Two oligonucleotide primers corresponding to the 5′ and 3′ end of the *Dhfr* coding sequence were used as primers for the amplification of full-length *Dhfr* cDNA from S3 and S3Mtx cells. The 550-bp amplified products (Fig. 3) were subcloned into the pBluescript SK^+ and subsequently sequenced on both strands from three individual transformants.

The sequence of *Dhfr* cDNA from S3 cells was identical to that established for the adult *Dhfr* cDNA (19). The sequence of three S3MTX cDNA differed from the S3 and fly cDNA by a T to A transversion at position 89. Translation of the codon results in a substitution of Gln for Leu at residue 30.

Kinetic Properties of the Enzymes

Both S3 and S3MTX *Dhfr* cDNAs were expressed in *E. coli* TOP 10 cells from a pTrcHis expression vector. The recombinant enzymes were purified and used for steady-state kinetics and ligand binding experiments (Table 1). The Michaelis constant (K_m) for the cofactor, NADPH, is the same for both DHFRs but the K_m for the substrate, dihydrofolate, shows a twofold increase for the S3Mtx DHFR. There was a significant difference in the binding affinity for the inhibitors with a 15-fold increase in the K_i for TMP and in the K_d for MTX for the enzyme from the resistant cells compared to the S3 DHFR.

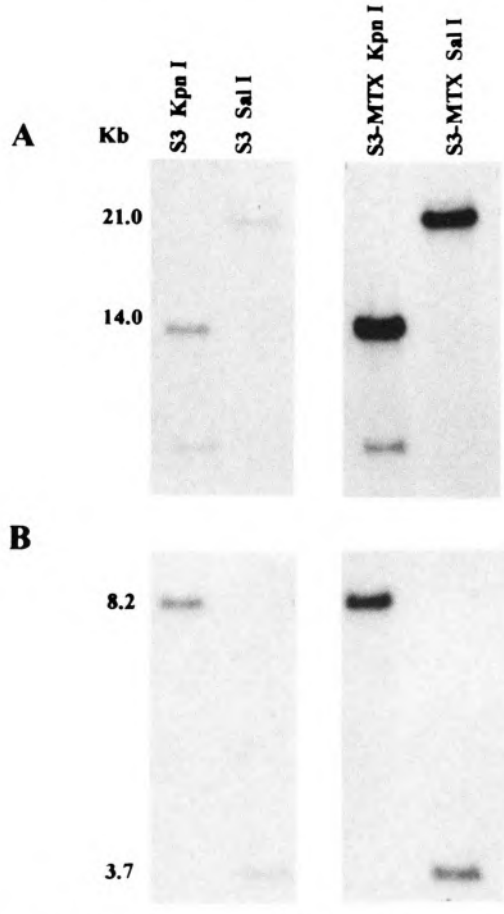

FIG. 2. Southern blot analysis of genomic DNA from S3Mtx and S3 cells. Genomic DNA (6 μg) from either S3 cells (lane 1, 2) or S3Mtx cells (lane 3, 4) was digested with *Kpn*I (lane 1, 3) and *Sal*I (lane 2, 4), electrophoresed, and transferred to a Hybond-N membrane and hybridized with *Dhfr* cDNA labeled by nick translation (A). The same blot was hybridized to *Drosophila* α-tubulin cDNA (31) to determine the relative DNA loading in the lanes (B).

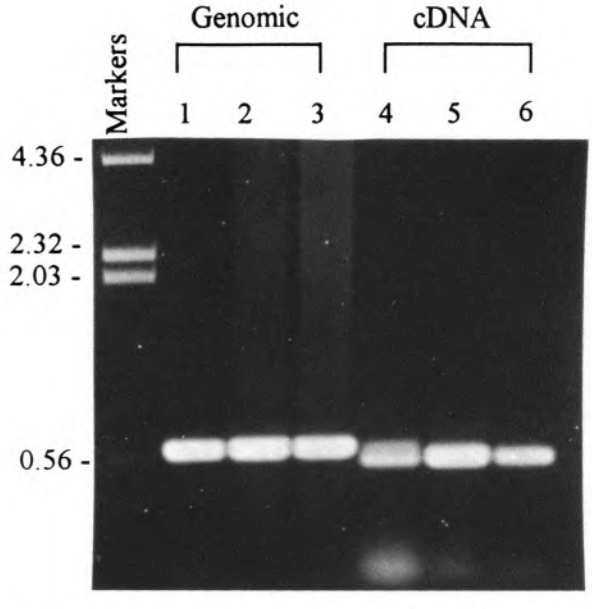

FIG. 3. PCR amplification of *Dhfr* from S3 and S3Mtx cells. Two oligonucleotide primers corresponding to the 5′ and 3′ end of the *Dhfr* coding sequence and Vent DNA polymerase were used to amplify (see Materials and Methods) *Dhfr* sequence with the following *Drosophila* template DNAs: lane 1, adult fly genomic DNA; lane 2, S3 genomic DNA; land 3, S3Mtx genomic DNA; lane 4, adult fly cDNA; lane 5, S3 cDNA 6, S3Mtx cDNA. *Hind*III digested λ DNA marker is shown on the left.

TABLE 1
KINETIC PROPERTIES OF *DROSOPHILA* S3 DHFR AND MUTANT S3Mtx DHFR RECOMBINANT ENZYMES PURIFIED FROM *E. COLI*

	K_m NADPH (μM)	K_m DHF (μM)	K_i TMP (μM)	K_d MTX (nM)
S3 DHFR	1.3 ± 2.1	4.7 ± 2.4	95.9 ± 16.3	0.30 ± 0.03
S3Mtx DHFR	12.2 ± 5.8	8.1 ± 4.1	1414.5 ± 313.1	4.33 ± 2.13

Molecular Models

The *Drosophila Dhfr* amino acid sequence has 38% identity with the vertebrate enzymes and an additional 22% of the residues are similar. Initial positioning of the MTX binding site was based on information from vertebrate high-resolution structures but amino acid sequence differences required minimization of the docked structures for side chain–ligand optimization. There are three gaps and one insertion for the *Drosophila* sequence relative to the vertebrate sequences. The first gap (one residue between Trp-24 and Arg-25) was in the region of a left-handed polyproline helix found in vertebrate, but not bacterial DHFRs (Fig. 4). The second gap (three residues between Glu-102 and Gln-103) occurs at the juncture of two adjacent α-helices found in vertebrate structures and results in the loss of the second helix in the *Drosophila* model. The third gap (one residue between Pro-149 and Asp-150) and the insertion (one residue at Leu-83) occur in surface loops and have no significant impact on the structure of the model (Fig. 4). A stylized version of the final model, overlaid on the chicken X-ray structure, is shown in Fig. 4.

FIG. 4. Ribbon-coil views of the *Drosophila* DHFR model (black) superimposed on the chicken DHFR X-ray structure (26) shown in gray. The arrows indicate regions of insertion and deletion.

Once the *Drosophila* DHFR model was developed, the model of the altered DHFR from the S3Mtx strain was made by changing Leu-30 to Gln. The mutation occurs in the hydrophobic cleft of the substrate binding site, the region that interacts with the benzoyl group of the MTX substrate (Fig. 5). This replacement could result in an increase in desolvation energy during ligand binding due to the greater affinity of glutamine for water of hydration, as well as to a decrease in van der Waals interactions between the hydrophobic region of the ligands and the enzyme binding site. In addition, examination of the ligand-free model suggested that slight rotation of the Gln-30 and Lys-63 side chains results in a strong water-mediated H bond bridge between these residues, which would effectively block access to the folate/MTX binding site. Binding these ligands would require breaking this bridge.

DISCUSSION

Schimke et al. suggested more than 15 years ago that drug resistance could be analogous to insecticide resistance (39). The observation that high levels of MTX resistance in mammalian cells was almost invariably associated with the amplification of *Dhfr* led to other investigations, which showed that increases in the gene copy number for organophosphate insecticide target proteins resulted in the development of resistant insect pest populations (32,37). To make direct comparisons with the mammalian systems and to provide a better understanding of the molecular basis of resistance in insect cells, a *Drosophila* cell line, S3Mtx, was selected for MTX resistance over a 4-year period by stepwise increases in drug concentration. Surprisingly, the 2000-fold increase in resistance was accompanied a mere 1.4-fold increase in the *Dhfr* copy number (Fig. 2), no significant increase in DHFR protein, as determined by Western blots, and an overall twofold decrease in specific activity (data not shown). Compared to some Mtx-

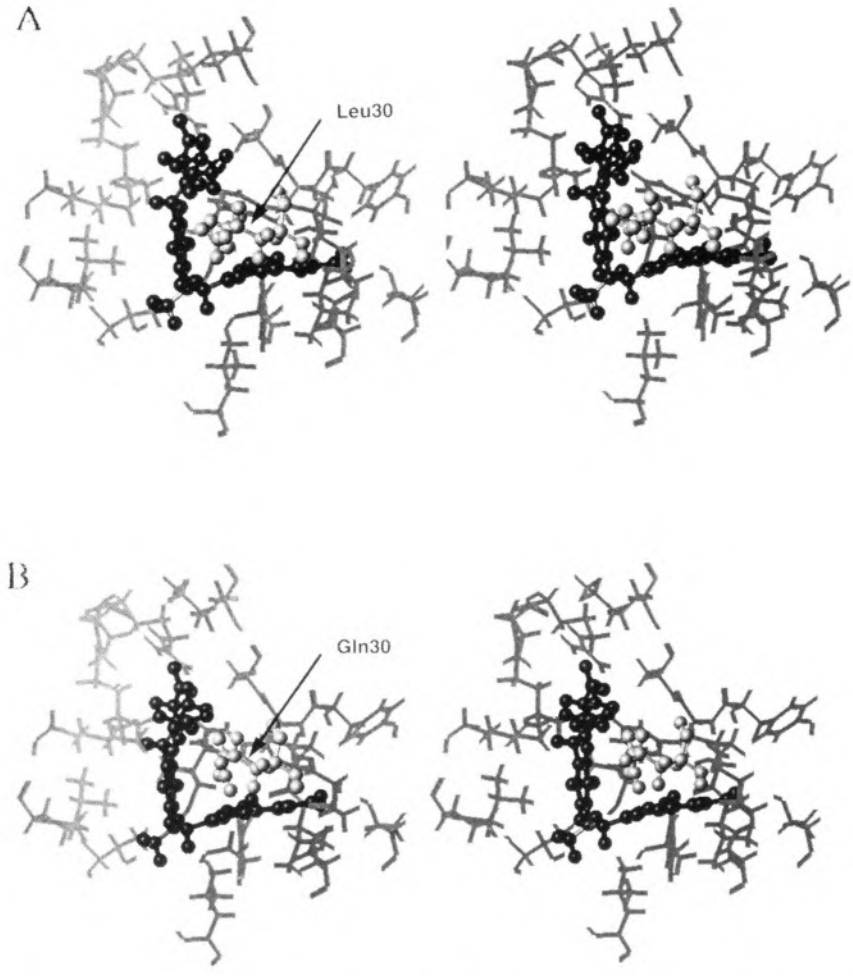

FIG. 5. Interaction of the *Drosophila* DHFR substrate binding region with MTX. (A) Stereo view of the interaction of Leu-30 in wild-type (adult fly and S3 cell) DHFR with MTX, with Leu-30 and MTX shown as ball and stick figures. (B) Stereo view of the interaction of Gln-30 in the mutant S3Mtx cells with MTX.

resistant mammalian cell lines (34), there was only a slightly reduced accumulation of [^{3}H]MTX in S3Mtx cells. Because MTX enters cells by passive diffusion at high drug concentrations, the slightly lower drug accumulation at 2×10^{-4} M MTX in S3Mtx cells may possibly result from an increased MTX efflux. However, this does not appear to be due to a differential amplification of the P-glycoprotein multidrug resistance genes (52) as the copy number in the two lines was the same (data not shown).

To explain the high level of MTX resistance, *Dhfr* cDNA was cloned from S3 and S3Mtx cells. Sequence analysis revealed a T to A transversion at nucleotide 89, resulting in a single amino acid substitution of Gln for Leu at amino acid 30 in the S3Mtx cells. In addition, no differences were noted in the 5′ upstream region of genomic DNA (to −900) sequenced from S3 and S3Mtx cells [(42); data not shown]. Most altered residues in DHFRs from resistant mammalian cells have been shown to be directly involved in substrate and inhibitor binding (8,21,27,42,43). Amino acid substitutions are not necessarily restricted to the active site (9), but the replacement of such residues in resistant cells invariably leads to a lower affinity for inhibitor. In the model of the wild-type or S3 DHFR (Fig. 5), the side chain of Leu-30 makes hydrophobic and van der Waals interactions with MTX. Replacement of Leu by the more polar Gln then should result in increased solvation and, in addition, H bond formation with Lys-63 likely would decrease access to the folate/MTX binding site. Thus, this single amino acid substitution ap-

pears to result in reduced affinity for the inhibitor. Indeed, similar importance for MTX interaction has been reported for Phe-31 of the mouse and human DHFR structures (33,45). The results from our kinetic study on recombinant S3 and S3Mtx DHFRs were consistent with this hypothesis because the mutation to Gln at residue 30 produced a 15-fold decrease in the binding affinity of the enzyme for MTX and TMP. Substrate binding (K_m DHF) was slightly diminished (twofold; Table 1), and may account for the decrease in DHFR activity observed in cell homogenates over the course of MTX selection.

The reduced affinity of the mutant DHFR for MTX and the slightly lower intracellular MTX concentration are associated with a 2000-fold increase in resistance to MTX in S3Mtx cells. In highly resistant (100–400 μM MTX) mammalian cells altered or unaltered DHFRs are almost invariably accompanied by a moderate or high levels of DHFR gene amplification. In contrast, *Drosophila* S3Mtx cells show an altered enzyme with a 15-fold decrease in affinity to MTX at 200 μM. Insect DHFRs appear to have a high K_d value for MTX; *Drosophila* DHFR shows a 30–6600-fold higher K_d than mammalian DHFRs (35). Therefore, the intrinsic insensitivity of *Drosophila* DHFR to MTX, coupled with a reduced affinity for MTX and a somewhat reduced intracellular MTX concentration, would give S3Mtx cells a selective advantage in drug-containing medium.

High levels of MTX resistance can thus be achieved in *Drosophila* cells without a concomitant increase in gene amplification. In contrast, MTX-resistant mosquito cells appear to behave more like mammalian cells in that they show a 1200-fold amplification of wild-type *Dhfr* (40,41). The kinetic characteristics of the mosquito enzyme are not known but it could be more susceptible to MTX inhibition than the *Drosophila* wild-type enzyme. Alternatively, *Drosophila* has a somewhat smaller genome than the *Aedes* mosquitoes and, perhaps more importantly, a long period dispersion pattern, possibly suggesting that the inherited reiteration of large amplicons may not be favored in this species. Coincidently, recent examples also show that organophosphate insecticide resistance can result from an altered, unamplified enzyme (12,15,51). Schimke et al.'s (39) suggestion that MTX-resistant cell lines be used as a model for insecticide resistance was prophetic. Nevertheless, insects are highly adaptable and perhaps it should not have been expected that they would respond to chemical stresses with a single resistance mechanism. Recent work on resistant pest populations coupled with this work on resistant cell lines has shown us that there is still much to be done in the understanding of the molecular mechanisms of resistance in insects.

ACKNOWLEDGEMENTS

We would like to thank Ms. T. Inasling for performing automated DNA sequencing, an anonymous friend for assistance with the molecular modeling, and Dr. Z. Jia for comments on the manuscript. Financial support came from a Queen's University bursary and graduate award to H.H. and a grant from the Natural Sciences and Engineering Research Council of Canada to V.K.W.

REFERENCES

1. Abole, E. E.; Bernstein, F. C.; Bryant, S. H.; Koetzle, T. F.; Weng, J. Protein data bank. In: Allen, F. H.; Bergerhoff, G.; Silvers, R., eds. Crystallographic databases, information content, software systems, scientific applications. Cambridge: Data Commission of the International Union of Crystallography; 1987.
2. Alt, F. W.; Kellems, R. E.; Bertino, J. R.; Schimke, R. T. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. 253:1357–1370; 1978.
3. Baccanari, D. P.; Stone, D.; Juyper, L. Effect of a single amino acid substitution on *Escherichia coli* dihydrofolate reductase catalysis and ligand binding. J. Biol. Chem. 256:1738–1747; 1981.
4. Benkovic, S. J.; Wagner, C. R. Dihydrofolate reductase: A paradigm for drug design. In: Hook, J. B.; Poste, G., eds. Protein design and the development of new therapeutics and vaccines. New York: Plenum Press; 1990:237–249.
5. Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. F., Jr.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. The protein data band: A computer based archival file for macromolecular structures. J. Mol. Biol. 112: 535–542; 1977.
6. Blundell, T. L.; Carney, D. P.; Gardner, S.; Hayes, F. R. F.; Howlin, B.; Hubbard, T. J. P.; Overington, J. P.; Singh, D. A.; Sibanda, B. L.; Sutcliffe, M. J. Knowledge-based protein modelling and design. Eur. J. Biochem. 172:513–520; 1987.

7. Davies, J. F.; Delcamp, T. J.; Prendergast, N. J.; Ashford, V. A.; Freisheim, J. H.; Kraut, J. Crystal structure of recombinant human dihydrofolate reductase complexed with folate and 5-deazofolate. Biochemistry 29:9467-9479; 1990.
8. Dicker, A. P.; Volkenandt, M.; Schweitzer, B. I.; Canerjee, D.; Bertino, J. R. Identification and characterization of a mutation in the dihydrofolate reductase gene from the methotrexate-resistant Chinese hamster ovary cell line Pro^{-}3 Mtx^{RIII}. J. Biol. Chem. 265:8317-8321; 1990.
9. Dicker, A. P.; Waltham, M. C.; Volkenandt, M.; Schweitzer, B. I.; Otter, G. M.; Schmid, F. A.; Sirotnak, F. M.; Bertino, J. R. Methotrexate resistance in an *in vivo* mouse tumor due to a non-active-site dihydrofolate reductase mutation. Proc. Natl. Acad. Sci. USA 90:11797-11801; 1993.
10. Dixon, K. H.; Brendan, C. L.; Chiu, J.; Kelley, K.; Cowan, K. H. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. J. Biol. Chem. 269:17-20; 1994.
11. Dolnick, B. J.; Berenson, R. J.; Bertino, J. R.; Kaufman, R. J.; Nunberg, J. H.; Schimke, R. T. Correlation of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells. J. Cell Biol. 83:394-402; 1979.
12. ffrench-Constant, R. H.; Steichen, J. C.; Rocheleau, T. A.; Aronstein, K.; Roush, R. T. A single-amino acid substitution in a τ-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in *Drosophila* populations. Proc. Natl. Acad. Sci. USA 90:1957-1961; 1993.
13. Flintoff, W. F.; Davidson, S. V.; Siminovitch, L. Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. Somatic Cell Genet. 2:245-261; 1976.
14. Flintoff, W. F.; Essani, K. Methotrexate-resistant Chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. Biochemistry 19:4321-4327; 1980.
15. Fournier, D.; Bride, J. M.; Hoffmann, F.; Karch, F. Acetylcholinesterase: Two types of modifications confer resistance to insecticide. J. Biol. Chem. 267:14270-14274; 1992.
16. Goldie, J. H.; Dedhart, S.; Krystal, G. Properties of a methotrexate-insensitive variant of dihydrofolate reductase derived from methotrexate-resistant L5178Y cells. J. Biol. Chem. 256:11629-11635; 1981.
17. Goldman, D. Membrane transport of methotrexate (NSC-740) and other folate compounds: Relevance to rescue protocols. Cancer Chemo. Rep. 6:63-72; 1975.
18. Haber, D. A.; Beverley, S. M.; Kiely, M. L.; Schimke, R. T. Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J. Biol. Chem. 256:9501-9510; 1981.
19. Hao, H.; Tyshenko, M. G.; Walker, V. K. Dihydrofolate reductase of *Drosophila*: Cloning and expression of a gene with a rare transcript. J. Biol. Chem. 269:15179-15185; 1994.
20. Hill, B. T.; Brenda, D. B.; White, J. C.; Goldman, I. D. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. Cancer Res. 39:2440-2446; 1979.
21. Howell, E. E.; Mayer, R. J.; Warren, M. S.; Villafranca, J. E.; Krant, J.; Benkovic, S. J. Active site mutations in dihydrofolate reductase. In: Protein engineering. New York: Alan R. Liss Inc.; 1987: 251-256.
22. Hugues, J. P.; Shen, W. C. Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cultured cells. Proc. Natl. Acad. Sci. USA 75:3867-3870; 1978.
23. Lewis, W. S.; Cody, V.; Galistsky, N.; Luft, J. R.; Pangborn, W.; Chunduru, S. K.; Spencer, H. T.; Appleman, J. R.; Blakley, R. L. Methotrexate-resistant variants of human dihydrofolate reductase with substitutions of leucine 22. J. Biol. Chem. 270: 5057-5064; 1995.
24. McCormick, J. I.; Susten, S. S.; Freisheim, J. H. Characterization of the methotrexate transport defect in a resistant L1210 lymphoma cell line. Arch. Biochem. Biophys. 211:311-318; 1981.
25. Mclvor, R. S.; Simonsen, C. C. Isolation and characterization of a variant dihydrofolate reductase cDNA from methotrexate-resistant murine L5178Y cells. Nucleic Acids Res. 18:7025-7032; 1990.
26. McTigue, M. A.; Davies, J. F., III; Kaufman, B. T.; Kraut, J. Crystal structure of chicken liver dihydrofolate reductase complexed with NADP^{+} and biopterin. Biochemistry 31:7264-7273; 1992.
27. Melera, P. W.; Davide, J. P.; Oen, H. Antifolate-resistant Chinese hamster cells. J. Biol. Chem. 263: 1978-1990; 1988.
28. Melera, P. W.; Lewis, J. A.; Biedler, J. L.; Hession, C. Antifolate-resistant Chinese hamster cells. J. Biol. Chem. 255:7024-7028; 1980.
29. Melera, P. W.; Davide, J. P.; Hession, C. A.; Scotto, K. W. Phenotypic expression in *Escherichia coli* and nucleotide sequence of two Chinese hamster lung cell cDNAs encoding different dihydrofolate reductase. Mol. Cell. Biol. 4:38-48; 1984.
30. Milbrandt, J. D.; Heintz, N. H.; White, W. C.; Rothman, S. M.; Hamlin, J. L. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA 78:6043-6047; 1981.
31. Mischke, D.; Pardue, M. L. Organization and expression of alpha tubulin multigene family is transcribed in both oogenesis and later in embryonic development. J. Mol. Biol. 156:449-466; 1982.
32. Mouches, C.; Pauplin, Y.; Agarwal, M.; Lemieux,

L.; Herzog, M.; Abadon, M.; Beyssat-Arnaouty, V.; Hyrien, O.; Vincent, B. R. D. S.; Georghiou, G. P.; Pasteur, N. Characterization of amplification core and esterase B1 gene responsible for insecticide resistance in *Culex*. Proc. Natl. Acad. Sci. USA 87:2574–2578; 1990.

33. Oefner, C.; D'Aray, A.; Winkler, F. K. Crystal structure of human dihydrofolate reductase complexed with folate. Eur. J. Biochem. 174:377–385; 1988.
34. Ohnuma, T.; Lo, R. J.; Scanion, K. J.; Kamen, B. A.; Ohnoshi, T.; Wolman, S. R.; Holland, J. F. Evolution of methotrexate resistance of human acute lymphoblastic leukemia cells in vitro. Cancer Res. 45:1815–1822; 1985.
35. Rancourt, S. L.; Walker, V. K. Kinetic characterization of dihydrofolate reductase from *Drosophila melanogaster*. Biochem. Cell Biol. 68:1075–1082; 1990.
36. Rancourt, S. L.; Walker, V. K. The purification of dihydrofolate reductase from *Drosophila melanogaster*. Biochim. Biophys. Acta 1039:261–268; 1990.
37. Raymond, M.; Beyssant-Arnaouty, V.; Sivasubramanian, N.; Mouches, C.; Georghiou, G. P.; Pasteur, N. Amplification of various esterase B's responsible for organophosphate resistance in *Culex* mosquitoes. Biochem. Genet. 27:417–421; 1989.
38. Schimke, R. T. Gene amplification in cultured animal cells. Cell 37:705–713; 1984.
39. Schimke, R. T.; Kaufman, R. J.; Alt, F. W.; Kellems, R. F. Gene amplification and drug resistance in cultured murine cells. Science 202:1051–1055; 1978.
40. Shotkoski, F. A.; Fallon, A. M. An amplified insect dihydrofolate reductase gene contains a single intron. Eur. J. Biochem. 201:157–160; 1991.
41. Shotkoski, F. A.; Fallon, A. Genetic changes in methotrexate-resistant mosquito cells. Arch. Insect Biochem. Phys. 15:79–92; 1990.
42. Simonsen, C. C.; Levinson, A. D. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA 80:2495–2499; 1983.
43. Srimatkandada, S.; Schweitzer, B. I.; Moronson, B. A.; Dube, S.; Bertino, J. R. Amplification of a polymorphic dihydrofolate reductase gene expressing and enzyme with decreased binding to methotrexate in a human colon carcinoma cell line, HCT-8R4, resistant to this drug. J. Biol. Chem. 264: 3524–3528; 1989.
44. Srimatkandada, S.; Medina, W. D.; Cashmore, A. R.; Whyte, W.; Engel, D.; Moronson, B. A.; Franco, C. T.; Dube S. K.; Bertino, J. R. Amplification and organization of dihydrofolate reductase genes in a human leukemic cell line, K-562, resistant to methotrexate. Biochemistry 22:5774–5781; 1983.
45. Stammers, D. K.; Champness, J. N.; Beddell, C. R.; Dann, J. G.; Eliopoulos, D. E.; Geddes, A. J.; Ogg, D.; North, A. C. T. The structure of mouse L1210 dihydrofolate reductase-drug complexes and the construction of a model of human enzyme. FEBS 218:178–184; 1987.
46. Stark, G. R. DNA amplification in drug resistant cells and in tumours. Cancer Surveys 5:1–23; 1986.
47. Sutcliffe, M. J.; Hayes, F. R. F.; Blundell, T. L. Knowledge-based modeling of homologous proteins. Part II: Rules for the conformation of substitute side chains. Prot. Eng. 1:385–392; 1987.
48. Sutcliffe, M. J.; Haneef, I.; Carney, D. P.; Blundell, T. L. Knowledge-based modeling of homologous proteins. Part I: Three dimensional frameworks derived from the simultaneous superposition of multiple structures. Prot. Eng. 1:377–384; 1987.
49. Tyler-Smith, C.; Alderson, T. Gene amplification in methotrexate-resistant mouse cells: I. DNA rearrangement accompanies dihydrofolate reductase gene amplification in a T-cell lymphoma. J. Mol. Biol. 153:203–218; 1981.
50. Underhill, T. M.; Williams, F. M. R.; Murray, R. C.; Flintoff, W. F. Molecular cloning of a gene involved in methotrexate uptake by DNA-mediated gene transfer. Somat. Cell Mol. Genet. 18:337–349; 1992.
51. Whyard, S.; Downe, A. E. R.; Walker, V. K. Characterization of a novel esterase conferring insecticide resistance in the mosquito *Culex tarsalis*. Arch. Insect Biochem. Physiol. 29:329–342; 1995.
52. Wu, C. T.; Budding, M.; Griffin, M. S.; Croop, J. M. Isolation and characterization of *Drosophila* multidrug resistance gene homologs. Mol. Cell. Biol. 11:3940–3948; 1991.