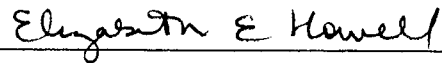


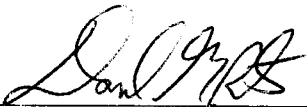
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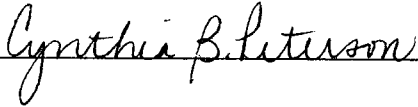
I am submitting herewith a thesis by Freddie West entitled "Production and characterization of two single tryptophan mutants of R67 Dihydrofolate reductase." I have examined the final copy of this thesis for form and content and recommend it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biotechnology.



Dr. Elizabeth E. Howell, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

PRODUCTION AND CHARACTERIZATION OF TWO SINGLE
TRYPTOPHAN MUTANTS OF R67 DIHYDROFOLATE
REDUCTASE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Freddie W. West

December 1997

This thesis is dedicated to my parents, Freddie W. West Sr. and Joan K. West, who have always been there with support and encouragement. I am very grateful and will never forget the sacrifices they made to further my education. Thanks Mom and Dad.

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ABSTRACT

One of the fundamental unanswered questions in biochemistry today is; how do proteins fold? R67 dihydrofolate reductase (R67 DHFR) is a well-characterized enzyme that is an excellent model for studying the structural and functional properties of proteins. R67 DHFR is a novel protein that provides clinical resistance to the antibacterial drug trimethoprim (TMP). R67 DHFR is active as a homotetramer with a molecular weight of 34,000. The monomer is 78 amino acids long and has no sequence or structural homology to chromosomal DHFRs. R67 DHFR contains two tryptophan residues per monomer. W45 and W145 as well as the symmetry related residues W245 and W345, occur at the monomer-monomer interfaces. W38 and W338, as well as their symmetry related residues are found at the dimer-dimer interfaces. The positioning of W45 and W38 residues at the monomer and dimer interfaces allow for the utilization of these tryptophans as intrinsic probes to follow the folding/unfolding reactions of R67 DHFR.

In these studies, the equilibrium unfolding transitions of R67 DHFR were monitored by following changes in tryptophan fluorescence. Fluorescence spectroscopy is a powerful technique for monitoring conformational changes in proteins. A change in tryptophan environment as monitored by fluorescence generally coincides with a change in the oligomeric state of the protein or a global loss of tertiary and secondary structure, as the unfolding of many proteins are highly cooperative reactions. The dependency of the steady-state fluorescence properties on the concentration of denaturant can provide values for the free energy of folding.

The unambiguous assignment of the fluorescent properties to an individual tryptophan residue is necessary for the interpretation of the observed fluorescence signals at a molecular level. There are difficulties however, in resolving the individual contributions of chromophores in multichromophore containing proteins. This limits ones ability to interpret the structural and dynamic properties of these side chains during the unfolding reaction. To over come this limitation the two native tryptophans of R67 DHFR were mutated to the weakly fluorescent amino acid phenylalanine.

The goal of this research is to correlate the observed equilibrium unfolding/folding profiles with the individual contributions of the tryptophan residues at each interface. Thus each of the two tryptophans of R67 DHFR were mutagenized to phenylalanine to produce the single Trp mutants, W45F and W38F. The kinetics, oligomeric state, fluorescence and CD-spectral properties of these mutants were then compared to the wild type enzyme.

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LIST OF ABBREVIATIONS

DHFR	dihydrofolate reductase
R67 DHFR	R67 dihydrfolate reductase
wtR67 DHFR	wild-type R67 dihydrofolate reductase
TMP	trimethoprim
MTX	methotrexate
DHF	7, 8-dihydrofolate
THF	5,6,7,8-tetrahydrofolate
NADPH	nicotinamide adenine dinucleotide phosphate
Trp	tryptophan
His	histidine
Gln	glutamine
Ser	serine
Phe	phenylalanine
K_M	Michealis-Menton constant
F_{app}	fraction unfolded form of the protein
TE	10mM Tris-HCl, 1mM EDTA
GdnHCl	guanidine-HCl
ΔG_{H_2O}	free energy of unfolding in $k_{cal} \text{ mol}^{-1}$
K_{av}	$((\text{elution volume}-\text{void volume})/(\text{bed volume}-\text{void volume}))$
NLIN	non-linear least squares fitting
CD	circular dichroism
K_d	dissociation constant

M_G	slope of the unfolding transition in $\text{k}_{\text{cal}} \text{mol}^{-1} \text{M}^{-1}$
$\langle \lambda \rangle$	intensity averaged emission wavelength
UV	ultraviolet
T	tetrameric
D	dimeric
M	monomeric

CHAPTER 1

INTRODUCTION AND BACKGROUND

Fluorescence techniques provide powerful tools that can be used to obtain thermodynamic and kinetic information about protein folding transitions. The combination of fluorescence spectroscopy and site-directed mutagenesis provides a means not only to study the mechanisms by which enzymes catalyze chemical reactions, but also for investigating how the primary sequence of a protein is transduced into its three dimensional native conformation. One of the fundamental unanswered questions in biochemistry today is; how do proteins fold? R67 dihydrofolate reductase is a well-characterized enzyme that has become an excellent model for studying the structural and functional properties of enzymes.

Dihydrofolate Reductases

Dihydrofolate reductase (DHFR, EC 1.5.1.3) is a commonly occurring oxidoreductase found in all organisms. It catalyzes a vital reaction in one-carbon metabolism, the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). DHFR is a central enzyme in folate metabolism since the generation of THF is required for the synthesis of thymidylate, purine nucleosides, methionine and other metabolic intermediates which are required for cell viability (Blakley, 1969). Rapidly growing cells require DHFR activity to survive. Inhibition of this enzyme results in a blockage of DNA synthesis, due to the inability to maintain the intracellular pool of

THF that is necessary for the production of intermediates, and eventually results in cell death (Kraut and Matthews, 1987).

Chromosomal DHFRs have been the target of various folate analogs that inhibit the enzyme by binding at the active site. Several clinically important inhibitors of chromosomal enzymes include the antitumor drug, methotrexate (MTX), the antibacterial drug, trimethoprim (TMP) and the antimalarial drug, pyrimethamine (Hitchings, and Baccanari, 1987). Although there is only 25% sequence identity between the chromosomal DHFRs from mammals and bacteria, the tertiary structure of these proteins is quite similar (Kulmar *et al.*, 1980; Lai *et al.*, 1982). Chromosomal DHFRs are monomeric proteins with molecular weights of 18,000-22,000 daltons (Blakley, 1984). They generally contain 159-189 amino acid residues and can be structurally described as an eight stranded β -sheet with four α -helices that serve to connect the successive β -strands (Bolin *et al.*, 1982; Filman *et al.*, 1982; Bystroff *et al.*, 1990). Although there is high structural homology between the mammalian and bacterial DHFRs, strong species selectivity for antifolate drugs has been seen, resulting from slight sequence differences. Some active site inhibitors of chromosomal DHFRs exhibit species selectivity allowing them to be used as broad-spectrum antibiotics. The best example of this is trimethoprim (TMP), which has been widely used as a broad-spectrum antibiotic since it only weakly affects the mammalian DHFR, while it strongly inhibits bacterial enzymes. The affinity of TMP for the *E. coli* enzyme has been shown to be $\sim 10,000$ times greater than that for the mammalian enzyme (Kraut and Matthews, 1987).

R-Plasmid Encoded DHFRs

In 1972, it was found that certain strains of *E. coli* and *K. aerogenes* had developed high levels of tolerance to TMP (Fleming *et al.* 1972; Datta and Hedges, 1972; Amyes and Smith, 1974). The resistant strains were shown to carry resistance plasmids (R-plasmids), which coded for novel DHFRs with greatly reduced sensitivity to TMP (Amyes and Smith, 1974). These novel DHFRs were shown to function in the presence of levels of TMP that would completely inhibit the chromosomal enzyme, thus allowing cells harboring these plasmids to grow in the presence of the antibiotic (Amyes and Gemmel, 1992). Pattishall *et al.*, originally classified these R-plasmid resistance enzymes into two groups based on the sensitivity of these enzymes to inhibitors. The type I enzyme typified by the R483 enzyme is synthesized in amounts several fold higher than the chromosomal enzymes, while type II R-plasmid encoded DHFRs were produced in amounts that were equivalent to the chromosomal enzyme but are practically insensitive to TMP, MTX, and aminopterin. This difference in sensitivity can generally be correlated with the affinity with which it binds to the active site of the enzyme. Currently, at least twelve groups of R-plasmid encoded DHFRs (designated I-XII) have been isolated and characterized (Pattishall, *et al.*, 1977; Broad and Smith, 1982; Joyner *et al.*, 1984; Young and Amyes, 1986; Sundstrom *et al.*, 1987; Houvinen, 1995; Wylie *et al.*, 1988; Amyes *et al.*, 1992; Jansson and Skold, 1991; Parsons *et al.*, 1991; Singh *et al.*, 1992).

While the origin of R-plasmid encoded DHFRs is not known for certain, it has been shown that types I, III, IV, VI and IX share regions of high homology with the *E. coli* chromosomal DHFR gene sequence (Jannsson and Skold, 1991; Joyner *et al.*, 1984; Sundstrom *et al.*, 1987). These regions of high homology have been shown to correspond to the folate binding regions of chromosomally encoded DHFRs. Citing this as evidence, it is thought that types I, III, IV, VI, and IX are evolutionarily related to chromosomally encoded DHFRs. Interestingly however, the type II R-plasmid encoded DHFRs show no sequence or structural homology to any of the chromosomal or other R-plasmid encoded DHFRs (Amyes and Gemmel, 1992; Matthews *et al.*, 1986). Due to its uniqueness, it has been suggested that the type II R-plasmid encoded enzymes were evolutionarily derived from some other bacterial oxidoreductase and that under TMP pressure, this NADPH-linked oxidoreductase was recruited to carry out the required reaction (Stone and Smith, 1979). A second possibility is that the type II R-plasmid encoded DHFRs evolved from a non-catalytic protein or from a single domain taken from another protein. In such a selection, resistance to TMP would be more important than the catalytic efficiency of the enzyme. This idea is supported by the fact that type II DHFRs are almost insensitive to TMP, with a K_i in the millimolar range as compared to the nanomolar range for the *E. coli* chromosomally encoded DHFR (Amyes, 1989). Although the K_m values for substrate and cofactor are similar between the *E. coli* DHFR and the type II R-plasmid encoded DHFRs, the catalytic efficiency (k_{cat}/K_m) of the type II enzyme is about 100 times less than that for the *E. coli* enzyme (Joyner *et al.*, 1984; Smith and

Burchall, 1983). In addition, when hydride transfer rates are compared, R67 DHFR is 180 fold slower at pH 7.0 (Dion-Schultz and Howell, 1997).

Type II DHFRs

Three type II DHFRs have been identified and named R67, R388, and R751 (Broad and Smith, 1982). The type II enzymes have been sequenced and shown to have greater than 78% sequence homology with each other (Amyes and Towner, 1990). They have also been shown to be active as a tetramer with molecular weights of ~34,000 daltons and are composed of four identical monomers, each with molecular weight of approximately 8,500 daltons (Smith *et al.*, 1979). The monomer is encoded as a 78 amino acid polypeptide. The R388 and R751 type II R-plasmid encoded DHFRs have identical protein sequences from amino acids 22 to 78, and the R67 protein only differs by six amino acid residues in this region (Flensburg and Steen, 1986; Stone and Smith, 1979; Swift *et al.*, 1981; Zolg and Hanggi, 1981).

R67 DHFR Structure

R67 DHFR, the focus of this research, has been crystallized in both dimeric and tetrameric forms (Matthews *et al.*, 1986; Narayana *et al.*, 1995). The crystal structure of dimeric R67 DHFR was solved to a resolution of 2.8 Å and has a calculated R-factor of 28% for all data between 8 and 2.8 Å. Matthews *et al.* were only able to make unambiguous assignments of amino acid residues 18-78, with the first 17 having densities that were too weak to define (Matthews *et al.*, 1986). With

the exception of Val - 8, the first ten amino acid residues of R67 DHFR are hydrophilic, so it is thought that these residues project into the solvent resulting in a disordered amino terminal end. To determine if the first 16 amino acids were necessary for the function of R67 DHFR, Reece *et al* removed them by digestion with chymotrypsin (Reece *et al.*, 1991). This truncated protein was shown to be fully active, confirming the idea that the functional core of the protein is defined by the residues 27-78, which form a β -barrel structure. The truncated enzyme was crystallized in the tetrameric form and was solved to a resolution of 1.7 Å (Narayana *et al.*, 1995). It was found that each of the four monomers crystallized identically and formed a tetramer with exact 222 symmetry.

Figure 1-1 shows the structure of dimeric R67 DHFR. Each R67 DHFR monomer has a core structure that folds to form an up and down six-stranded β -barrel, except that the fifth strand is missing. The five antiparallel β -barrel strands sequentially labeled as a, b, c, d, and e are connected by three short loops and a turn of 3_{10} helix. Strand b is the longest β -strand in R67. It contains 10 residues and has a classic β -bulge near its midpoint that bends the strand by 90°, distorting the β -barrel into two nearly orthogonal β -sheets (Narayana *et al.*, 1995).

The dimeric form is stabilized by the interactions of β -strands b, c and d from one monomer with that of another monomer to form a third β -barrel. Strands b, c and d of one monomer align in an antiparallel manner with the symmetrically related strands from the adjacent monomer to form a third (six-stranded) antiparallel β -barrel. The interactions that stabilize the dimeric form of R67 DHFR are substantial,

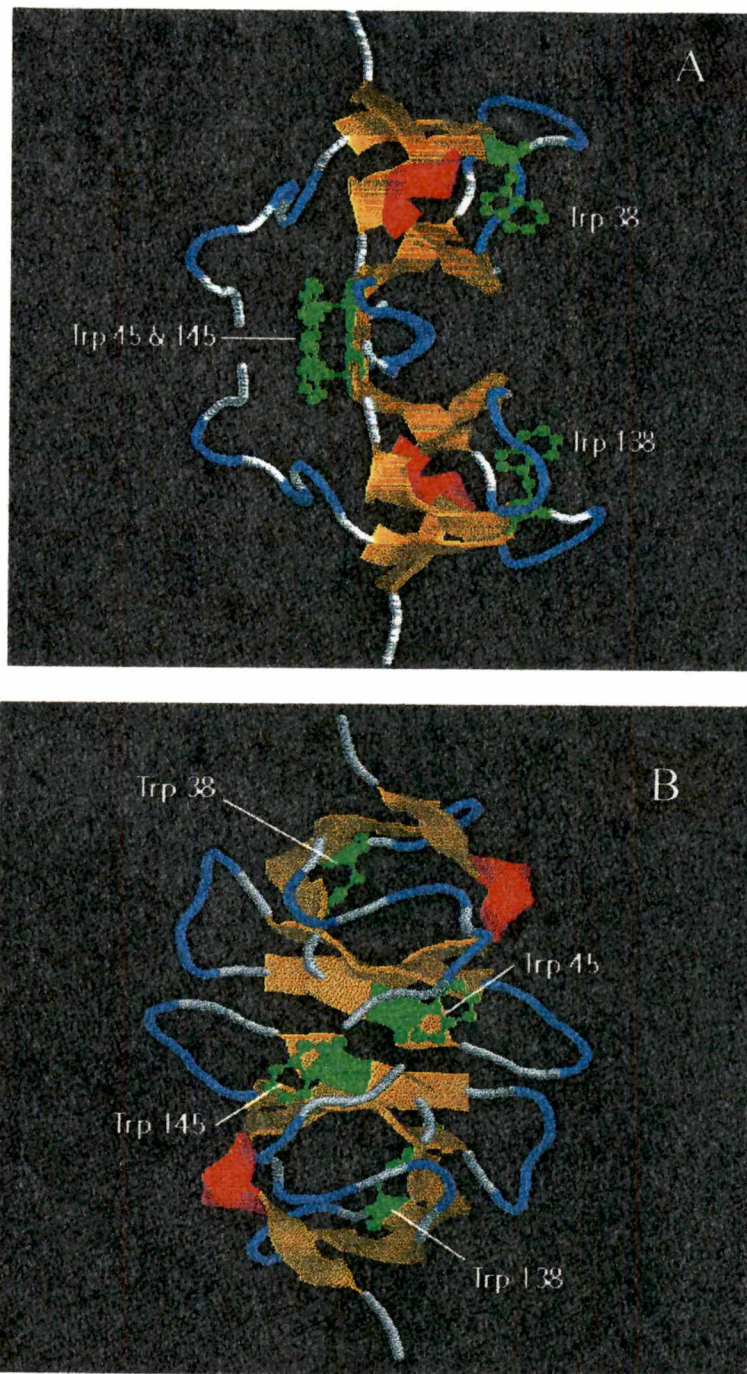


Figure 1-1. Ribbon diagram of dimeric R67 DHFR taken from the structure of Narayana., Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) *Nat. Struct. Biol.* 2, 1018-1025. Panel (A) views the structure down the inter-subunit β -barrel. Panel (B) is a 90° rotation about the y-axis from panel (A), such that the structure is viewed down the monomer β -barrels. Tryptophan side chains are shown in green.

including the packing of hydrophobic side chains within the interior of the intersubunit β -barrel, hydrogen bonds between the strands of the intersubunit β -barrel, and van der Waals interactions between the side chain of Met 26 of one monomer and residues 45-47 of the second monomer (Narayana *et al.*, 1995). The six-stranded inter-subunit β -barrel is imperfect. An 8 Å "gap" occurs between the symmetry related d strands preventing its hydrogen bonding with the juxtaposed strand on the second subunit, see Figure 1-1. Interestingly, dimeric R67 DHFR has one of the smallest buried surface areas (535 Å²) of any stable dimeric protein presently known (Narayana *et al.*, 1995).

The tetramer, which is the enzymatically active species, is formed primarily through the interactions of residues in the loop connecting β -strands c and d (59-62), and four residues just preceding strand b, as well as stacking interactions between Trp 38 & 338 and Trp 138 & 238 and the formation of hydrogen bonds between the symmetry related Gln 67 residues, see Figure 1-2. Two pairs of symmetry related histidines (62 & 362, 162 & 162) are in close van der Waals contact across the dimer-dimer interface. In the tetramer, His 62 accepts a hydrogen bond (at the ϵ 2 nitrogen) from the hydroxyl of the nearby Ser 259 from the symmetry related monomer. It is the only hydrogen bond acceptor close enough to interact with the buried serine hydroxyl. The protonation of His 62 (pKa ~6.8) disrupts tetramer formation, since a buried positively charged imidazole is unstable and juxtaposition of two positive charges is repulsive (Nichols *et al.*, 1993; Narayana *et al.*, 1995). This leads to a tetramer to two-dimers transition going from pH 8 to 5.

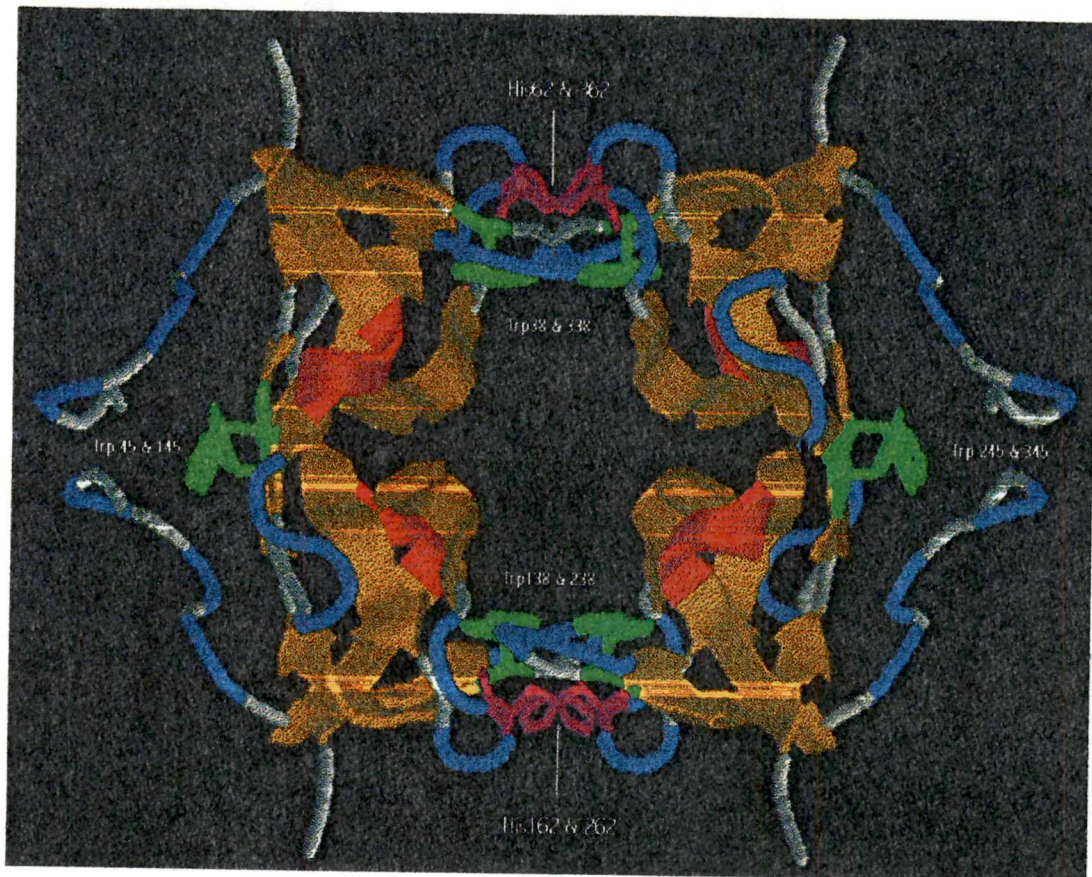


Figure 1-2: Ribbon diagram of tetrameric R67 DHFR taken from the structure of Narayana. Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) *Nat. Struct. Biol.* 2, 1018-1025. The structure is viewed down the center of the central pore. The side chains of the symmetry-related trp38 and trp45 and Histidine 62 residues are shown. Helices are shown in (red), β -sheet (yellow), turns (blue) and disordered (white)

The R67 DHFR homotetramer is a dimer of dimers that shows exact 222 symmetry. It is composed almost entirely from two unique interfaces and is hollow. The homotetramer is a roughly doughnut shaped molecule that measures 30 X 35 X 40 Å and has a unique pore that runs through the middle of the molecule, see Figure 1-3. This unusual pore runs through the full length of the molecule (25 Å) and has an elliptical cross-section. The mouth of the pore measures 24 X 18 Å, while at the center of the molecule, the dimensions are reduced to 12 X 9 Å respectively (Narayana *et al.*, 1995).

Narayana *et al.* also crystallized R67 DHFR in complex with either thio-NADP⁺ or folate and found electron density corresponding to ligand binding in the pore. However, co-crystals of enzyme plus thio-NADP⁺ plus folate did not show density in the pore (Matthews, Xuong, Narayana; personal communication). This was most likely due to all the possible combinations available for binding, which results from having a symmetrical active site. Difference density maps indicate that this pore is the binding site for either thio-NADP⁺ or folate. These results as well as solution binding studies suggest that all four subunits contribute to the formation of a single active site (Narayana *et al.*, 1995; Bradrick *et al.*, 1996).

Mechanism of Catalysis

The symmetry related Gln 67 residues project into the center of the pore reducing its diameter near the center. These side chains are hydrogen bonded to each other and form the roof and floor of the pore, forming the active site of the enzyme. The center of the pore is ideally suited for the stacking of flat rings and maximizing van der

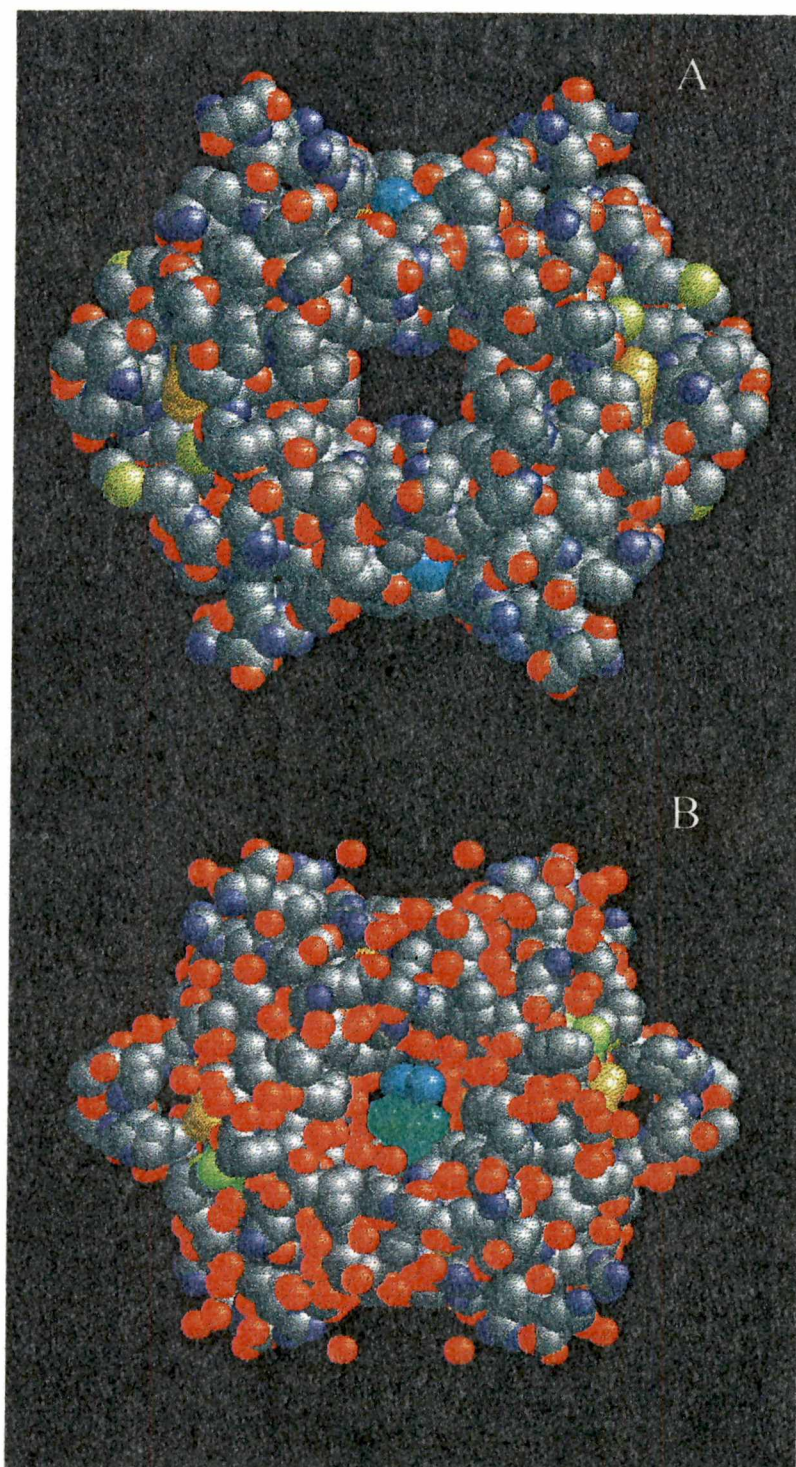


Figure 1-3: Space-filling structure of tetrameric R67 DHFR taken from the structure of Narayana., Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) *Nat. Struct. Biol.* 2, 1018-1025. Panel (A) shows the space filling model of tetrameric R67 DHFR. Panel (B) shows a model of tetrameric R67 DHFR in ternary complex with NADPH docked (cyan) and folate (green) bound as in the binary complex crystal structure. Tryptophans are shown in orange. Ordered water molecules (red) are additionally shown in Panel (B).

Waals edge on ring interactions (Narayana *et al.*, 1995). The relative positions of these residues can be seen in Figure 1-4. The simplest model for catalysis that is consistent with both the crystal structure and the binding studies that have been carried out on R67 DHFR suggests that NADPH occupies half the pore and DHF occupies the other half (Bradrick, *et al.*, 1996). The pteridine ring of DHF and the nicotinamide ring of NADPH are proposed to stack between the hydrogen bonded Gln 67 side chains at the center of the pore where the reaction occurs.

In the reduction of DHF to THF, a hydride ion is transferred from the C4 carbon of NADPH to the C6 carbon of DHF and a proton is added to N5 of DHF, although the sequence of hydride and proton transfer is not clear. In the *E. coli* chromosomal DHFR, a proton donor, Asp 27, greatly increases the rate of catalysis. However, no proton donor has been identified for R67 DHFR. It is thought that protonated DHF is the actual substrate for R67 DHFR. In the absence of a proton donor, R67 DHFR could still catalyze the reaction if the enzyme mediated protonation step was bypassed by binding preprotonated DHF (pK_a for N5 nitrogen of DHF = 2.59; Maharaj *et al.*, 1990).

This was shown to be the case for an *E. coli* chromosomal DHFR mutant in which the active site proton donor Asp 27 was mutated to asparagine (D27N) (Villafranca *et al.*, 1983; Howell *et al.*, 1986). The catalytic efficiency of the mutant enzyme was dramatically reduced at pH 7, but it could still function by using preprotonated substrate from solution, since a increase in the rate of reduction of DHF was seen as the pH is lowered. This results from increasing the population of protonated DHF present in solution as the pH is lowered.

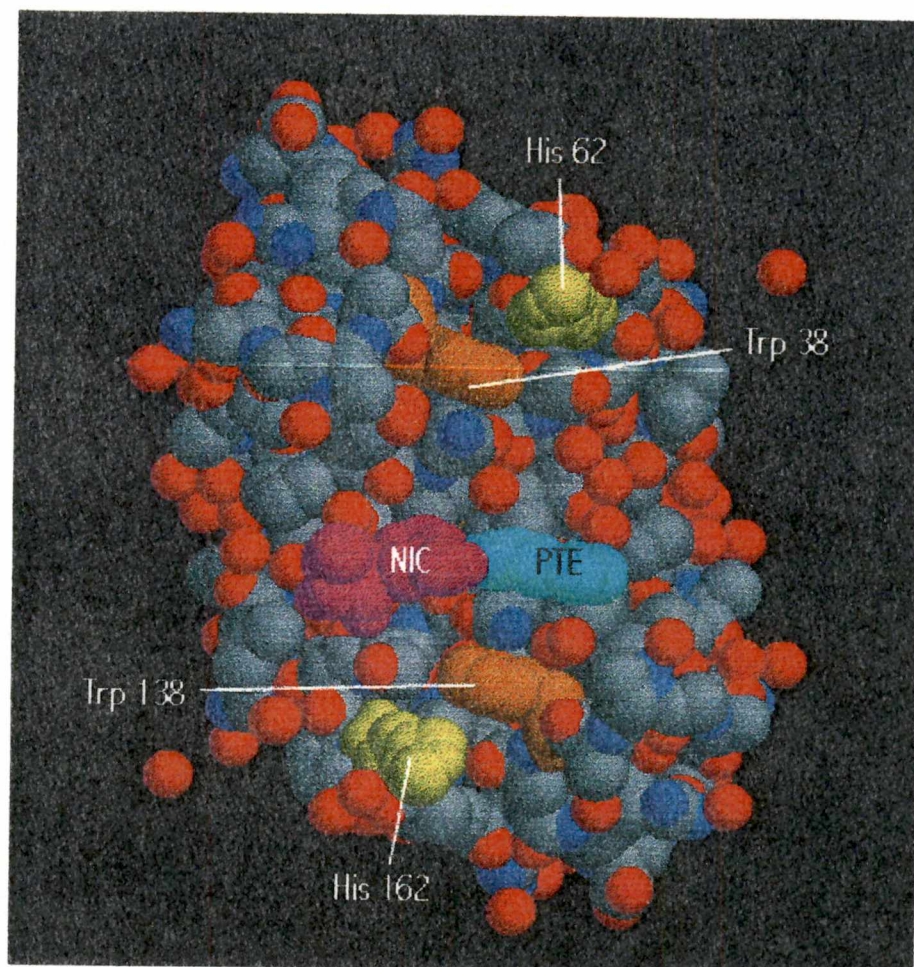


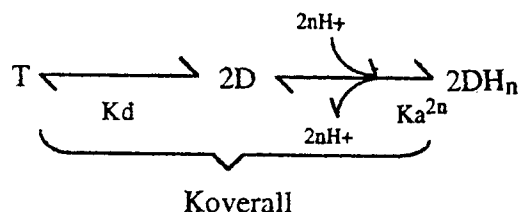
Figure 1-4: Space filling structure of R67 DHFR derived from the a model of the ternary complex with NADPH and folate. Taken from the structure of Narayana., Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) *Nat. Struct. Biol.* 2, 1018-102. The structure is viewed perpendicular to the central pore. (NIC) represents the nicotinamide ring of NADP⁺ and (PTE) represents the pteridine ring of folate. Only half the enzyme is shown for clarity.

Oligomeric States of R67 DHFR

Unlike the D27S mutant *E. coli* chromosomal DHFR, a bell-shaped pH profile is seen for R67 DHFR when monitored by enzyme activity. It has an apparent basic pKa of ~ 7.3 and an apparent acidic pKa that is protein concentration dependant. The latter was shown to be due to the ionization of His 62 resulting in the formation of dimers, which have only slight activity (Park *et al.*, 1997).

The following model was proposed to show the pH dependence of the tetramer to 2 dimers transition:

Scheme 1



where $K_{\text{overall}} = ([\text{T}][\text{H}]^{2n})/([\text{DH}_n]^2) = K_a^{2n}/K_d$ (**Eq 1**) in units of M or M³ for n = 1 or 2, respectively, and K_d is the dissociation constant for the unprotonated tetramer $\rightleftharpoons$ 2 dimers equilibrium. This is the simplified model used to describe the linkage between protonation and the tetramer $\rightleftharpoons$ 2 dimers equilibrium. This T $\rightleftharpoons$ 2D equilibrium can be removed from R67 DHFR by constructing a H62C mutant that allows disulfide cross-linking. In this mutant, the pH profile for k_{cat} shows increasing rates as the pH is decreased, constant with R67 DHFR using protonated DHF as the productive substrate (Park *et al* 1997).

The structure of R67 DHFR allows for the alteration of association complexity as a function of pH. At pH 5.0, R67 DHFR is dimeric and equilibrium-unfolding studies have shown unfolding to be a two-state process according to the following model:



where D is dimeric R67 DHFR and U is the unfolded monomer (Reece *et al.*, 1991). However, with increasing pH, dimeric R67 DHFR undergoes a pH dependent association to form the active tetramer that predominates at pH 8.0. Equilibrium unfolding studies indicate that at pH 8.0, unfolding is no longer a two-state process as an intermediate occurs. The unfolding of tetrameric R67 DHFR at pH 8.0 can be described by the following model:



where T is tetrameric R67 DHFR, D is the dimeric form and U represents the unfolded monomer (Zhuang *et al.*, 1994).

Fluorescence Spectroscopy

In the above studies, the equilibrium unfolding transitions were monitored by following the loss of tryptophan fluorescence intensity at 340nm. It is pertinent here to note that R67 DHFR contains two tryptophans per monomer. As can be seen in Figure 1-1, W45 and W145 as well as the symmetry related residues W245 and

W345, occur at the monomer-monomer interfaces. W38 and W338, as well as their symmetry related residues are found at the dimer-dimer interfaces. The positioning of W45 and W38 residues at the monomer and dimer interfaces allow for the utilization of these tryptophans as intrinsic probes to follow the unfolding of R67 DHFR.

Fluorescence spectroscopy is a very powerful technique for monitoring conformational changes in proteins. In particular, tryptophan residues are suitable intrinsic probes because of the sensitivity of the indole ring to the polarity of its environment and other molecular interactions within the protein. In order for a spectroscopic method to be used to monitor a conformational transition in a protein, there must be a measurable difference in some signal between two or more of its conformational states. Fluorescence measurements are multi-dimensional as signal changes that can be monitored include the following: fluorescence intensity (at some emission wavelength, $em\lambda$), $F_{em\lambda}$; quantum yield, Φ ; emission maximum, λ_{max} ; fluorescence lifetime, τ_f ; pre-exponential factor, α_f ; fluorescence anisotropy, r ; and rotational correlation time, ϕ (Eftink, 1994). This multi-dimensionality makes fluorescence a powerful tool, since it is likely that one of these signals will be sensitive to the conformational state of the protein.

The primary chromophore found in proteins is tryptophan. A change in tryptophan environment as monitored by fluorescence generally coincides with a change in the oligomeric state of the protein or a global loss of tertiary and secondary structure, as the unfolding of many proteins are highly cooperative reactions. The dependency of the steady-state fluorescence intensity on the concentration of denaturant can provide values for the free energy of folding (Eftink, 1994).

The unambiguous assignment of the fluorescent properties to an individual tryptophan residue is necessary for the interpretation of the observed fluorescence signals at a molecular level. There are difficulties however, in resolving the individual contributions of chromophores in multichromophore containing proteins. This limits the ability to interpret the structural and dynamic properties of these side chains during the unfolding reaction (Eftink, 1994). To overcome this limitation, site-directed mutagenesis has been widely used to construct single-tryptophan containing mutants (Vos and Engelborghs, 1995; Dijkstra *et al.*, 1997; Kombo *et al.*, 1996; Szpikowska *et al.*, 1994; Weitzman *et al.*, 1995; Mann *et al.*, 1993; Royer *et al.*, 1993). In most cases, the tryptophans are replaced individually with the weakly fluorescent aromatic amino acid phenylalanine.

Equilibrium Unfolding Studies

Equilibrium denaturation followed by fluorescence spectroscopy has been very useful in understanding the structure, stabilization, and folding of many monomeric proteins (Vos and Engelborghs, 1995; Dijkstra *et al.*, 1997; Kombo *et al.*, 1996; Szpikowska *et al.*, 1994; Weitzman *et al.*, 1995; Mann *et al.*, 1993; Royer *et al.*, 1993). The range of stabilities calculated for monomeric proteins are generally seen to fall within the range of 1.5 to 7 kcal/mol. This represents the small difference between the multiple noncovalent interactions and the small number of covalent (and disulfide linkages if present) interactions favoring the folded protein state versus the unfavorable entropic terms, favoring the unfolded state. Such studies can provide useful information about the packing forces present in the interior of a protein and the

functional interactions of residues. The application of these techniques to oligomeric proteins can provide information on subunit interactions and the stability of the oligomeric state. Dimeric proteins that show a single transition from folded dimer to 2 unfolded monomers, have stabilities that range from 10 to 27 kcal/mol, which are significantly larger than those seen for monomeric proteins (Neet and Timm, 1994). The large stabilization that is seen for oligomeric proteins is primarily due to the intersubunit interaction and may provide a rationale for the evolutionary formation of dimers.

In most cases, single-domain monomeric proteins show a single fluorescence-transition in equilibrium unfolding studies. The basis for the thermodynamic analysis of the equilibrium denaturation in this case is the assumption that at equilibrium only two states exist, the native state and the unfolded monomer. That is the unfolding can be represented by a two-state model. Multi-domain monomeric proteins and oligomeric proteins may follow a two-state unfolding mechanism or exhibit one or more stable intermediates in the equilibrium unfolding profile. This is seen as an unfolding profile with more than one transition. For example, a biphasic-unfolding curve is indicative of unfolding by a three-state mechanism. Alternately, three state unfolding mechanisms can be observed when using more than one technique to investigate the unfolding process. For example, one technique may not be sensitive to the presence of the intermediate resulting in a single-transition, while a second technique may detect its presence and result in a biphasic curve or non-overlapping transitions can be observed indicating a three-state process.

Equilibrium Unfolding Studies R67 DHFR

The equilibrium unfolding characteristics of wild-type R67 DHFR (wtR67) have been studied in some detail (Nichols *et al.*, 1993; Zhuang *et al.*, 1994). At pH 5.0, unfolding in guanidine-HCl followed by fluorescence yielded a single transition that represents the loss of the monomer-monomer interface and exposure of the symmetry related Trp 45s to solvent (Nichols *et al.*, 1993). At pH 8.0 however, a biphasic fluorescence transition was not seen. When the unfolding transitions monitored by CD and fluorescence spectroscopy were compared however, they were seen to be nonsuperimposable with the unfolding transition monitored by enzyme activity, which suggests that the folding proceeds by a three-state mechanism (Zhuang *et al.*, 1994). This suggests that either the folded dimeric state is not a significantly populated state during unfolding or that perhaps the amplitude of the T $\leftrightarrow$ 2D fluorescence signal is much smaller than that describing the 2D $\leftrightarrow$ 4U transition.

The goal of this research is to correlate the observed equilibrium unfolding/folding profiles with the individual contributions of the tryptophan residues at each interface. Thus each of the two tryptophans of wtR67 DHFR were mutagenized to the weakly fluorescent aromatic amino acid phenylalanine to produce the single Trp mutants, W45F and W38F. The kinetics, fluorescence and CD-spectrai properties of these mutants were then compared to the wild type enzyme.

CHAPTER II

MATERIALS AND METHODS

As discussed in Chapter I, R67 DHFR has two tryptophan residues per monomer at positions 38 and 45. Figure 1-2 shows their relative positions. W45 and W145, as well as the symmetry related residues W245 and W345, occur at the monomer-monomer interfaces. W38F and W338, as well as their symmetry related residues, W138 and W238, occur at the dimer-dimer interfaces and are involved in stacking interactions that help to stabilize these interfaces. The goal of this thesis is to correlate the observed unfolding/folding profile with the individual contributions of the tryptophan residues at each interface. To this end, it was necessary to utilize site-directed mutagenesis methodologies to individually replace each tryptophan in R67 DHFR with the weakly fluorescent amino acid phenylalanine.

DNA Manipulations

The full length synthetic Met-Ile variant R67 DHFR gene sequence cloned into the polycloning site of the bacteriophage M13mp19 under the control of a mutant (up) promoter was available in our lab (Reece *et al.*, 1991). The mutagenic oligonucleotides:

5'-ATTTGTGCAGTAGAAACCCGACAATATG – 3': (W45F)

5' – AATCTGACCTTGGAAGGCGCC – 3': (W38F)

were used as primers for site directed mutagenesis reactions to replace the codon for tryptophan (TGG) with that for phenylalanine. A Muta-Gene[®] in vitro Mutagenesis kit from BIO-RAD Laboratories was utilized to carry out the in vitro mutagenesis using the above mutagenic oligonucleotides as primers and following the procedures outlined in the kit. Due to the low efficiency of mutagenesis, the W45F mutant was identified by dot-blot hybridization using standard procedures (production of the W45F mutant was carried out by Han-Seok Seo) and then sequenced, while the W38F mutant was screened by sequencing (Maniatis *et al.*, 1982). Upon confirmation of the mutation by sequencing, the mutant genes were cloned into pUC8 and transformed into *E. coli* (strain SK383) to increase expression levels. Standard cloning procedures were used to digest, anneal and transform the cassette from M13mp19 to pUC8 and to transform this vector into the host (Maniatis *et al.*, 1982). Single- and double-stranded DNA sequencing was performed using a Sequenase kit from United States Biochemical Corp. Primers for dideoxy sequencing included a universal primer (Promega) as well as the mutagenic oligonucleotides used to generate the mutation.

Protein Purification

The mutant R67 DHFRs were expressed and purified according to the following procedure. The modified version of TB media that was utilized to culture the bacteria, consisted of 0.017 M KH₂PO₄, 0.07 M K₂HPO₄, 12 g/L Bacto-yeast extract, 24/ g/L Bacto-yeast extract, 4 ml/L glycerol, and 2 g/L NaCl (Tartof and Hobbs, 1987). Cells were grown to late stationary phase (to an OD₆₆₀ of a 1/10 dilution =

0.45) at 37° in the presence of 200 µg/ml ampicillin plus 50 µg/ml TMP. Cells were harvested by centrifugation and lysed by addition of 1 M NaOH until a pH of 12.5 was reached (Vermersch *et al.*, 1986). Cellular debris was removed by centrifugation, and the cell lysate was titrated to pH 2.0. Precipitated material was again removed by centrifugation and the solution returned to pH 6.5. The proteins remaining in the cell lysis solution were then precipitated with 55% ammonium sulfate. This salt pellet was then resuspended in PE buffer (50 mM KH₂PO₄, and 1 mM EDTA at pH 8.0) and applied to a 3.5 X 80 cm G-75 Sephadex chromatography column. Proteins were eluted with PE buffer, fractions were collected, and peaks were monitored by following the absorbance at 280nm. Fractions were analyzed by assaying for enzymatic activity (see below for details) and Coomassie Blue-stained SDS-PAGE gels. Fractions containing R67 DHFR were pooled and applied to a DEAE Fractogel column and eluted with PE, pH 8.0. R67 DHFR does not bind to this column due to the high ionic strength of the buffer and the type of counter ion present, but many of the contaminating proteins will. Fractions were again collected and analyzed and those containing R67 DHFR were pooled. The pooled fractions were dialyzed against TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) and applied to a DEAE Sepacel column. Elution utilized a 0 – 0.2 M KCl gradient, pH 8.0. The fractions were again assayed for enzymatic activity and purity. If necessary, a final purification step using a Pharmacia FPLC equipped with a Mono-Q anion exchange column was employed. Elution was achieved with a 0 – 4% gradient between TE and TE with 1 M KCl at pH 8.0. Pure fractions were dialyzed against distilled, deionized water, lyophilized and stored at 4° C. The purified enzyme concentrations were

determined by the biuret method and extinction coefficients were determined (Gornall *et al.*, 1949).

Kinetic Analysis

A Perkin-Elmer lambda 3a spectrophotometer interfaced with an IBM PS/2 was used to obtain steady-state kinetic data (Howell *et al.*, 1987). After the addition of substrate and cofactor, the assay was initiated by the addition of enzyme. The initial rates of reaction (1 minute) were measured spectrophotometrically by measuring the loss of absorbance at 340nm (A_{340}) using the computer program UVS (Softways). A change in A_{340} corresponds to the loss of reduced cofactor and DHF. All assays were performed at 30° C in a polybuffer containing (50 mM MES (2-[N-Morpholino] ethanesulfonic acid, 100 mM Tris, 50 mM acetic acid and 10mM β -mercaptoethanol) (MTA buffer), and pH 7.0. This buffer has been shown to maintain a constant ionic strength between pH 4.5 –9.5 (Ellis and Morrison, 1982). The kinetic parameters K_m (DHF), K_m (NADPH) and k_{cat} were determined for the W45F mutant using saturating concentrations of either DHF or NADPH while varying the other at subsaturating levels. The resulting data were then fit to the Michaelis-Menton equation using the non-linear regression program, Enzfitter (Elsevier-Biosoft). The determination of the kinetic values for the W38F mutant required a more rigorous approach, because this mutant has K_m values much larger than either those seen for the wild type R67 DHFR or the W45F mutant DHFR (see Table 1). This results in the inability to saturate with either substrate or cofactor, as it would require concentrations that would be out of the linear range of the spectrophotometer. The

method used to determine the kinetic values in this case, is to measure the initial reaction velocity while holding one reactant constant and varying the other. This is repeated for four subsaturating concentrations of the fixed substrate. The resulting data were then plotted according to the method described by (Cleland, 1963).

Gel Filtration Studies

Gel filtration studies were carried out at 4° C with a Pharmacia FPLC using a Superose 12 (HR 10-30) column (Pharmacia) with a flow rate of 1.0 ml/min. TE buffer, at either a pH of 8.0 or 5.0 was used. The $K_{av} = ((\text{elution volume} - \text{void volume})/(\text{bed volume} - \text{void volume}))$ was monitored as a function of pH for wild-type R67 DHFR (wtR67) and the W45F and W38F mutant DHFRs. Standard curves at pHs 5.0 and 8.0 were produced by plotting the log molecular weight of protein standards (Pharmacia LMW standards) versus K_{av} . This allowed for the determination of the molecular weight of the three proteins.

pH Dependence of Oligomeric State

In order to compare the pH dependent equilibrium between tetramer and two dimers for R67 DHFR and the two single-tryptophan mutants, the intrinsic protein fluorescence was monitored at room temperature (~23° C) as a function of pH. Fluorescence measurements were made using a Perkin-Elmer LS-5B spectrometer interfaced to an IBM PS/2 compatible computer. Tryptophan residues were excited at 295nm (slit 10nm) and emission was monitored from 300 – 500nm (slit 10nm), with a cell path length of 1cm. For the pH titrations, the concentration of the three proteins,

R67 DHFR and the two single tryptophan mutants W45F and W38F, were held constant at (2 μ M dimer) in MTA polybuffer without β -mercaptoethanol. The samples were titrated individually with small aliquots of 2M HCl and pH measurements were performed directly in the cuvettes using a microelectrode (Microelectrodes Inc.).

The experimental data were fit to the model previously described by (Nichols *et al*, 1993). This model describes the behavior of R67 DHFR as a function of pH. It shows the linkage between the protonation-state and the tetramer $\leftrightarrow$ 2 dimers equilibrium. In scheme 1, T is tetrameric while D and DH_n are dimeric forms of unprotonated and protonated R67 DHFR, K_d is a dissociation constant, while K_a is an ionization constant. This scheme was used to derive the following equation to which the experimental data were fit:

$$F_{(obs)} = (DH_n/P_{tot})(Lim_1 - Lim_2) + Lim_2 \quad \text{Eq 4}$$

where Lim₁ and Lim₂ are the asymptotic values for dimer and tetramer respectively, [DH_n] = [D*H²ⁿ]/[K_a²ⁿ] and P_{tot} = the total protein concentration in terms of dimer. This fit was performed with a nonlinear least-squares fitting program (NLIN) in the Statistical Analysis Systems package (SAS Institute Inc., Cary, NC).

Circular Dichroism

Circular dichroism (CD) spectra were recorded at 22° C in 10 mM phosphate buffer, pH 8.0 for 10 μ M wtR67, W45F and W38F DHFRs. Data were recorded from 190nm to 250nm using a Jasco J720 spectropolarimeter at Vanderbilt University with a cuvette pathlength of 0.2 cm. A buffer baseline was subtracted from each protein scan. The CD data were collected in millidegrees. Selected data were converted to molar ellipticity (θ) by taking 108g/mole as the mean residue molecular weight. θ is the molar ellipticity expressed in degrees cm²/decimole.

Steady-State Fluorescence Unfolding at pH 5.0

Tryptophan fluorescence was employed to monitor the equilibrium unfolding as a function of guanidine-HCl (GdnHCl) concentration (Zhuang, *et al* 1993; Reece *et al.*, 1991). Changes in tryptophan fluorescence were monitored using a Perkin-Elmer LS-5B fluorimeter. Tryptophans were excited at 295nm (slit 5) and the emission spectra measured from 300 to 500nm at increasing concentrations of denaturant using either a cell path length of 1cm or 0.5cm. Measurements were performed at room temperature (~23° C), in MTA buffer pH 5.0, without β -mercaptoethanol. The unfolding of wtR67 DHFR, W45F and W38F mutant DHFRs was induced by addition of guanidine hydrochloride at various concentrations between 0 and 5.0 M. A Zeiss refractometer was used to measure the refractive index of the stock GdnHCl solutions. GdnHCl concentrations were calculated using an equation relating the refractive index to concentration (Pace *et al.*, 1990). The samples were allowed to incubate for 45 min in order to reach equilibrium. Both

fluorescence intensity at 340nm and the spectral center of mass for fluorescence emission were used to monitor the protein folding transitions. Several different concentrations of each protein were evaluated in order to determine the concentration dependence of the unfolding reaction. Thermodynamic values were obtained by fitting data describing the fluorescence intensity at 340nm and the spectral center of mass. The spectral center of mass is the intensity averaged emission wavelength $\langle\lambda\rangle$, which is calculated according to the following equation:

$$\langle\lambda\rangle = \Sigma(I_i\lambda_i)/\Sigma(I_i) \quad \text{Eq 5}$$

where I is intensity and λ is the wavelength (Royer *et al.*, 1993). The center of mass is an integral measurement and is less sensitive to noise than the change in fluorescence intensity at a fixed wavelength. This method is based on the assumption that the quantum yields of the folded and unfolded states of the proteins are similar. If the quantum yield of the folded and unfolded states are not comparable then the center of mass may not accurately track the population of states, resulting in the data being weighted toward the more intensely emitting species (Eftink, 1994).

The data for the equilibrium unfolding of wtR67 DHFR and the two single Trp mutants at pH 5.0 were fit to a two-state model of unfolding (Eq 2), where the protein goes from folded dimer to 2 unfolded monomers with no stable intermediate state. The Gibbs free energy change, ΔG_{H_2O} , between the folded and unfolded states was then calculated by nonlinear regression of the following equation:

$$Y_{\text{obs}} = \left[\left(Y_U + M_U [\text{GdnHCl}] \right) - \left(Y_N + M_N [\text{GdnHCl}] \right) \right] \times \left[\exp \left(-(\Delta G_{\text{H}_2\text{O}} + M_G [\text{GdnHCl}]) / RT \right) \right]^2 + 8 \left\{ \exp \left\{ -\Delta G_{\text{H}_2\text{O}} + M_G [\text{GdnHCl}] / RT \right\} [P_{\text{tot}}] \right\}^{1/2} - \left\{ \exp \left[(\Delta G_{\text{H}_2\text{O}} + M_G [\text{GdnHCl}]) / RT \right] \right\} / 4 [P_{\text{tot}}] + Y_N + M_N [\text{GdnHCl}] \quad \text{Eq 6}$$

where R is the gas constant, T is the temperature, $\Delta G_{\text{H}_2\text{O}}$ is the free energy change between the native and unfolded protein in the absence of GdnHCl, M_G is the slope describing the dependence of ΔG on denaturant concentration, Y_N and Y_U are the concentration-independent optical values, and M_N and M_U are the slopes of the pre- and post-transition regions, respectively (Reece *et al.*, 1991; Zhuang *et al.*, 1994). SigmaPlot (Jandel Scientific) was used to fit the data to this equation using a nonlinear least-squares algorithm.

To facilitate the comparison between the various unfolding curves, the data were normalized by fitting to the following equation:

$$F_{\text{app}} = (Y_N - Y_{\text{obs}}) / (Y_N - Y_U) \quad \text{Eq 7}$$

where F_{app} is the fraction of the unfolded protein, Y_{obs} is the observed value, and Y_N and Y_U are the values associated with the native and unfolded forms of the protein (Reece *et al.*, 1991).

Global Fits

Global fits using Scientist (MicroMath Scientific Software) were performed on the unfolding data obtained for wtR67 DHFR and the two single Trp mutants using the two-state model discussed above. In a global fit, all the data from a series of unfolding curves are fit concurrently to yield a single set of values. This typically results in a fit value possessing a lower error.

CHAPTER 3

RESULTS

Steady State Kinetic Analysis

The enzymatic activities of W45F and W38F mutant R67 DHFRs were assessed at pH 7.0 and 30° C. The kinetic parameters, k_{cat} and K_m for DHF, for the W45F mutant DHFR were determined by using saturating levels of NADPH, and then measuring the rate of the reaction at varying concentrations of DHF. Likewise, the k_{cat} and K_m values for NADPH were determined by saturating the enzyme with DHF, and then varying NADPH. The data points were fit to the Michaelis-Menton equation using the non-linear regression program, Enzfitter (Elsevier-Biosoft) to yield the kinetic values.

The determination of the kinetic values for the W38F mutant DHFR required a more rigorous approach, because this mutant has K_m values that are much higher than that seen for either the wild-type enzyme or the W45F mutant. This resulted in the inability to saturate with either substrate or cofactor, as it would require concentrations that would be out of the linear range of the spectrophotometer. In this case, the accepted method for determining the kinetic parameters requires four subsaturating concentrations of both ligands (Cleland, 1963). In this procedure, the concentration of NADPH was held constant at a subsaturating level, while the concentration of DHF was varied. This process was repeated, for three more concentrations of NADPH, until four lines were obtained. In brief, the data were plotted as 1/velocity versus 1/[DHF] and 1/velocity versus 1/[NADPH]. Plotting the

y-intercepts versus the concentrations of the fixed substrate then generated two secondary plots. From these plots, the kinetic parameters were calculated as described by (Cleland, 1963). Figure 3-1 shows the data plots for the W38F mutant DHFR, along with a brief set of definitions used in calculating the kinetic values.

Table 3-1 summarizes the kinetic parameters for the two mutant R67 DHFRs and gives the wtR67 DHFR values for comparison. It can be seen that the kinetic parameters of the W45F mutant DHFR approximate those of the wild-type enzyme. However, the W38F mutant R67 DHFR was shown to have K_m values that are much larger than those seen for the wild type or the W45F mutant R67 DHFR, while having a significantly lower k_{cat} . Also shown in Table 3-1 are the extinction coefficient (ϵ_{280}) values determined for each of the three proteins at 280nm. It is seen that the ϵ_{280} values for the two single tryptophan mutants are significantly lower than that of the wild-type R67 DHFR. This is not unexpected as tryptophan is the primary absorbing species at this wavelength.

Gel Filtration Studies

Molecular sieving chromatography was utilized to assess the oligomeric state of the mutant R67 DHFRs. In these studies, the apparent molecular weight of the proteins was determined by measuring the retention time at pH 5.0 and 8.0 on a calibrated Superose 12 column. This column was calibrated using proteins of known molecular weight. Standard curves were generated by plotting the K_{av} for each protein versus the log of its molecular weight. A regression line was generated in SigmaPlot, which was used to calculate the apparent molecular weight of the

Brief definitions

- (1) $-1/K_m$ = x-intercept of intercept vs. $1/\text{fixed substrate}$ plot
- (2) $1/V_{\max}$ = y-intercept of intercept vs. $1/\text{fixed substrate}$ plot
- (3) $k_{\text{cat}} = V_{\max}/\text{concentration of enzyme}$

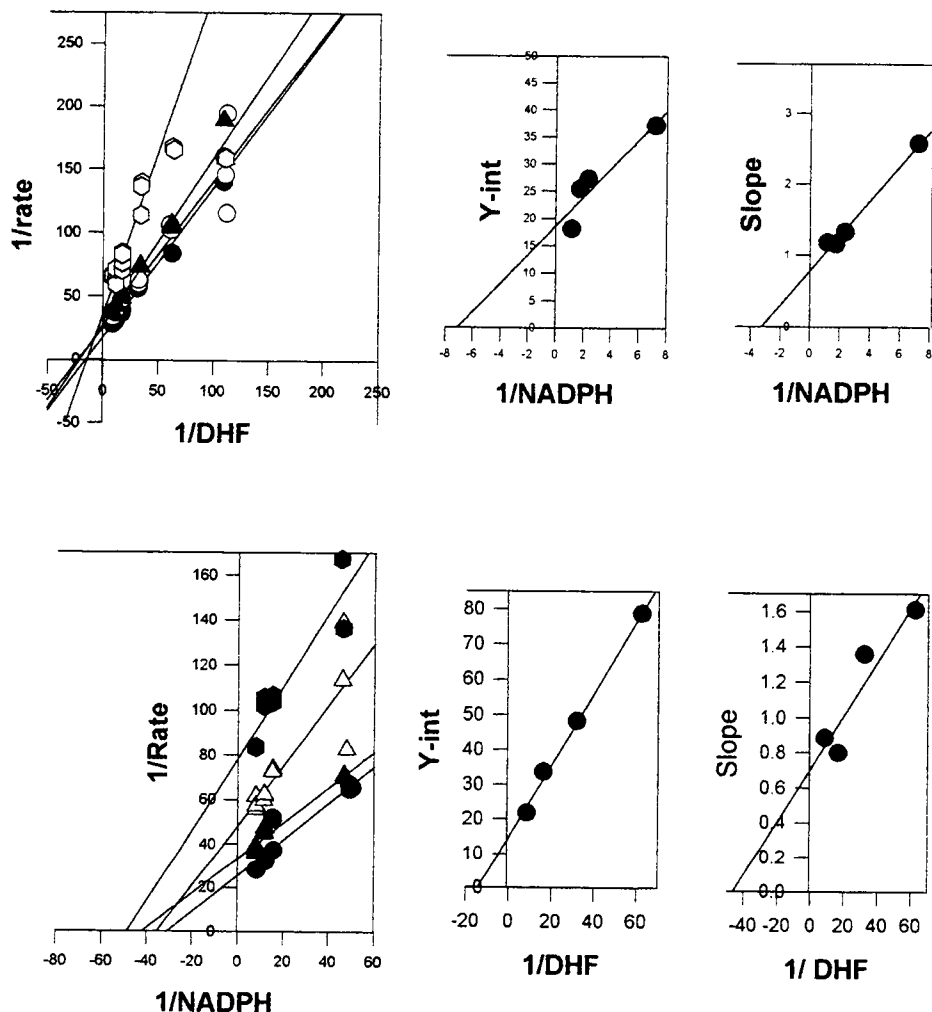


Figure 3-1: Reciprocal plots of the bisubstrate reaction for W38F R67 DHFR. (a) Primary plots (reciprocal initial velocity vs. $1/\text{variable substrate}$); (b) secondary plots (y-intercepts and slopes vs. $1/\text{fixed substrate}$). Also included is a brief set of definitions showing how the kinetic data were extracted.

Table 3-1 Kinetic Values at pH 7.0

<i>Enzyme</i>	$\epsilon_{280\text{nm}}$	$k_{\text{cat}} (\text{s}^{-1})$	$K_m \text{ DHF}(\mu\text{M})$	$K_m \text{ NADPH} (\mu\text{M})$
wtR67 DHFR*	1.82	1.3 ± 0.073	5.8 ± 0.015	3.0 ± 0.06
W45F DHFR	1.26	0.90 ± 0.04	7.6 ± 0.5	4.0 ± 0.41
W38F DHFR	0.89	0.013 ± 0.007	65 ± 9.5	141 ± 44

Note: Summary of kinetic values determined for wt, W45F and W38F R67 DHFRs. $\epsilon_{280\text{nm}}$ represents the extinction coefficient at 280nm with units of ml/mg.

* The kinetic values for wt R67 DHFR were reported previously by Reece et al., 1991.

unknown proteins. Figure 3-2, shows the standard curves with data points for wt R67 DHFR and the two single Trp mutants at both pH 5.0 and pH 8.0. Table 3-2 summarizes the apparent molecular weights that were determined for wt, W45F and W38F R67 DHFRs.

From Table 3-2, it can be seen that at pH 5.0, the two mutant R67 DHFRs have apparent molecular weights that are very similar to the value for the wild-type enzyme. At pH 5.0, wtR67 DHFR exists as a dimer, due to the protonation of the symmetry-related histidine 62s. At pH 8.0, the W45F mutant DHFR has an apparent molecular weight that is similar to that seen for the wild-type enzyme, which is tetrameric at this pH. However, at pH 8.0, the W38F R67 DHFR has an apparent molecular weight that is similar to that seen at pH 5.0. This suggests that the W38F mutant DHFR remains dimeric at this pH. Since the tryptophan 38 occurs at the dimer-dimer interface, it is not surprising that this mutation might disrupt the stability of the tetramer.

pH Dependence of the Oligomeric State

The oligomeric state of the mutant proteins was further characterized by investigating the pH dependent equilibrium between tetramer and two dimers for wtR67 and the two single tryptophan mutants. The intrinsic protein fluorescence was monitored as a function of pH. Figure 3-3(A), plots the change in fluorescence intensity as a function of pH. Figure 3-3(B), plots the change in fluorescence center of mass as a function of pH. Interpretation of Figure 3-3(A) is difficult due to the large pre- and post-transition slopes that are seen. However, the center of mass versus pH

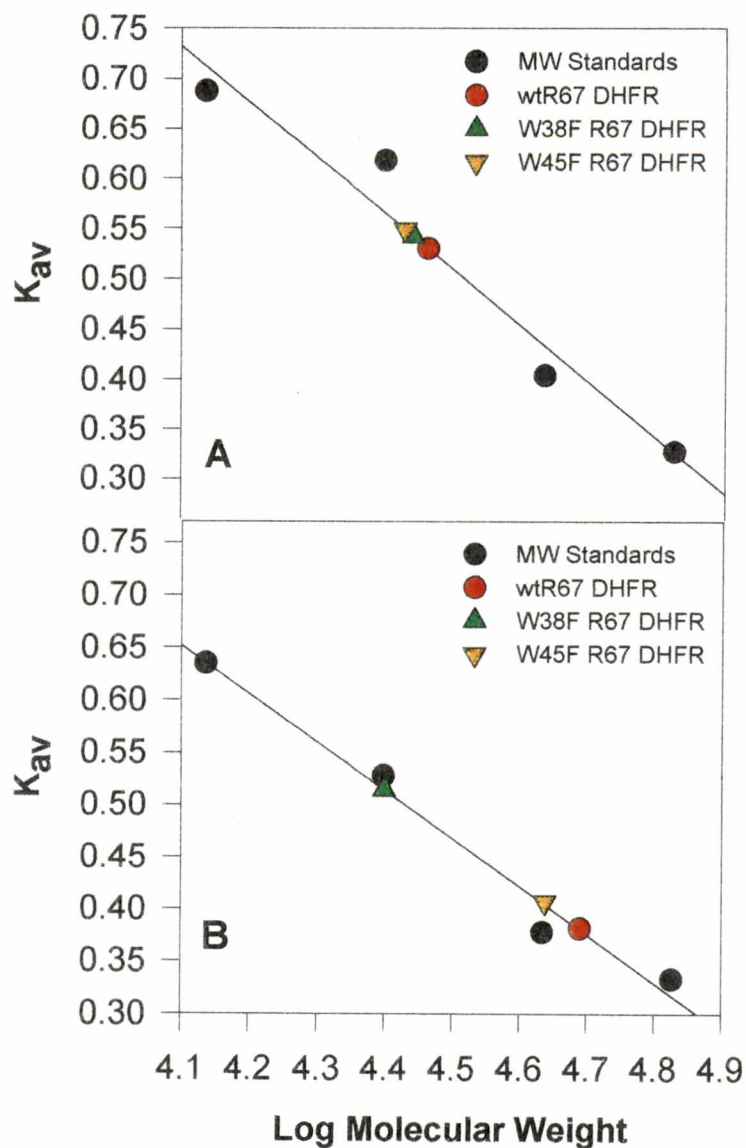


Figure 3-2: FPLC gel filtration standard curves. The elution positions for wtR67 DHFR, W38F DHFR and W45F DHFR are shown. Standard curves were generated using a Superose 12HR 10-30 column equilibrated in TE buffer at pH 5.0 (Panel A) or pH 8.0 (Panel B). Error bars are not shown, as they are smaller than the symbols used.

Table 3-2: Molecular Sieving Data pH 5.0 and 8.0

A (pH 5.0)	Retention Time (ml)*	K_{av}	Log MW	MW(Da)
Rnase	18.32	0.688	4.1367	13,700
Chymotrypsinogen	17.18	0.618	4.3979	25,000
Ovalbumin	13.7	0.404	4.6335	43,000
BSA	12.5	0.328	4.8261	67,000
Blue-Dextran	7.12	0	6.3010	2,000,000
WtR67 DHFR	16.1	0.549	4.4272	26,700±70
W38F R67 DHFR	15.8	0.530	4.4613	28,900±150
W45F R67 DHFR	16.0	0.543	4.4393	27,500±700

B (pH 8.0)	Retention Time(ml)*	K_{av}	Log MW	MW
Rnase	17.6	0.635	4.1367	13,700
Chymotrypsinogen	15.9	0.528	4.3979	25,000
Ovalbumin	13.6	0.378	4.6335	43,000
BSA	12.9	0.333	4.8261	67,000
Blue-Dextran	7.6	0	6.3010	2,000,000
WtR67 DHFR	13.63	0.382	4.670	49,000±650
W38F R67 DHFR	15.73	0.5145	4.399	25,000±640
W45F R67 DHFR	13.98	0.4073	4.637	43,500±1400

Note: Molecular sieving data used to estimate the molecular weight of wtR67 DHFR and the two single tryptophan mutants W38F and W45F. Standard curves were generated using a Superose 12HR 10-30 column equilibrated in TE buffer pH 5.0 or 8.0. (Panel A) shows the data at pH 5.0 and (panel B) shows the data at pH 8.0

* All points were reproduced minimally in duplicate and the average value is presented. Error values were calculated only for the molecular weight (MW) of unknowns.

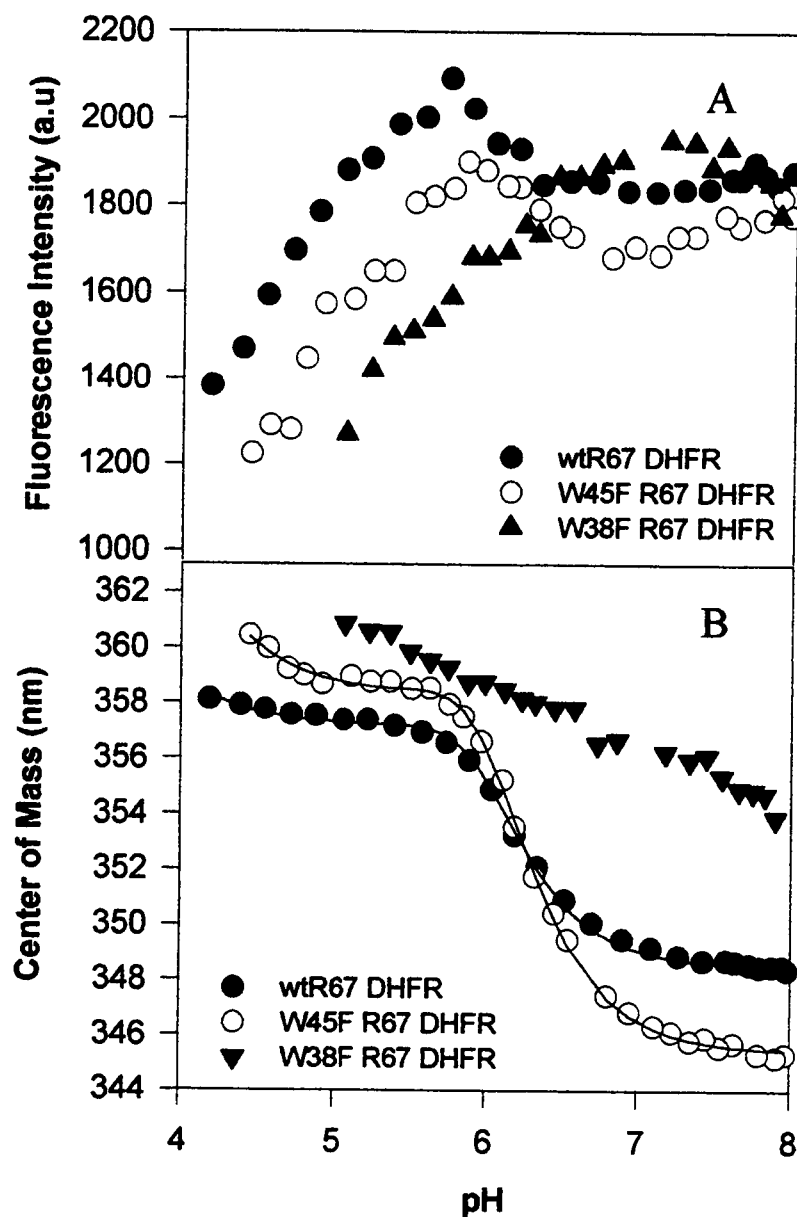


Figure 3-3: pH titration data for wt, W45F and W38F R67 DHFR. Data are presented in terms of the change in fluorescence intensity at 340nm and in terms of the fluorescence center of mass.

plots yield a single clear transition for both the wt and W45F mutant DHFRs. The W38F mutant R67 DHFR plot yields no transition and shows only a linear decrease from 361 to 354nm, consistent with it remaining dimeric from pH 5.0 to 8.0.

The experimental data were fit to Equation 4, using a nonlinear least-squares fitting program (NLIN) in the SAS package. The fits were repeated using the following values of $(2n) = 2, 3$ and 4, which correspond to the possible number of protons acquired by the symmetry related His 62 residues at the dimer-dimer interface. The best fit to the experimental data resulted when a value of $(2n = 3)$ was used for the center of mass data as evaluated by a comparison of chi-squared values. SAS was unable to fit the fluorescence intensity as a function of pH data with a reasonable error. The best-fit lines for the center of mass data are plotted in Figure 3-3(B) and the resulting parameters are summarized in Table 3-3.

As can be seen in Table 3-3, the pH titration data for the wt and W45F R67 DHFRs yielded K_{overall} values that are within experimental error. This suggests the W45F mutation does not effect the dependency of the oligomeric state on the pH, consistent with its position in the structure distant from the dimer-dimer interface.

Fluorescence Spectra and relative yields, pH 5.0 and 8.0

The effects of the W45F and W38F single-tryptophan mutations on the total protein fluorescence in R67 DHFR were determined by fluorescence spectroscopy (excitation 295nm). Steady-state emission spectra (300nm to 450nm) were obtained in the absence and presence of 4M GdnHCl for wt, W45F and W38F R67 DHFRs, all at 4 μ M concentration. Data were collected in MTA buffer at either pH 5.0 or 8.0 at

Table 3-3**Comparison of pH titration parameters**

	Koverall (M)	LIM1 (nm)	LIM2 (nm)	SLOPE B	SLOPE A
WtR67 DHFR	1.42 (± 0.85) E-13	357.1 ± 0.1	348.5 ± 0.1	18.3 ± 3.9	2885 ± 1828
W45F R67 DHFR	1.50 (± 0.5) E-13	358.2 ± 0.2	345.4 ± 0.1	62.5 ± 7.4	6648 ± 1389

Note: Summary of the fitting parameters resulting from the nonlinear least-squares fit of the pH titration data for wt and W45F R67 DHFRs, to Equation 4 using SAS. Data were collected at room temperature in MTA buffer. A protein concentration of 2 μ M was utilized. The samples were individually titrated with small aliquots of 2M HCl.

room temperature. Figures 3-4 and 3-5, show these data plotted as fluorescence intensity versus wavelength. Both mutations show a decrease in fluorescence intensity, consistent with the loss of a tryptophan from the structure. The effect on intensity is more dramatic in the W38F mutant, suggesting tryptophan 38 contributes more to the total fluorescence signal than tryptophan 45. It should be recalled that the W45F mutant R67 DHFR contains only Trp 38, while the W38F mutant R67 DHFR contains only Trp 45.

In Figure 3-4(A), it can be seen that the W45F mutant R67 DHFR has a fluorescence scan that has approximately four times the intensity of the W38F mutant R67 DHFR at pH 5.0, 0M GdnHCl. At pH 5.0 in the absence of denaturant, not only is the W38F R67 DHFR scan of much lower intensity, it is also slightly blue shifted as compared to either the wild-type or the W45F R67 DHFRs scans. This result is consistent with the sequestering of Trp 45 at the monomer-monomer interface. The scans for W45F and W38F R67 DHFRs are added to yield the curve shown in black. It can be seen that this closely approximates the wild-type scan, suggesting that the effects are additive. Figure 3-4(B), shows the pH scans at pH 5.0 in the presence of 4M GdnHCl, which results in complete unfolding. In the presence of 4M GdnHCl, it can be seen that the three proteins show an overall decrease in fluorescence intensity and have similar red-shifts, indicating that the tryptophan residues are in similar environments (*i.e.* exposed equally to solvent). The addition of the fluorescence scans for W45F and W38F R67 DHFRs in 4M GdnHCl do not approximate the wild-type scan.

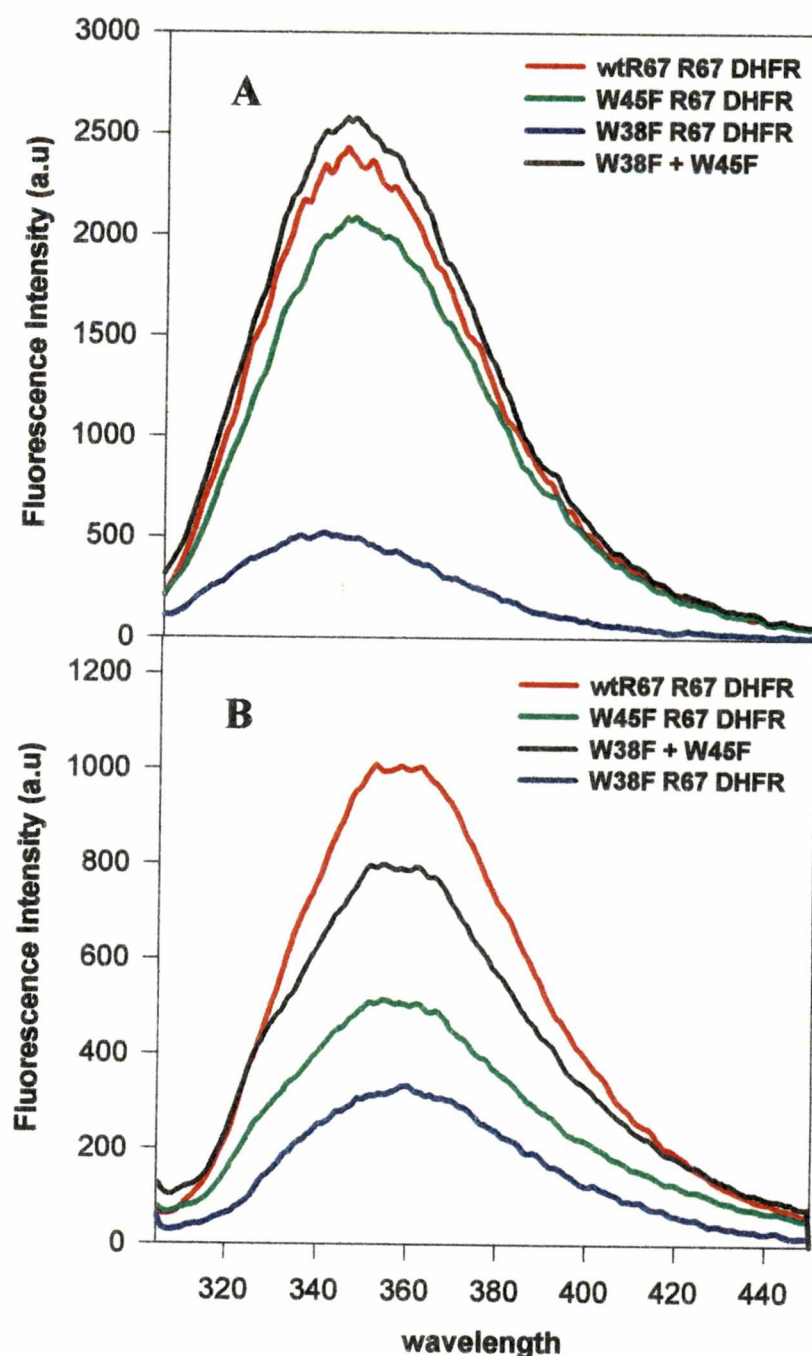


Figure 3-4: Fluorescence emission scans, pH 5.0. Panel (A), shows the fluorescence intensity scans for wt, W45F and W38F R67 DHFRs all at 4 μ M concentration in MTA buffer, pH 5.0. Panel (B) shows the fluorescence intensity scans for the same three proteins in MTA buffer, pH 5.0 and 4M GdnHCl. The curves for the W45F mutant and the W38F mutant were added in SIGMA PLOT to yield the black plot.

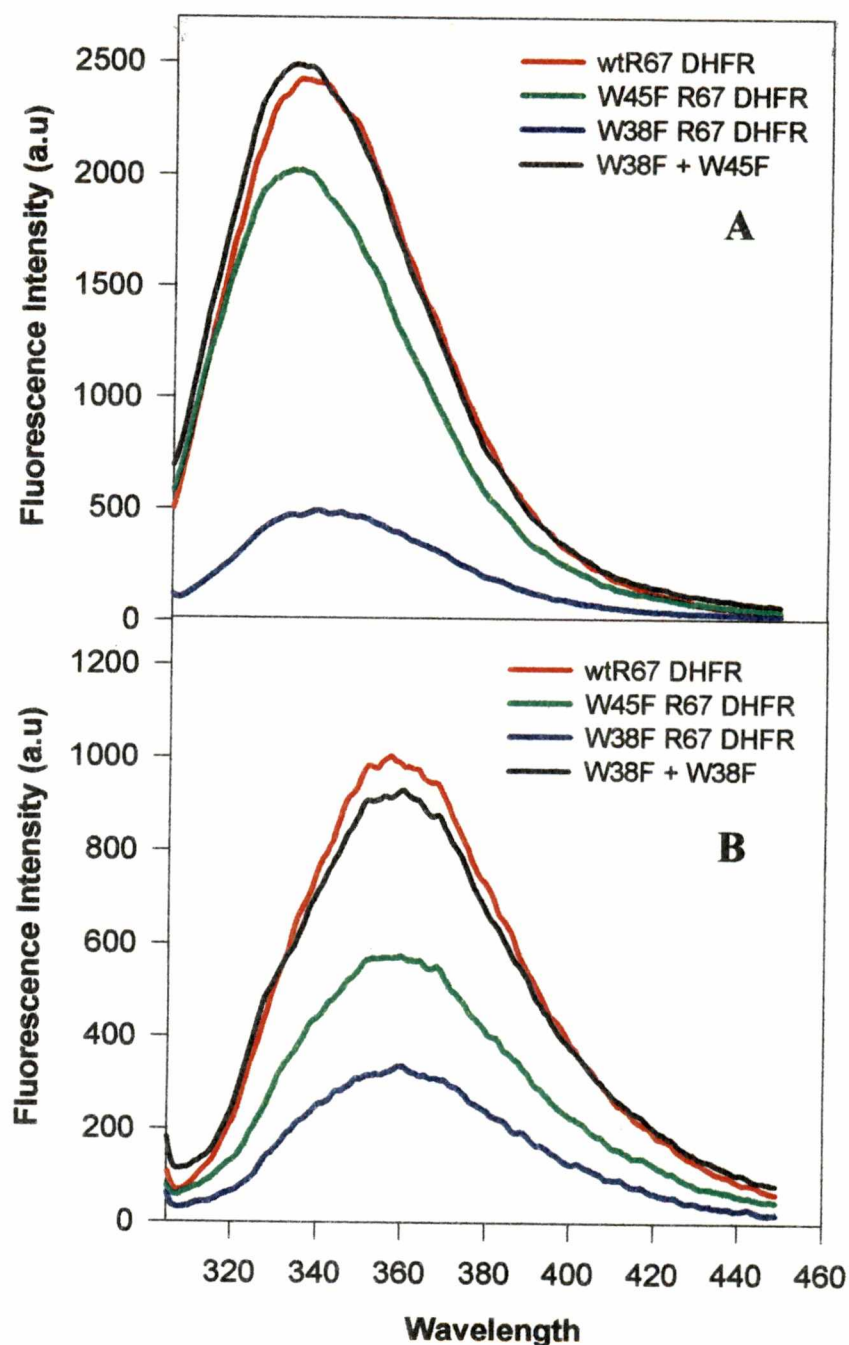


Figure 3-5: Fluorescence emission scans, pH 8.0. Panel (A), shows the fluorescence intensity scans for wt, W45F and W38F R67 DHFRs all at 4 μ M concentration in MTA buffer, pH 8.0. Panel (B) shows the fluorescence intensity scans for the same three proteins in MTA buffer, pH 8.0 and 4M GdnHCl. The curves for the W45F mutant and the W38F mutant were added in SIGMA PLOT to yield the black plot.

The changes seen in the fluorescence spectrum as the proteins are denatured in 4M GdnHCl result from the exposure of the indole ring of tryptophan to solvent. The fluorescence emission spectra of many fluorophores are sensitive to the polarity of their surrounding environment. For example, if the emission spectrum of tryptophan is examined in solvents of differing polarity, it is seen that the emission spectrum shifts to shorter wavelengths (blue shifts) as the solvent polarity is decreased. Conversely, increasing solvent polarity generally results in a shift to longer wavelengths (red shifts). Red shifts are often, but not always, accompanied by a decrease in the quantum yield of the fluorophore (Lakowicz, 1986).

In Figure 3-5(A), it is seen that at pH 8.0 in the absence of denaturant, the fluorescence intensity scan for W45F R67 DHFR is of slightly lower intensity than that seen for wtR67. In addition, the W38F R67 DHFR seems to have a much lower quantum yield than the wild type protein. At pH 5.0, the W45F mutant is seen to have a slight blue shift as compared to the wild type and the W38F mutant R67 DHFR, consistent with the sequestering of Trp 38 at the dimer-dimer interface. The W45F and W38F mutant R67 DHFR scans were added to generate the black curve, which again closely approximates that seen for the wild type.

Figure 3-5(B), shows the fluorescence scans at pH 8.0 in the presence of 4M GdnHCl for wt, W45F and W38F R67 DHFRs. Under these conditions, the scans are similar to what is seen in the absence of GdnHCl, except that the total intensities are lower. In the presence of 4M GdnHCl, it can be seen that the three proteins have similar red shifts, indicating that the tryptophan residues are also in similar

environments at pH 8.0 (i.e. exposed equally to solvent). The W45F and W38F R67 DHFR scans also appear to be additive under these conditions.

Circular Dichroism Scans, pH 8.0

In order to assess the effects of the W45F and W38F mutations on the secondary structure of R67 DHFR, CD spectra were recorded at 22° C in 10 mM phosphate buffer, pH 8.0 for 10μM wt, W45F and W38F R67 DHFR. The CD data are presented in Figure 3-6, as the molar ellipticity versus wavelength. In Figure 3-6, striking differences in the CD scans of the three proteins are seen. The wild type and W38F R67 DHFRs both show strong signals in the far-UV (below 230nm). The CD spectrum of the W38F mutant R67 DHFR is somewhat shifted to shorter wavelengths and has a strong peak at ~201nm. This region of the CD spectra is sensitive to the backbone conformational states. Surprisingly, the W45F mutant R67 DHFR has almost no CD signal, although the protein is folded and fully active as shown by the accompanying kinetic and fluorescence studies. Loss of CD signal is normally associated with the loss of secondary structure.

Equilibrium Unfolding Studies

To investigate the effects of these mutations on the structural stability of R67 DHFR, equilibrium unfolding studies were performed at increasing concentrations of GdnHCl. Tryptophans were excited at 295nm and the emission spectra were measured from 300 to 500nm at increasing concentrations of denaturant. All measurements were made at room temperature in MTA buffer, pH 5.0 without

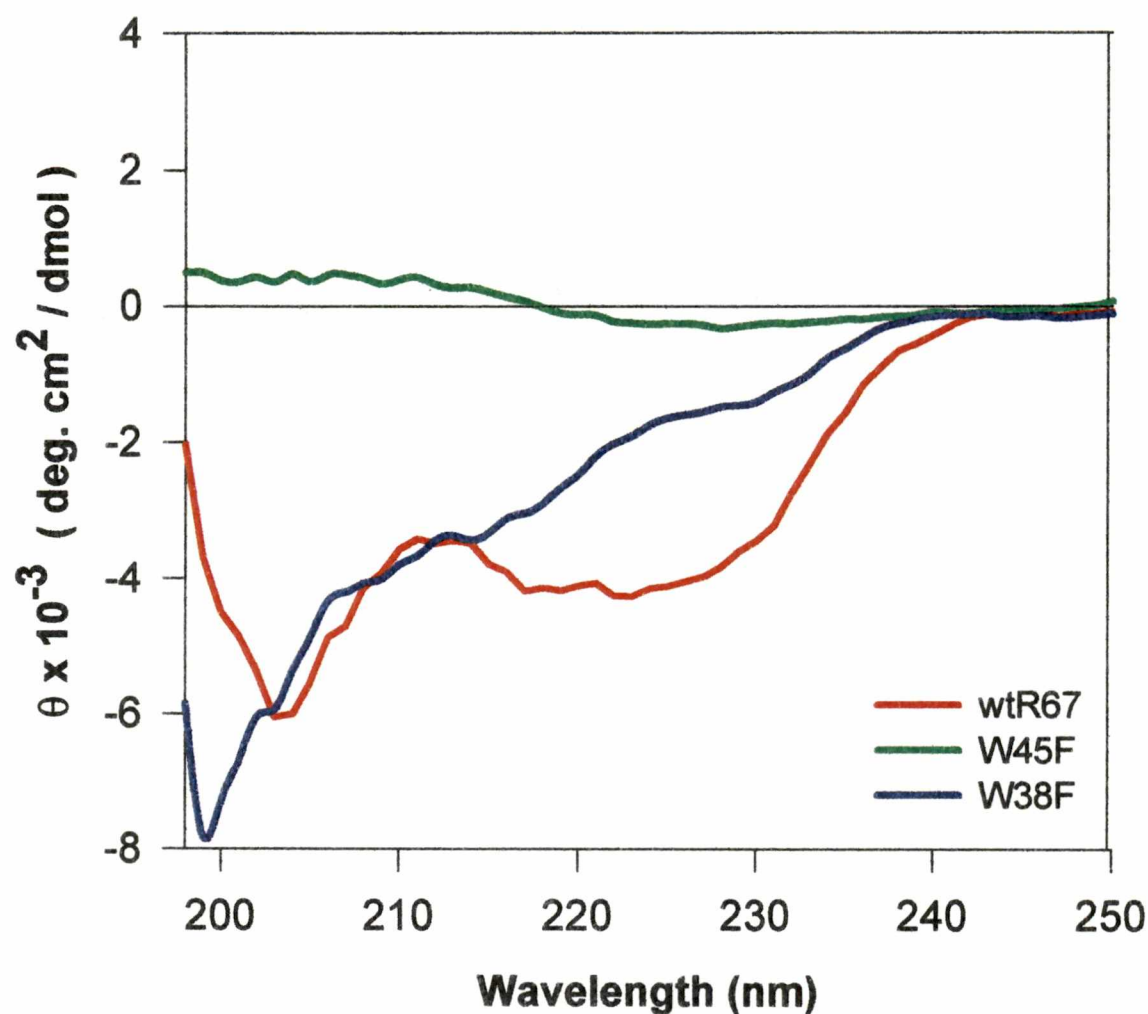


Figure 3-6: CD scans for wt, W45F and W38F R67 DHFR. Scans were conducted in 10mM phosphate buffer, pH 8.0. The data are presented in terms of the mean residue ellipticity by taking 108g/mole as the mean residue molecular weight. θ is the molar ellipticity expressed in degrees $\text{cm}^2/\text{decimole}$. The data below 197nm are not presented due to noise.

β -mercaptoethanol. The equilibrium unfolding of both tryptophan mutants were monitored using various protein concentrations, in order to verify the protein concentration dependence of the unfolding reaction. The unfolding transitions were monitored by both the loss of fluorescence intensity at 340nm and the fluorescence center of mass. Thermodynamic values were obtained by fitting this data to Equation 6, as described in the materials and methods section.

W45F R67 DHFR

Figure 3-7(A) shows the equilibrium unfolding transitions of W45F R67 DHFR at [2 μ M], [5 μ M] and [20 μ M] monitored by following the change in fluorescence intensity at 340nm. Data were obtained at pH 5.0 and fit to a two-state unfolding model (Eq 2). The best-fit lines for each plot are shown. Figure 3-7(B), shows the conversion to F_{app} for easy comparison. In these plots, a clear dependence on the concentration of protein is seen. As the protein concentration is increased, it shifts the equilibrium such that it requires a higher concentration of denaturant to unfold the protein, consistent with the increase in the concentration of dimer.

Figure 3-8(A), shows the equilibrium unfolding transitions of W45F R67 DHFR at [2 μ M], [5 μ M] and [20 μ M] monitored by following the change in fluorescence center of mass. Data were obtained at pH 5.0 and fit to a two-state unfolding model (Eq 2). The best-fit lines for each plot are shown. Figure 3-8(B), shows the conversion to F_{app} for easy comparison. The fluorescence center of mass is often used as it is an integral measurement and as such is less prone to noise. In this plot, we also see the concentration dependence of the unfolding reaction.

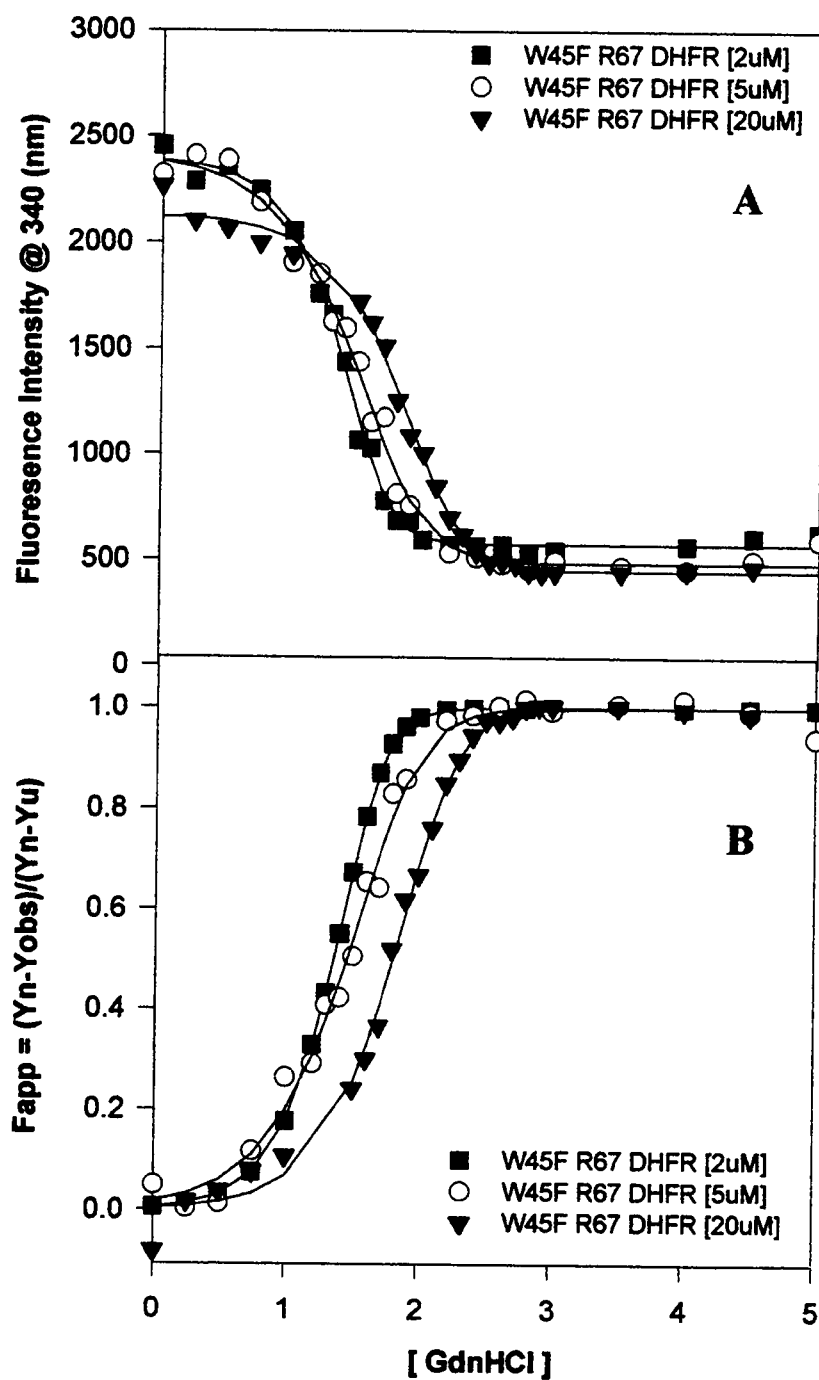


Figure 3-7. Equilibrium unfolding of W45F R67 DHF followed by the change in intensity at 340nm. [2 μ M], [5 μ M] and [20 μ M] were utilized. Unfolding transitions were monitored by the loss of fluorescence intensity at 340nm Panel (A). Data were obtained at pH 5.0 and fit to a two-state model and converted to F_{app} for easy comparison in Panel (B).

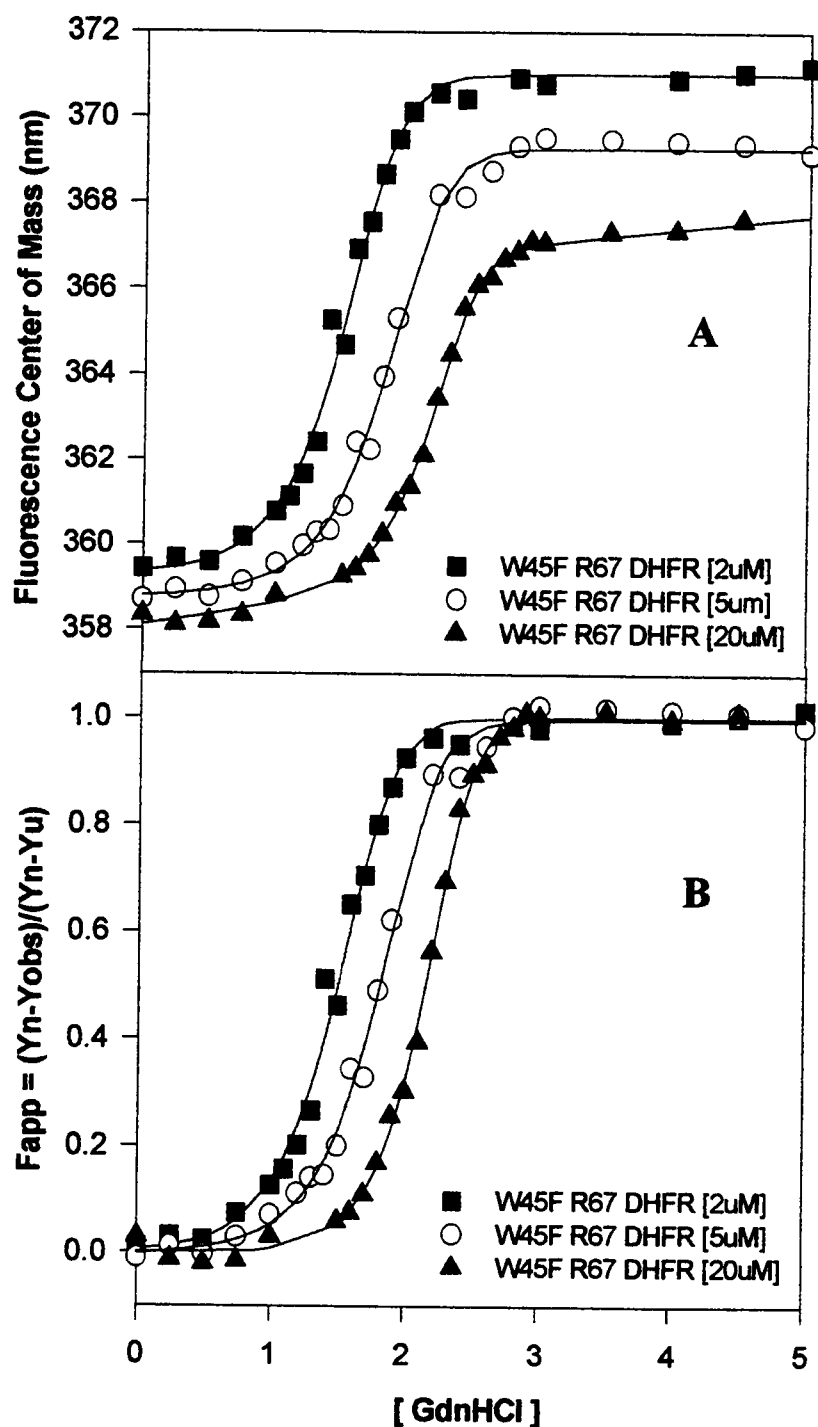


Figure 3-8. Equilibrium unfolding of W45F R67 DHFR followed by the change in center of mass. [2μM], [5μM] and [20μM] were utilized. Unfolding curves monitored by fluorescence center of mass are shown in Panel (A). Data were obtained at pH 5.0 and fit to a two-state model and converted to F_{app} for easy comparison in Panel (B).

W38F R67 DHFR

Figure 3-9(A), shows the equilibrium unfolding transitions of W38F R67 DHFR at [8 μ M] and [12 μ M] monitored by following the change in fluorescence intensity at 340nm. Data were obtained at pH 5.0 and fit to a two-state unfolding model (Eq 2). The best-fit lines for each plot are shown. Figure 3-10(B), shows the conversion to F_{app} for easy comparison. Figure 3-10(A), shows the equilibrium unfolding transitions of W38F R67 DHFR at [8 μ M] and [12 μ M] monitored by following the change in fluorescence center of mass. Data were obtained at pH 5.0 and fit to a two-state unfolding model (Eq 2). The best-fit lines for each plot are shown. Figure 3-10(B), shows the conversion to F_{app} for easy comparison. In both of these plots, the unfolding transition is seen to be dependent on the protein concentration.

Tables 3-4 and 3-5, summarize the values derived from fitting the equilibrium unfolding transitions followed by the change in fluorescence intensity and fluorescence center of mass, respectively. The data were fit to Equation 6 independently as previously described, to yield the free energy change between the native and unfolded conformational states of the three proteins (ΔG_{H2O}) in the absence of denaturant. As can be seen, the ΔG_{H2O} values determined from the change in fluorescence intensity at 340nm as a function of GdnHCl concentration are in the range of 12 to 14 Kcal/mol. ΔG_{H2O} values determined from the change in fluorescence center of mass as a function of GdnHCl concentration are also in the range of -12 to -14 Kcal/mol, but give slightly higher estimates of the ΔG_{H2O} values than those seen for the fits to the intensity at 340nm data. The ΔG_{H2O} values

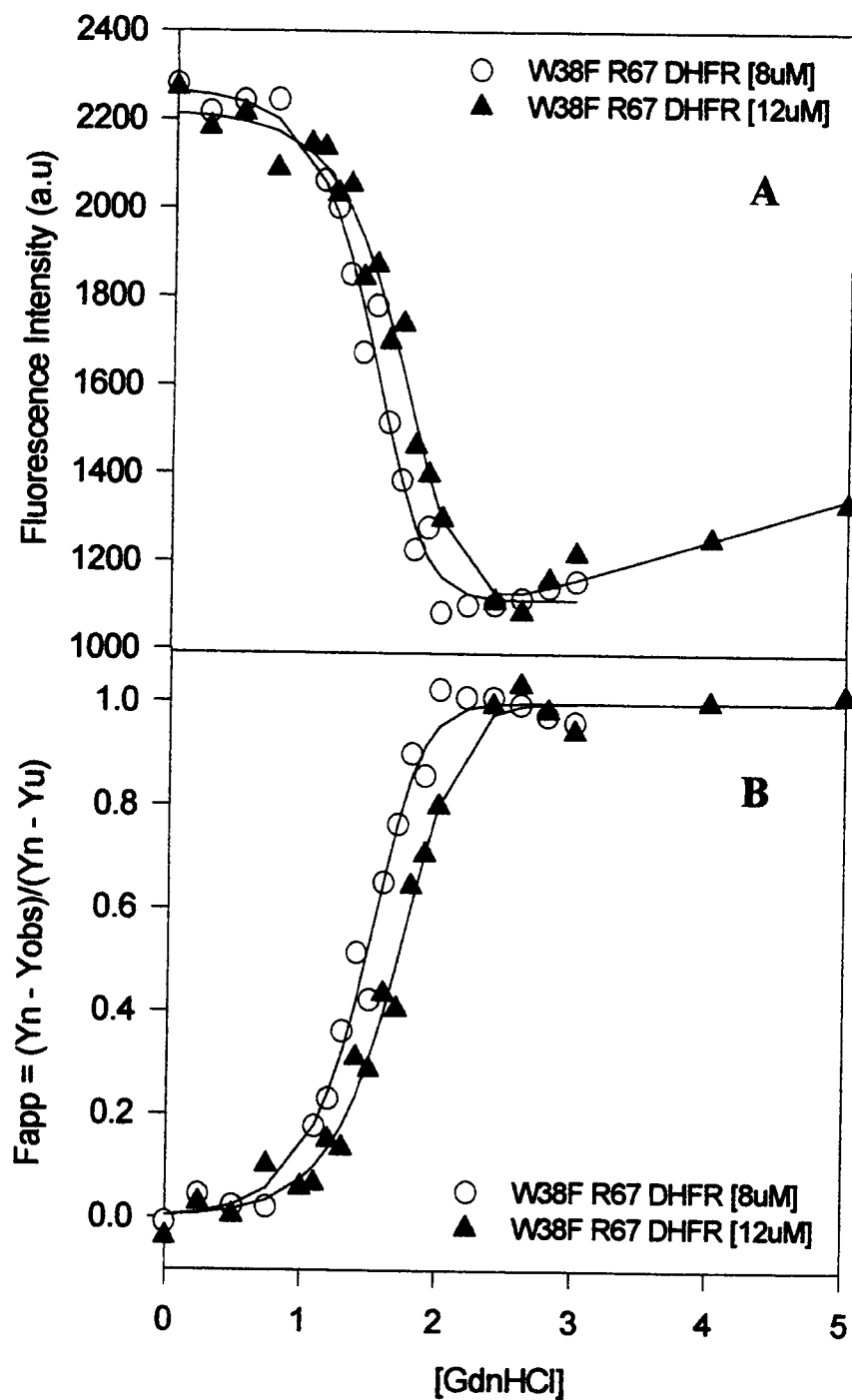


Figure 3-9: Equilibrium unfolding of W38F R67 DHFR followed by the change in intensity at 340nm. Data in (panel A) were obtained at pH 5.0 and fit to a two-state model. (Panel B) shows the conversion to F_{app} for easy comparison.

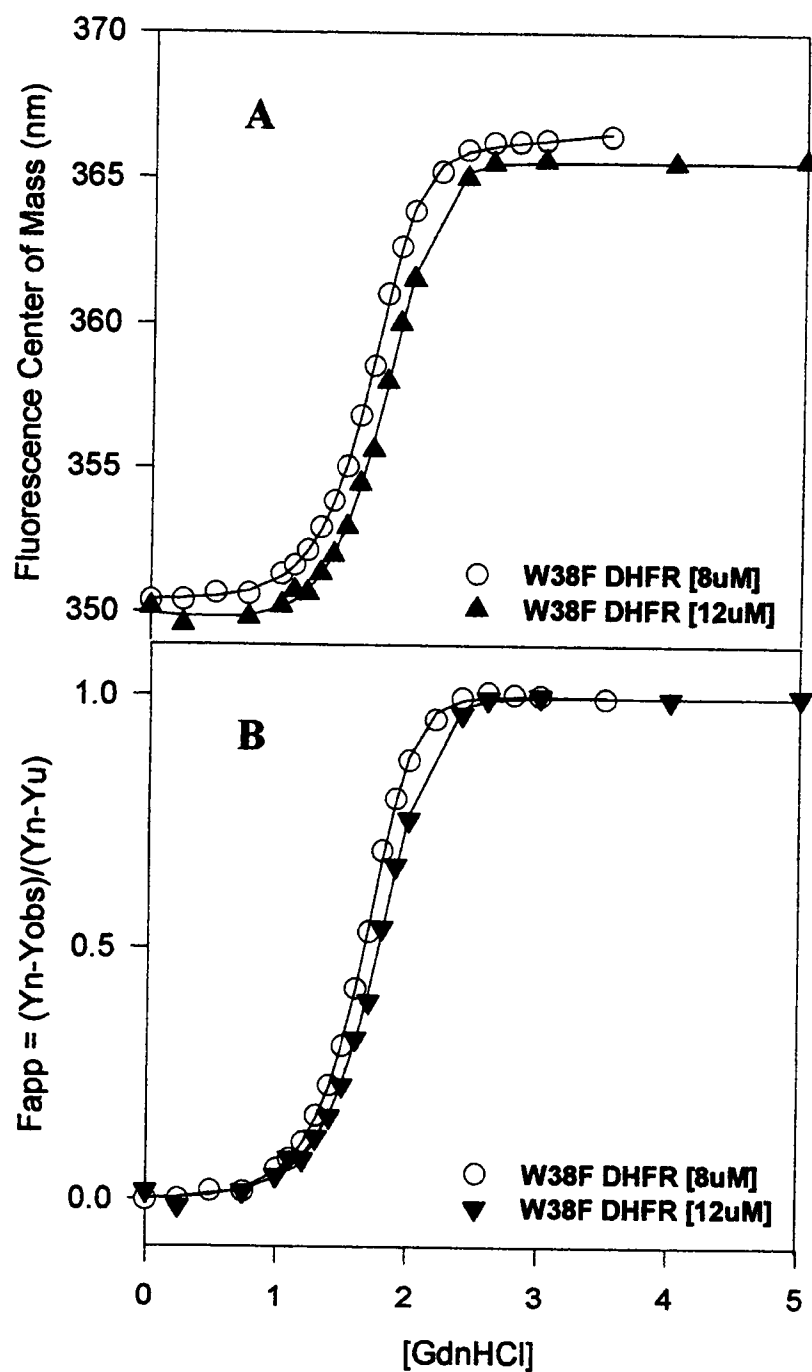


Figure 3-10: Equilibrium unfolding transitions of W38F R67 DHFR followed by the change in center of mass. Data in (panel A) were obtained at pH 5.0 and fit to a two-state model. (Panel B) shows the conversion to F_{app} for easy comparison.

Table 3-4: Fitting Parameters from Intensity at 340nm vs. [GdnHCl] plots

Condition	YF (a.u)	YU (a.u)	ΔG_{H2O} (Kcal/mol)	M_G (Kcal/mol/M)
WtR67 (4 μ M)	2451 $\pm$ 28	802 $\pm$ 13	14.4 $\pm$ 0.4	-3.2 $\pm$ 0.2
W45F (2 μ M)	2393 $\pm$ 30.5	573 $\pm$ 14.9	13.6 $\pm$ 0.4	-4.3 $\pm$ 0.3
W45F (5 μ M)	2274 $\pm$ 44.9	486 $\pm$ 23	12.2 $\pm$ 0.5	-3.3 $\pm$ 0.3
W45F (20 μ M)	2199 $\pm$ 28.1	457 $\pm$ 13	14.4 $\pm$ 0.8	-4.2 $\pm$ 0.4
W38F (8 μ M)	2268 $\pm$ 35.4	1113 $\pm$ 26.0	13.0 $\pm$ 0.8	-4.1 $\pm$ 0.5
W38F (12 μ M)	2218 $\pm$ 31.0	878 $\pm$ 89	13.1 $\pm$ 0.8	-3.7 $\pm$ 0.4

Note: Comparison of the thermodynamic fitting parameters for wtR67 DHFR, W38F DHFR and W45F DHFR at various protein concentrations, taken from the fluorescence intensity at 340nm vs. [GdnHCl] plots at pH 5. These are single fits to each curve.

Table: 3-5: Comparison of Fitting Parameters from Center of Mass Curves

Condition	YF (nm)	YU (nm)	ΔG_{H2O} (Kcal/mol)	M_G (Kcal/mol/M)
WtR67 (4 μ M)	357.8 $\pm$ 0.12	369.1 $\pm$ 0.8	15.6 $\pm$ 0.3	-5.0 $\pm$ 0.23
W45F (2 μ M)	359.3 $\pm$ 0.3	371 $\pm$ 0.2	13.6 $\pm$ 0.5	-3.8 $\pm$ 0.3
W45F (5 μ M)	358.8 $\pm$ 0.2	369.3 $\pm$ 0.1	14.0 $\pm$ 0.5	-3.7 $\pm$ 0.3
W45F (20 μ M)	358.1 $\pm$ 0.1	366.1 $\pm$ 0.2	14.7 $\pm$ 0.4	-4.5 $\pm$ 0.2
W38F (8 μ M)	350.4 $\pm$ 0.1	365.0 $\pm$ 0.4	14.5 $\pm$ 0.2	-4.5 $\pm$ 0.1
W38F (12 μ M)	349.9 $\pm$ 0.2	365.6 $\pm$ 0.1	14.6 $\pm$ 0.4	-4.2 $\pm$ 0.2

Note: Comparison of the thermodynamic fitting parameters for wtR67 DHFR, W38F DHFR and W45F DHFR at various protein concentrations, taken from the fluorescence Center of mass vs. [GdnHCl] plots at pH 5. These are single fits to each curve.

determined from the change in fluorescence intensity at 340nm data show more variation than do those for the center of mass data. This most likely results from the intensity measurements being more prone to instrumental noise than fluorescence center of mass data, which is an integral measurement. The center of mass data however, can often yield a erroneously high estimate of the ΔG_{H2O} value, according o (Eftink, 1994). For this reason, more weight will be given to the ΔG_{H2O} values determined from the change in fluorescence intensity data.

Global Data Analysis

The equilibrium unfolding data for wt and mutant DHFRs were fit globally as discussed in the materials and methods. An advantage of this approach is that data obtained at different protein concentrations can be fit simultaneously to a two-state model to obtain one pair of ΔG_{H2O} and M_G values, which should have a lower error, due to the increase in the number of observations. The values obtained from fitting the equilibrium unfolding data individually to the two-state equation were used as initial guesses for the global fits. The equilibrium unfolding data followed by both fluorescence intensity and center of mass were fit separately to the global equation.

Figure 3-11 (A), shows the global fit plots for the equilibrium unfolding transitions of the W45F mutant R67 DHFR followed by the change in the fluorescence center of mass. The best fit lines are also shown here. These plots show fits that are similar to those seen for the individual fits to the two-state model. Similarly, Figure 3-12 shows the global analysis of the equilibrium unfolding data for the W38F mutant R67 DHFR in terms of the intensity at 340nm and the center of

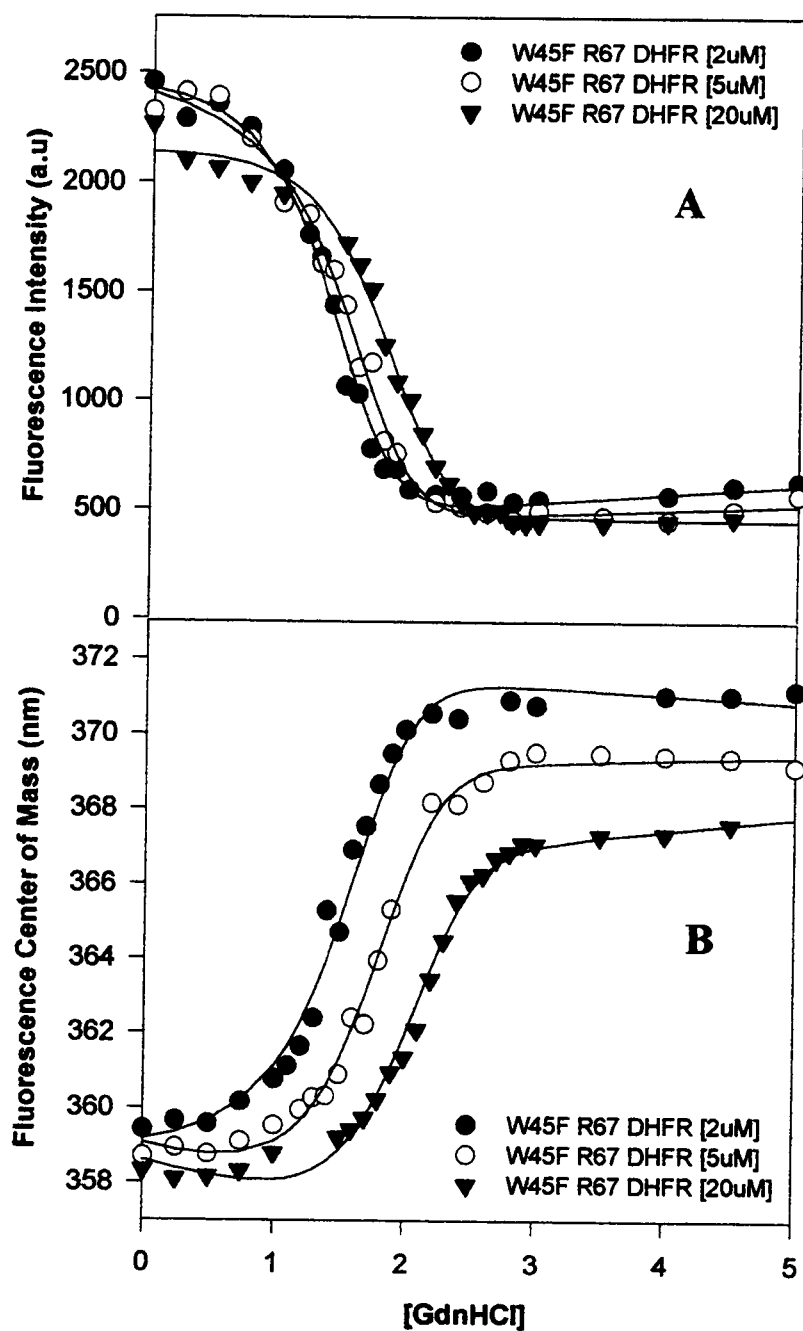


Figure 3-11: Global fits of the equilibrium unfolding transitions for W45F R67 DHFR. As monitored by fluorescence intensity (panel A) and center of mass (Panel B). Data in this plot were obtained at pH 5.0 and fit simultaneously to a two-state model.

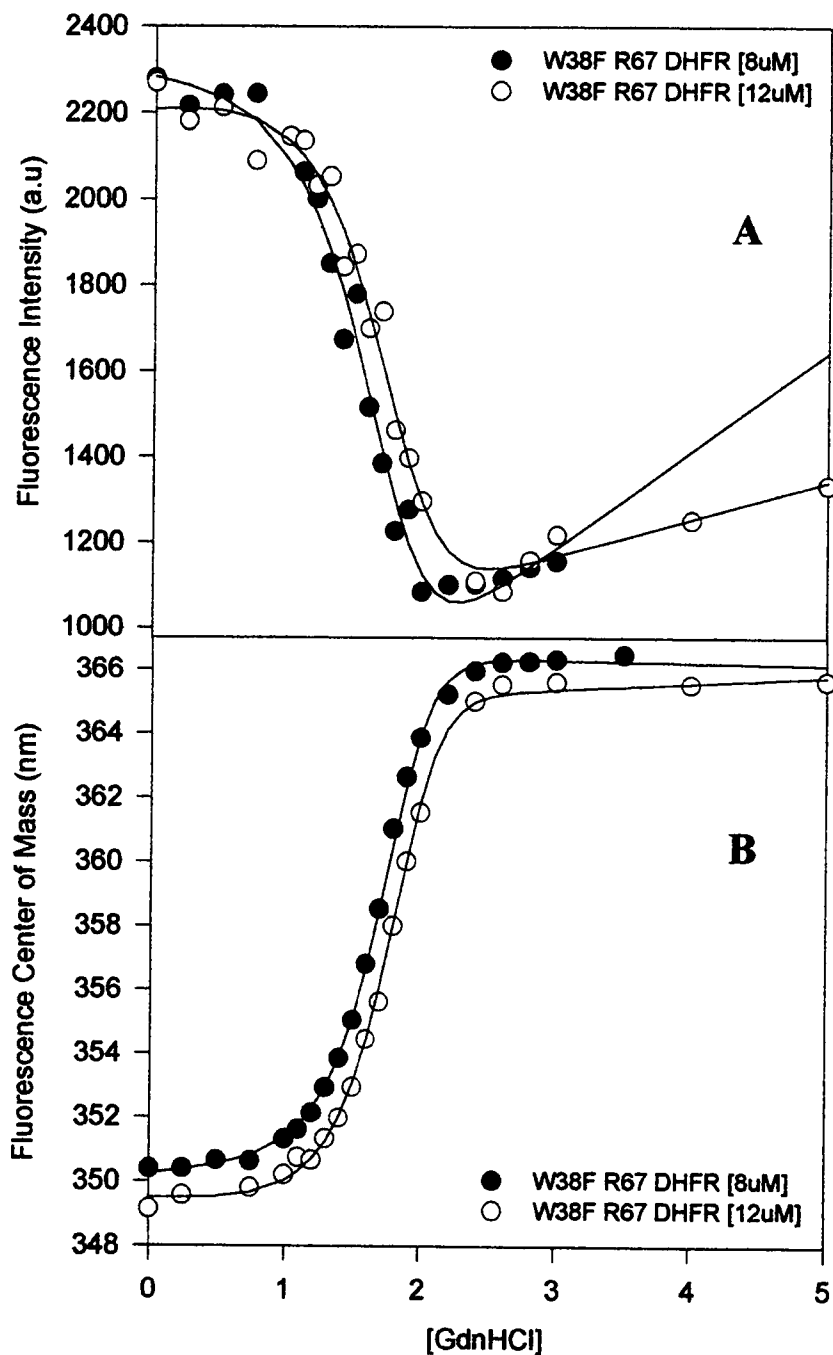


Figure 3-12: Global fits of the equilibrium unfolding transitions for W38F DHFR. As monitored by fluorescence intensity (panel A) and fluorescence center of mass. (Panel B). Data were simultaneously fit to a two-state model.

mass. The ΔG_{H2O} values determined for the W45F and W38F mutant R67 DHFRs by the global fits are shown in Table 3-6.

Table 3-6 summarizes the ΔG_{H2O} values determined by global analysis of the equilibrium unfolding data sets for wt, W45F and W38F R67 DHFRs to a two-state unfolding model. Values for the fluorescence intensity at 340nm and the center of mass are compared for each protein. The equilibrium unfolding data for wt R67 DHFR followed by the change in fluorescence intensity at 340nm were taken from the work of (Reece *et al*, 1991.) and fit globally. Unfortunately, the center of mass data were not available for the wtR67 DHFR. The ΔG_{H2O} values are all within error of for each other for the fluorescence intensity data. The center of mass data lack a wtR67 DHFR comparison value, also a comparison of the of the ΔG_{H2O} values for the W38F mutant obtained by the intensity and center of mass are different, consistent with Eftink's proposal of skewing of the ΔG_{H2O} values toward the more intensely emitting species.

Table 3-6: Summary of Global fit Values

Fluorescence Intensity at 340nm

	ΔG_{H2O} , Intensity at 340nm (Kcal mol ⁻¹)	M_G , Intensity at 340nm (Kcal/mol/M)
WtR67 DHFR*	13.2 ± 0.4	-3.5 ± 0.2
W45F R67 DHFR	12.4 ± 0.5	-3.4 ± 0.3
W38F R67 DHFR	12.6 ± 1.2	-3.6 ± 0.7

Fluorescence Center of Mass

	ΔG_{H2O} , Center of Mass (Kcal mol ⁻¹)	M_G , Center of Mass (Kcal/mol/M)
WtR67 DHFR	-----	-----
W45F R67 DHFR	12.6 ± 0.6	--3.1 ± 0.3
W38F R67 DHFR	14.4 ± 0.4	-4.4 ± 0.2

Note: Comparison of the thermodynamic fitting parameters for wt, W45F and W38F R67 DHFR. Data were fit simultaneously to a two-state unfolding model.

* Data for R67 DHFR (1.2 and 12μM) were from Reece et al 1991.

CHAPTER 4

DISCUSSION

Fluorescence Spectroscopy

Fluorescence techniques provide sensitive and potentially informative methods to monitor both equilibrium and kinetic properties of protein folding reactions. The fluorescence emission spectrum of tryptophan is highly sensitive to the polarity of its environment. The emission spectrum of tryptophan shifts to shorter wavelengths (blue shifts) as the polarity of its environment decreases. Conversely, as the environment becomes polar, the emission spectrum shifts to longer wavelengths (red shift). Therefore, the intensity and emission maximum can differ based on the degree of exposure to solvent (Lakowicz, 1986).

Due to the hydrophobic character of tryptophan, it is often sequestered in the interior in native protein conformations. The exposure of these residues to the polar environment of solvent during global unfolding reactions often leads to observable changes in emission properties. Analysis of these changes can yield kinetic and thermodynamic information about the unfolding transition taking place. Unfortunately, it is often difficult to unambiguously assign these changes to a specific fluorophore in multi-fluorophore containing proteins, which is necessary to make structural interpretations. To overcome this problem, two single tryptophan mutants were constructed in R67 DHFR. As with any mutational study, care must be taken to fully characterize its effect on the global conformation of the protein to minimize the possibility of misinterpretation of the data.

Kinetic Comparison

Table 3-1 summarizes the kinetic values determined for wt, W45F and W38F R67 DHFRs. It is seen that the W45F mutant has K_m values that are similar to those of the wild-type enzyme. The k_{cat} value determined for the W45F mutant R67 DHFR is slightly smaller than that of the wild-type, but not significantly so. This suggests that this mutation is not having a significant effect on the global conformation of the enzyme.

The W38F mutant R67 DHFR was shown to be a very poor enzyme with K_m values that are much higher than those seen for either the wt or W45F R67 DHFRs. The K_m (DHF) is approximately ten times larger, while the K_m (NADPH) is nearly 50 times that of the wild-type. The k_{cat} value determined for the W38F mutant R67 DHFR was 100 times smaller than that seen for the wild-type. These values were not unexpected as it was observed that the gene carrying this mutant provided much less resistance to TMP upon host *E. coli*. This data suggests that the W38F mutation causes a major change in the structure of R67 DHFR, resulting in a loss of activity. It is seen from figure 1-2, that this residue is found at the dimer-dimer interface, suggesting that this mutation might cause a disruption that prohibits the formation of a stable tetramer.

In support of this hypothesis is the kinetic data from R67 DHFRs that were either modified or engineered to remain in the dimeric form. WtR67 DHFR treated with diethyl pyrocarbonate (DEPC), a reagent that modifies histidine residues by adding a bulky *N*-carbethoxy group, was shown to disrupt the dimer-dimer interface

by modifying His 62. This resulted in the formation of stable dimers at pH 8.0, where R67 DHFR is normally tetrameric (Nichols *et al.*, 1993). The DEPC treated wt R67 DHFR yielded the following kinetic values, K_m (DHF) = 40 ± 4.0 μ M, K_m (NADPH) = 72 ± 5.5 and a k_{cat} of 0.052 ± 0.007 sec^{-1} (Nichols *et al.*, 1993). The second dimeric species of R67 DHFR is seen in a H62C mutant which when reduced, was shown to form only the dimeric species with kinetic parameters very similar to those of the DEPC treated wt R67 DHFR (Park *et al.*, 1997). These values are similar to those seen for the W38F mutant R67 DHFR, indicating that this mutant may only form dimers.

Gel Filtration Studies

In order to determine the oligomeric state of W38F R67 DHFR, molecular sieving chromatography was performed at both pHs 5.0 and 8.0. The data in Figure 3-2 and Table 3-2 show the W38F mutant R67 DHFR to be dimeric at both pHs 5.0 and 8.0. The apparent molecular weight of the W38F mutant was calculated to be $25,000 \pm 640$ at pH 8.0 which is approximately one half that seen for the wild type. Little change was seen going from pH 5.0 to 8.0 for this mutant. In contrast, the W45F mutant R67 DHFR was tetrameric at pH 8.0 and dimeric at 5.0.

pH Dependency of the Oligomeric State

To further support the contention that the W38F mutant R67 DHFR is incapable of forming stable tetramers, the pH dependant equilibrium between tetramer and two dimers was investigated. Figure 3-3 shows the plots of pH versus

fluorescence intensity and center of mass. From the center of mass plot, it can be seen that the wt and W45F mutant R67 DHFR have clear monophasic transitions going from pH 5.0 to 8.0, while the W38F mutant R67 DHFR shows no inflection only a slight linear decrease in the center of mass. The latter is consistent with the W38F mutant remaining dimeric, as the transition that is seen in the wt and W45F mutant R67 DHFR corresponds to the sequestering of Trp 38 from solvent as the dimer-dimer interface is formed. The sequestering of Trp38 from solvent results in the observed increase in fluorescence intensity that is seen in Figure 3-3(A) and the blue-shift that is seen in Figure 3-3(B) as the pH goes from 5.0 to 8.0. The K_{overall} values determined by fitting the pH profiles of wt and W45F R67 DHFRs are shown in Table 3-3. The values are within experimental error of each other, suggesting that the wt and W45F R67 DHFRs undergo similar dimer to tetramer transitions.

Fluorescence Spectra and Relative Quantum Yields

From the fluorescence scans of the three proteins at pH 5.0 and 8.0, it is seen that tryptophan 38 provides the greatest contribution to the fluorescence spectra of the wild-type R67 DHFR. Tryptophan 45 contributes less than one-fifth of the intensity seen for the wtR67 DHFR. The spectra for W45F and W38F R67 DHFR appear to be additive except in 4M GdnHCl at pH 5.0. It is unclear why this exception occurs, perhaps there is some interaction with denaturant at this pH that slightly lowers the quantum yield of one of the tryptophans.

Circular Dichroism Scans, pH 8.0

The CD spectra of the three proteins show the unexpected result that the W45F mutant yields almost no CD signal. Some change in the CD spectra as compared to the wild-type R67 DHFR might be expected, as aromatic residues can have an effect on the near uv-CD and on occasion a slight effect can be seen in the far uv-CD (Woody, 1995; Chakrabartty *et al.*, 1992). The CD spectrum seen for the W38F mutant R67 DHFR has similar intensity to that seen for the wtR67 DHFR, although it is of different shape. In the case of the W38F mutant, this might be explained as it remains dimeric at pH 8.0.

CD at a global level provides a measure of molecular asymmetry. In other words, to see a CD spectrum there must be an asymmetrical component in the molecular structure, such that the molecule is not superimposable on its mirror image. An optically active molecule must have an electric-dipole transition moment and a magnetic-dipole transition that are not perpendicular to each other. This can only happen for molecules that are not superimposable on their mirror image (Tinoco, Sauer and Wang, 1995).

These facts lead to a possible explanation for the near lack of a CD signal for W45F R67 DHFR. As is seen in the crystal structure, R67 DHFR has exact 222 symmetry, *ie.* 180° rotation about any axis yields the same structure. The fact that the wt and W38F R67 DHFRs display CD signals suggests that in solution, the 3D-structure is not as symmetrical as the crystal structure suggests. The loss of CD signal upon removal of Trp 45 might be explained by the mutant assuming a more symmetrical structure such that the electric and magnetic dipole moments cancel out,

resulting in the loss of CD signal. If this is true, it suggests that the symmetry related Trp 45s are somehow involved in generating a slight asymmetry in the R67 DHFR structure. From the crystal structure it appears as if the symmetry related Trp 45s might be involved in an edge-on stacking interaction. If so, this might introduce a slight asymmetry into the backbone conformation if an analogous interaction is precluded in the symmetry related pair of Trp 45s. This phenomena needs to be further investigated to determine the nature of the interaction, if any. The best fit of the pH titration data occurred when using a value of ($2n = 3$). This is interpreted as having three protons adding to the dimer-dimer interface. This is another potential cause of molecular asymmetry.

Equilibrium Unfolding Studies

Equilibrium unfolding studies were carried out to determine the effects of the W45F and W38F mutants on the structural stability of R67 DHFR. The unfolding curves were carried out at least two different protein concentrations in order to assess the dependence of the unfolding reaction on the concentration of protein at pH 5.0. Oligomeric proteins should show a protein concentration dependence, since increasing the protein concentration should shift the equilibrium toward the folded dimer and thus require more denaturant to produce an unfolding transition. A similar protein concentration dependence was seen for both mutant proteins.

Table 3-4 summarizes the fitting parameters derived from fitting the change in fluorescence intensity at 340nm to a two-state unfolding mechanism (Eq 2). Comparison of the ΔG_{H2O} values from the individual fits to the two-state model

suggests that there is no great difference in the stability when compared to the wild-type R67 DHFR. The data for the change in center of mass as a function of GdnHCl concentration fit to the two-state model individually are more consistent than those as monitored by fluorescence intensity. When compared, it is seen that in general the ΔG_{H_2O} values from the center of mass plots are higher than those determined from the intensity plots. This fits the observations of Eftink, in which he showed that if there is a large change in the quantum yield between two folding states of a protein, then the center of mass might not accurately track the population of states. He showed that the ΔG_{H_2O} would be skewed toward the state with the higher fluorescence (Eftink, 1994). Thus, the fits from the fluorescence intensity data are preferred for the purpose of comparison.

Global Analysis

In an attempt to get a better approximation of the ΔG_{H_2O} values for the three proteins, the unfolding curves from the change in fluorescence intensity were fit simultaneously to the two-state equation, to yield a single value of ΔG_{H_2O} . This was repeated for the center of mass data for W45F and W38F R67 DHFRs. The results of the global fits are shown in Table 3-6. Unfortunately, no data were available for the wtR67 DHFR in terms of the center of mass.

In Table 3-6, it can be seen that the global fits for W45F and W38F mutant R67 DHFRs yield ΔG_{H_2O} values that are within error of each other for the change in fluorescence intensity as a function of GdnHCl data. This suggests that these mutations are not having a major effect on the stability of dimeric R67 DHFR.

Since the W38F and W45F mutant R67 DHFRs show the same ΔG_{H2O} as wtR67 DHFR, this result indicates that the local environments are equivalent and support a 2-state model of unfolding. To determine if a transient intermediate exists, kinetic studies of unfolding/refolding rate will need to be determined.

Studies of single tryptophan mutants in other proteins have shown the ability of the mutants to monitor local folding environments. For example in lactate dehydrogenase (Smith *et al.*, 1991), nine single tryptophan mutants were constructed. In this enzyme, a 4-state unfolding mechanism was proposed as three different single tryptophan unfolding profiles were observed.

The present study has shown that W38 contributes more to the fluorescence signal than W45 (See Figures 3-4, 3-5). This result is surprising as previous studies of unfolding monitored by fluorescence at pH 8.0 showed a single, not a biphasic, transition (Zhuang *et al.*, 1994). The transition in fluorescence is equivalent to that observed by CD, but not that by enzyme activity. Thus a 3-state model of unfolding $T \rightleftharpoons 2D \rightleftharpoons 4U$ was proposed where the first transition was monitored by enzyme activity and the second by CD and fluorescence. Since the environment of W38 monitors the $T \rightleftharpoons 2D$ signal, Zhuang *et al.*(1994) proposed that the fluorescence signal monitoring the $T \rightleftharpoons 2D$ was small and was over-shadowed by the $2D \rightleftharpoons 4U$ transition. However, this thesis shows that W38 contributes more to the fluorescence than W45. Thus, the previous interpretation of the fluorescence signals at pH 8.0 seems unlikely. This data would suggest that at pH 8.0 the unfolding is highly cooperative with the dimeric state not being highly populated. Therefore, these two

tryptophan mutants should allow further clarification of the folding pathway of R67 DHFR at pH 8.0.

Future Studies

Equilibrium unfolding transitions need to be monitored at pH 8.0 to assess what effects can be seen at this pH. At pH 8.0, the transitions will again be monitored by fluorescence intensity and fluorescence center of mass. At this pH, it is expected that the W38F R67 DHFR will have a much lower ΔG_{H2O} when compared to the wt and W45F R67 DHFRs, due to the inability to form stable tetramers.

The equilibrium unfolding transition followed by the change in CD signal needs to be examined, at least for the wt and W38F R67 DHFRs at both pH 5.0 and 8.0. It is unclear if the W45F mutant could be evaluated by this technique at pH 8.0 due to the weakness of the signal. At pH 5.0 the W45F R67 DHFR shouldn't be symmetrical as a dimer and may show an increase in the CD signal. In addition, equilibrium unfolding studies followed by enzyme activity are possible. This method gives another way to visualize the tetramer to 2-dimer transition. The suggested interactions between the symmetry related trp 45s might be studied by the use of fluorescence anisotropy and quenching studies to determine the exposure of the Trps to solvent. The kinetics of unfolding will be probed by following the time dependence of total emission above 320nm utilizing stopped flow spectroscopy.

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