PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

A Structure--Function Study of Dihydrofolate Reductase by Protein Engineering

J. E. Villafranca, Elizabeth E. Howell, S. J. Oatley, M. S. Warren and J. Kraut

Phil. Trans. R. Soc. Lond. A 1986 **317**, 405-413
doi: 10.1098/rsta.1986.0050

Phil. Trans. R. Soc. Lond. A **317**, 405–413 (1986)
Printed in Great Britain

A structure–function study of dihydrofolate reductase by protein engineering

By J. E. Villafranca,[1] Elizabeth E. Howell,[1] S. J. Oatley,[2] M. S. Warren[2] and J. Kraut[2]

[1] *The Agouron Institute, 505 Coast Boulevard South, La Jolla, California* 92037, *U.S.A.*
[2] *The Chemistry Department, The University of California, San Diego, La Jolla, California* 92093, *U.S.A.*

Several mutants of the enzyme dihydrofolate reductase (DHFR) have been engineered by oligonucleotide-directed mutagenesis of the cloned *E. coli* gene. The mutations were designed to address specific questions about DHFR structure–function relations that arose from the analysis of the high-resolution structure. Mutations at the active site have revealed that the invariant residue aspartate-27 is involved in substrate protonation, and not in transition-state stabilization as previously thought. The 2.0 Å (1 Å = 10^{-1} nm = 10^{-10} m) refined structures of the Asn-27 and Ser-27 mutant enzymes reveal that the enhanced binding observed for the 2,4-diamino pteridine and pyrimidine inhibitors is probably not attributable to the charge interaction between Asp-27 and a protonated N-1 of the inhibitor.

Substitution of a cysteine for a proline at position 39 places two sulphydryls within bonding distance, and under certain oxidation conditions they will quantitatively form a disulphide bond. The refined 2.0 Å structures of both reduced and oxidized forms of this mutant show that only minor conformational changes occur for disulphide bond formation. The crosslinked enzyme is significantly more conformationally stable to denaturants such as guanidine hydrochloride and urea.

The technique of site-directed mutagenesis has made a great impact in the way we can approach a study of structure and function relations in proteins. It is especially powerful when applied to the study of a protein whose high-resolution crystal structure is known. Indeed the combination of protein crystallography and directed mutagenesis seems perfectly complementary. While the crystal structure provides a geometric 'blueprint' from which ideas for mutations can be rationally conceived, the ability to engineer specific mutations means that crystallographic studies are no longer limited to proposing mechanistic hypotheses which, in general, have been difficult to test directly.

The usefulness of this combined approach is illustrated by the study of the enzyme dihydrofolate reductase (DHFR) from *E. coli*. The three-dimensional structure of this enzyme has been extensively characterized, with the crystal structures of DHFR from three species (two bacterial, one vertebrate) now known to better than 2.0 Å† resolution. From this detailed description, the critical amino-acid residues in the active site have been identified, and their role in the catalytic mechanism and in inhibition by tight-binding substrate analogues such as methotrexate have been proposed (Bolin *et al.* 1982). Specific mutations have been engineered by oligonucleotide-directed mutagenesis to test these earlier proposals (Villafranca *et al.* 1983). Moreover, the crystal structures of the mutant enzymes have also been determined,

† 1 Å = 10^{-1} nm = 10^{-10} m.

to verify from a geometric standpoint our interpretation of the new molecular properties of the mutant enzymes. Although elucidating the function of the selected residues was certainly interesting and important, perhaps a more fundamental objective of the experiment was to explore our new-found capabilities in protein engineering.

The role of aspartate-27 in catalysis

DHFR is a rather small cytosolic enzyme (from *E. coli*, $M_r = 18000$) which catalyses the reduction of dihydrofolate to tetrahydrofolate by using the coenzyme NADPH. The reaction is thought to involve protonation of the N-5 of the substrate and hydride transfer from the coenzymes nicotinamide C-4 to the substrate C-6 atom. Bolin *et al.* (1982) have proposed a model of the enzyme–substrate complex that is consistent with the stereochemistry of the reaction based on the known absolute configuration of enzyme-reduced tetrahydrofolate (figure 1*a*). Here, the role of aspartate-27, a functionally conserved residue, has been postulated to contribute to substrate protonation as well as to stabilization of a putative carbonium ion transition state. We have used two mutant enzymes with substitutions at position 27 to examine further the functional role of this aspartate. First, an asparagine was engineered at position 27 by oligonucleotide-directed mutagenesis. Then, by applying selective pressure to the bacteria carrying the Asn-27 mutant (plasmid) with increasing concentrations of the DHFR inhibitor trimethoprim, a mutant with serine at this position was obtained (Howell *et al.* 1986).

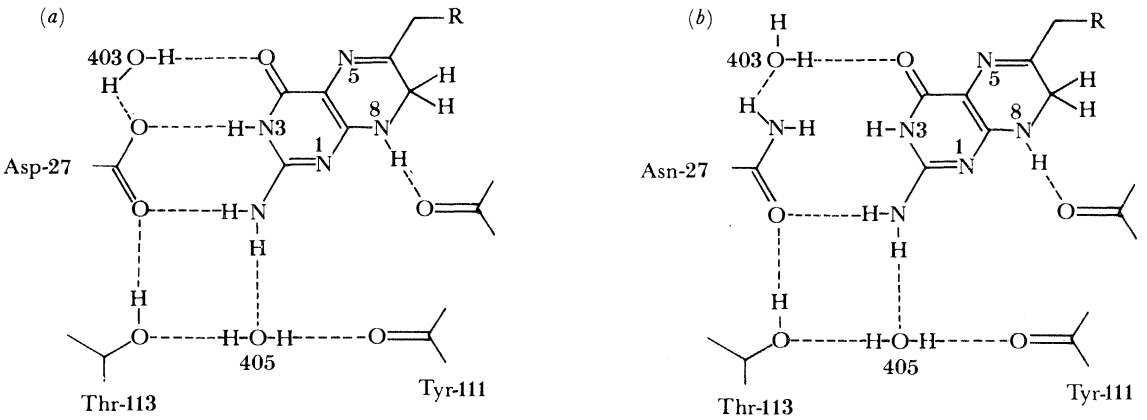

FIGURE 1. Schematic representation of the hydrogen bonding between (*a*) wild-type DHFR, and (*b*) Asn-27 DHFR and the substrate, dihydrofolate, as modelled in the crystal structures.

Kinetic analysis of these mutant enzymes indicates that the aspartate-27 is involved in protonation of the substrate N-5 but is not involved in transition-state stabilization. For both the Asn-27 and Ser-27 mutant enzymes, the pH–activity profile (figure 2) was shifted to lower pH with activity continuing to increase beyond the measurable lower pH limit. Because the most basic atom in the dihydrofolate molecule is N-5 with a pK_a of 3.8, it was reasonable to assume that the substrate for the mutant enzymes was a dihydrofolate molecule already protonated at N-5. Thus the catalytic rate is high at low pH where the protonated substrate becomes plentiful and it is lower at high pH, where a non-productive complex is formed with the unprotonated substrate.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

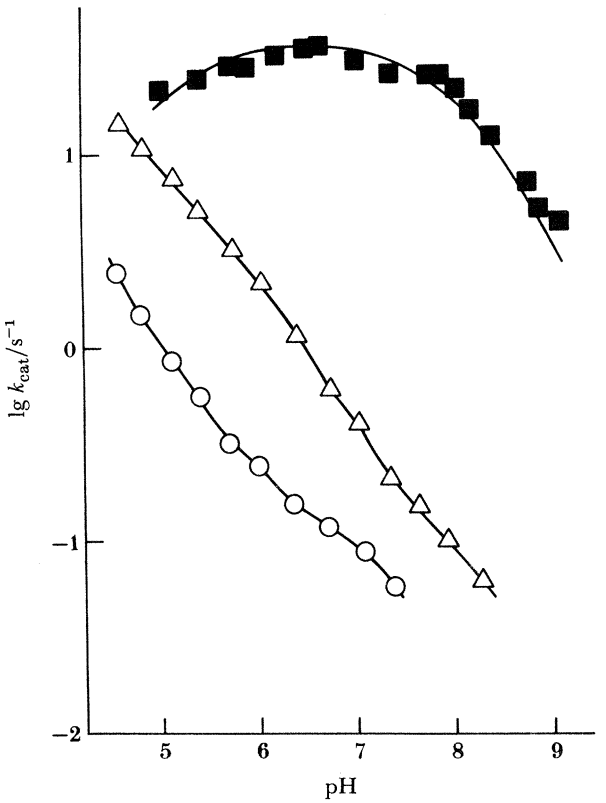

FIGURE 2. Profiles of lg k_{cat} against pH for (■) wild-type DHFR, (○) Asn-27 DHFR, and (△) Ser-27 DHFR.

Because the mutants appear to bypass the protonation step, measurement of isotope effects on the catalytic rate when using deuterated NADPH (NADPD) will characterize the rate of this step relative to hydride transfer. The ratios of k_{cat} with NADPH against NADPD (D_V) were 1.2, 2.2, 3.5 for the wild-type, Asn-27, and Ser-27 enzymes respectively. These results indicate that the protonation step is probably rate-limiting in the wild-type enzyme and that hydride transfer is partly rate-limiting in the mutants. This is consistent with the observation that extrapolation of the pH profiles to lower pH would show the mutant enzymes have catalytic rates approaching or even surpassing that of the wild-type enzyme. These results clearly indicate that the role of aspartate-27 is related to substrate protonation and its contribution to transition-state stabilization is negligible.

THE ROLE OF ASPARTATE-27 IN INHIBITOR BINDING

It has long been known that 2,4-diamino pteridine and pyrimidine analogues of folate such as methotrexate (MTX) and trimethoprim are extremely tight-binding inhibitors of DHFR, binding 10^3 or more times more tightly than the substrate. Spectroscopic studies of the DHFR–inhibitor complexes have shown that a charge interaction exists between the enzyme and the inhibitor, resulting in the protonation of the N-1 atom of the inhibitor's pteridine ring. The crystal structures of the DHFR–MTX binary and ternary complex (Matthews *et al.* 1977, 1979) have established that the crucial residue in the charge interaction is aspartate-27 (see figure 3*a*).

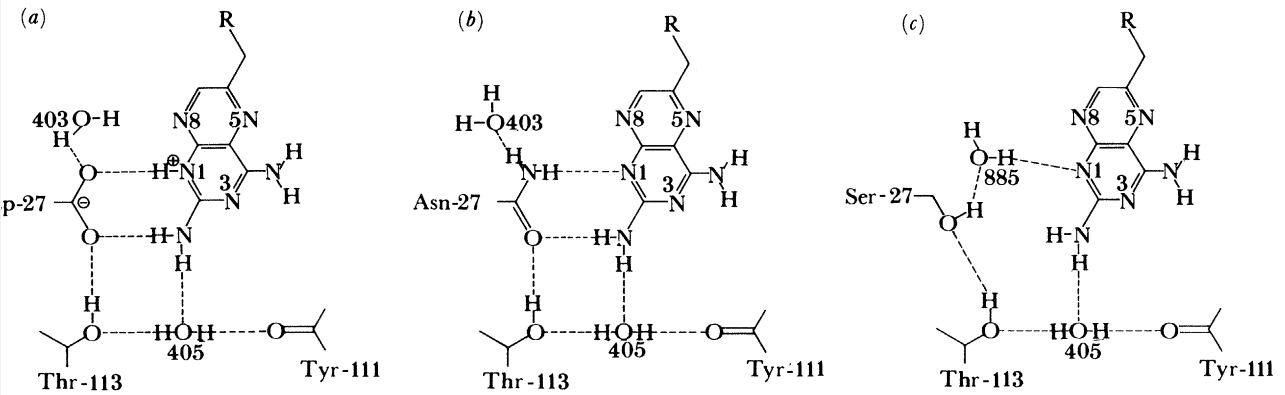

FIGURE 3. Hydrogen bonding between methotrexate and (*a*) wild-type DHFR, (*b*) Asn-27 DHFR, and (*c*) Ser-27 DHFR.

It was shown by molecular graphics modelling that we would obtain a nearly perfect isosteric replacement of aspartate-27 by asparagine in the DHFR–MTX binary structure. The asparagine-27 model showed little distortion in the binding geometry of the MTX and all the hydrogen bonds between the wild-type enzyme and the inhibitor would also be formed in the mutant enzyme–MTX complex. The ionic interaction would, of course, no longer be possible. The Ser-27 mutation also could not form the ionic interaction, but this substitution would almost certainly disrupt the hydrogen-bonding network.

As expected, u.v. difference spectra of the Asn-27 and Ser-27 enzymes complexed with MTX showed no protonation of the inhibitor (Howell *et al.* 1986). The higher dissociation constants were consistent with the anticipated loss in binding energy. The K_d values were 0.07, 1.9, and 210 nM for the wild-type, Asn-27, and Ser-27 enzymes respectively. The surprising result here is that the charge interaction seemed to contribute only 7.9 kJ mol^{-1} to the binding of MTX. One explanation for this could be that the MTX had adopted a different geometry from the one found in the wild-type, perhaps flipping over and binding more like the substrate (Bolin *et al.* 1982). This possibility was probably the most compelling reason to determine the structures of these enzymes.

THE CRYSTAL STRUCTURES OF ASN-27 AND SER-27 DHFR

Crystals of the Asn-27 and Ser-27 enzymes in the binary complex with MTX were isomorphous with those of the wild-type enzyme, so initial atomic coordinates for refinement were obtained from the interpretation of difference Fourier maps of the mutants against the wild-type enzyme. The refined 2.0 Å structure of the Asn-27–MTX complex was in general agreement with our graphics model (figure 3*b*). The main difference between the wild-type and Asn-27 structures was in the positions of two water molecules in the active site, and presumably results from the new hydrogen-bond geometry of the amido nitrogen of the asparagine. Because the geometry of the MTX is unchanged and it is not apparent how the new water positions would contribute additional binding energy to the DHFR–MTX complex, the conclusion that the charge interaction contributes only about 7.9 kJ mol^{-1} is inescapable. A low binding-energy contribution by this ionic interaction has also been measured by Stone

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

& Morrison (1982). By determining the pH dependence of DHFR–MTX binding constants, they estimate a difference in K_d between protonated and unprotonated MTX of about 11. This corresponds to 5.9 kJ mol^{-1} in binding energy.

The results of the Asn-27 analysis is supported by the 2.0 Å structure of the Ser-27 mutant (figures 3*c*, 4). Here we found, in addition to no charge interaction, the loss of at least one hydrogen bond and the formation of a van der Waals gap in the Ser-27–MTX complex. The analysis was facilitated by difference Fourier maps showing no significant geometric distortions of the MTX with respect to interacting residues in the active site, other than Ser-27. The destabilizing interactions arise from the increased distance between MTX and the sidechain of Ser-27 (see figure 4). The Ser-27 Oγ atoms lies 4.0 Å from the 2-amino group of MTX, too far to form a hydrogen bond and creating a 1 Å van der Waals gap. A new water molecule is found positioned roughly in the place of the aspartate-27 Oδ2 of the wild-type. Here it can form a hydrogen bond with the N-1 of MTX and the Oγ of Ser-27. If one assumes the charge interaction is worth about 8 kJ mol^{-1}, the 19.7 kJ mol^{-1} loss in binding energy in the Ser-27–MTX complex can be reasonably accounted for by including the unfavourable dispersion energy of the van der Waals gap and the loss of one hydrogen bond.

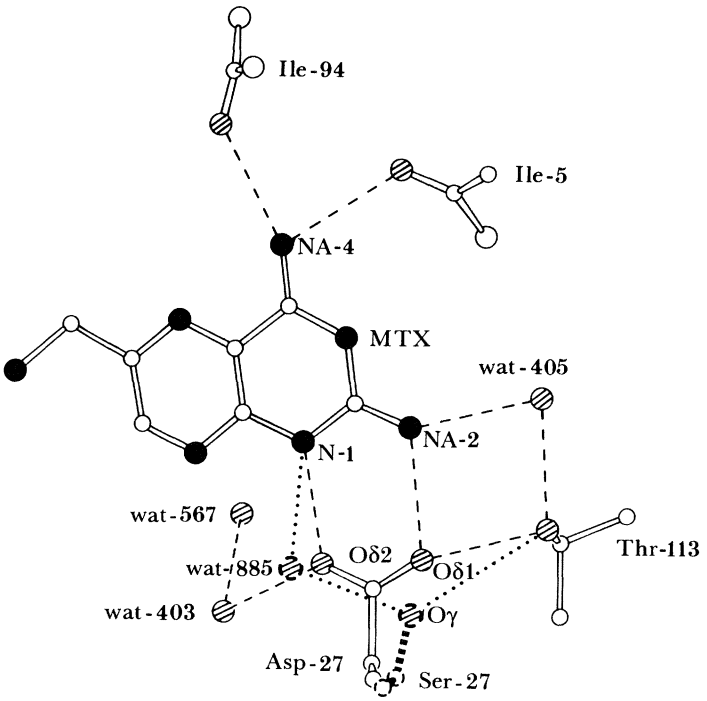

FIGURE 4. Depiction of the DHFR active site showing the geometry of the serine-27 in the mutant enzyme (broken lines) relative to that of the aspartate-27 (solid lines). Nitrogens are solid circles, oxygens are shaded circles and carbons are open circles.

That binding interactions with the substrate have also been lost in the mutant enzymes is evident in their K_M values. The K_M for wild-type Asn-27 and Ser-27 enzymes are 1.2, 44, and 140 μM respectively. These interactions can be reasonably modelled for Asn-27 (figure 1*b*), where it appears that only one hydrogen bond may have been lost. In the Ser-27 enzyme, however, it is difficult to speculate on the structural basis for the higher K_M. The possible

distortions in the active-site water structure of this enzyme leads to an ambiguous modelling of the substrate interactions, although intuitively one would expect the rather drastic change to serine at position 27 to result in a significant loss in substrate affinity.

Disulphide bond engineering

The function of disulphide bonds in proteins is thought to be mainly structural. These covalent crosslinks enhance conformational stability by destabilizing the unfolded (denatured) form of the protein (Anfinsen & Scheraga 1975). Unfortunately, little is known about the selection of a particular region in a protein with respect to disulphide bond formation and crosslinking stabilization. Nevertheless we have relied on some rather basic guidelines to engineer a disulphide bond in DHFR (Villafranca *et al.* 1983). A search of all the α-carbon positions having interatomic distances of about 6 Å between them yielded a position which could form a disulphide bond to an already existing cysteine, Cys85. There are two cysteines in *E. coli* DHFR: Cys-85 and Cys-152. By modelling a substitution of the proline residue 39 with a cysteine, it was found that little or no distortion would be expected by just a simple replacement. It was not clear, however, whether formation of a disulphide bond and its attendant 2.0 Å reduction of the sulphur to sulphur distance would cause some structural changes. One obvious advantage of engineering a disulphide bond here was that only a single mutation was required, but additionally, the anticipated small conformational distortion at this site would probably result in a marginal effect on the enthalpic thermodynamic component of the protein's stability, thus enhancing the probability of an overall positive effect on the stability through the entropic contribution of the crosslink.

By using olignucleotide-directed mutagenesis, proline-39 was replaced by a cysteine. When precautions are taken to prevent air oxidation, the purified Cys-39 DHFR was found to have all three cysteines in the free sulphydryl form. The disulphide bond was not formed *in vivo*, consistent with the notion of a high reducing potential in the cytosol (Ziegler 1985). Nevertheless, the disulphide could be formed nearly quantitatively *in vitro* by a disulphide exchange reaction by using dithionitrobenzoate (Villafranca *et al.* 1986). Both reduced and oxidized forms of the Cys-39 enzyme showed catalytic activity identical with that of the wild-type.

The structural stability of the wild-type and of the reduced and oxidized Cys-39 enzymes was studied by denaturation with the use of guanidine-HCl and urea. The reduced form of Cys-39 was found to have the same conformational stability as the wild-type. The oxidized form was more stable to both denaturants, although it showed a loss in folding cooperativity (see figure 5). An analysis of the denaturing curves has yielded a lower limit estimate of the increase in the conformational stability for the oxidized Cys-39 enzyme of 7.5 kJ mol^{-1} (Villafranca *et al.* 1986). This value compares relatively well with the estimated 10.9 kJ mol^{-1} expected from theoretical considerations of the entropy effect of a disulphide bond which crosslinks a 46-residue polypeptide loop (Poland & Scheraga 1965). These estimates suggest that both forms of the Cys-39 enzyme had not changed significantly in conformation. This was expected from our modelling studies and supported by the behaviour of the Cys-39 mutant on native polyacrylamide gels.

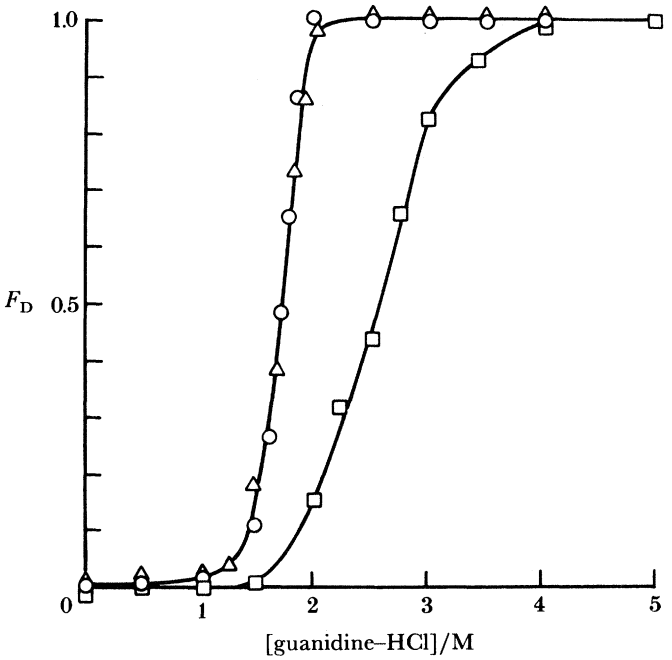

FIGURE 5. Profile of the fraction of denatured (unfolded) protein, F_D, against the concentration of guanidine-HCl; F_D was measured as $\epsilon_{obs}-\epsilon_N/\epsilon_D-\epsilon_N$, where ϵ_N and ϵ_D are the extinction coefficients at 292 nm for native (N) and denatured (D) forms of the protein; ○, wild-type; △, Cys-39 (reduced); □, Cys-39 (oxidized).

CRYSTAL STRUCTURES OF THE CYS-39 DHFR

As with the residue-27 site mutations, crystals of the reduced and oxidized forms of the Cys-39 enzyme were isomorphous with the wild-type. The 2.0 Å difference Fourier maps of both forms of the Cys-39 enzyme against the wild-type were readily interpretable and showed that indeed very little structural adjustment had occurred. The refined structures showed (in great detail) that the sulphydryl of Cys-39 was not bonded to that of Cys-85 but was in van der Waals contact (4.0 Å), and that in the oxidized form the disulphide bond had adopted a left-handed spiral geometry (see figure 6). In the reduced form the Cys-39 sulphydryl atom is partly exposed to solvent and appears to form a hydrogen bond with the backbone carbonyl of residue 85. On formation of the disulphide bond the Cys-39 sulphydryl becomes inaccessible to solvent, buried beneath the sidechain of lysine-58. This solvent inaccessibility of the disulphide bond accounts for its observed resistance to reduction.

Conformational changes were examined by comparison of the backbone torsion angles, ϕ and ψ, between the mutant and wild-type structures. Although there were some minor changes in these dihedral angles, they were confined to residues in the site of substitution (residues 38, 39, and 85) and were no larger than 12° (residue 38: $\Delta\psi = 12°$). The most significant change appears in the C_α–C_β dihedral (χ_1) of Cys-39, twisting by $-64°$ in the reduced form and by another $-82°$ in the oxidized form. The resulting disulphide dihedral angles closely resemble those of known left-handed protein disulphides and thus probably represent a 'natural' stable conformation.

From inspection of the structures of the two Cys-39 forms, there were no apparent structural constraints to formation of the disulphide bond, thereby providing strong support for the

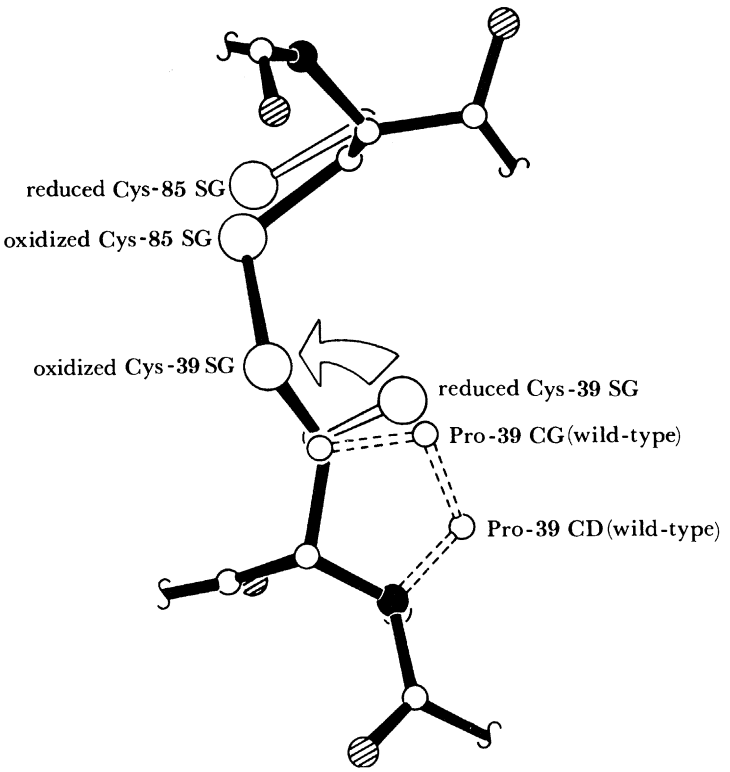

FIGURE 6. Depiction of the geometry in the vicinity of residues 39 and 85 for the wild-type (broken bonds) the reduced form of Cys-39 DHFR (open bonds) and the oxidized form of Cys-39 DHFR (solid bonds). Atoms are as described in figure 4.

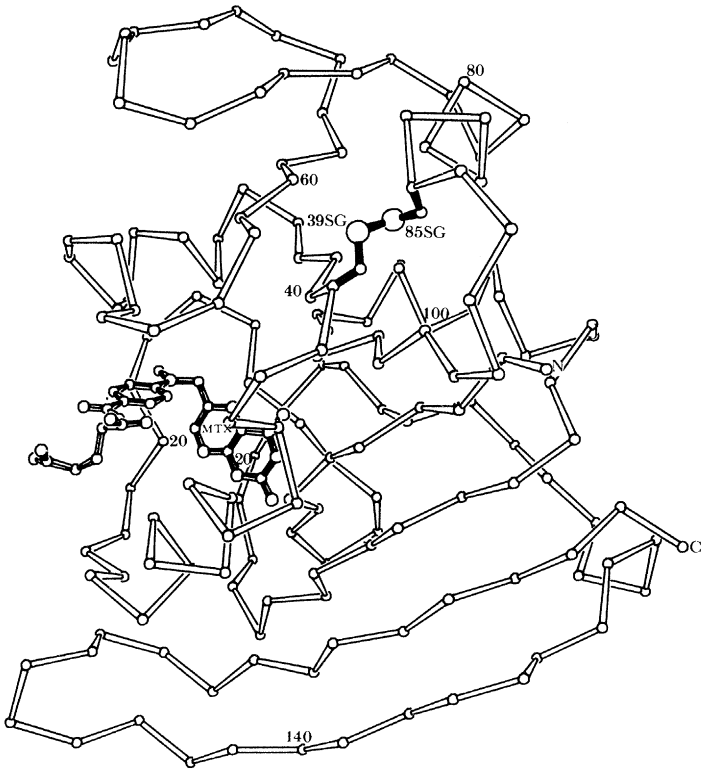

FIGURE 7. Alpha-carbon drawing of oxidized form Cys-39 DHFR showing the disulphide crosslink relative to the overall structure. Methotrexate is shown in striped bonds.

existence of a highly reducing environment in the bacterial cytosol, which prevents disulphide formation. It is also reasonable to suggest from the observed lack of conformation changes that the stabilization provided by the disulphide here is indeed mainly from entropic considerations. The loss of folding cooperativity in the oxidized Cys-39 enzyme probably reflects the presence of stable intermediates in the folding pathway. It is perhaps not too surprising to find a significant perturbation in folding when a covalent link has been introduced into an evolutionarily fine-tuned folding mechanism. No doubt many engineered mutations will be found to exhibit this kind of structural effect.

Conclusion

Although many questions concerning the DHFR mutant enzymes remain to be addressed, what is certainly clear from these studies is that the combination of protein crystallography and site-directed mutagenesis to study protein structure–function relations provides a particularly productive experimental approach. Current advances in crystallographic methodology at many levels have allowed increasingly rapid structural analyses to be performed. As we have discussed here, it is especially efficient in the analysis of mutations which cause only minor structural changes. This should apply in most situations because a general protein engineering strategy is likely to be one which will result in a highly focused specific modification. Although it is highly improbable that the crystallographic analysis of the mutant proteins will keep pace with the rate at which mutations can be engineered, it seems reasonable to expect that the full benefit of the experiment can only be achieved by correlating the functional properties of the new molecules with their new structures.

References

Anfinsen, C. B. & Scheraga, H. A. 1975 *Adv. Protein Chem.* **29**, 205–299.
Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C. & Kraut, J. 1982 *J. biol. Chem.* **257**, 13663–13672.
Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J. & Kraut, J. 1986 *Science, Wash.* (In the press.)
Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N.-h., Kraut, J., Poe, M., Williams, M. & Hoogstein, K. 1977 *Science, Wash.* **197**, 452–455.
Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N.-h. & Kraut, J. 1979 *J. biol. Chem.* **254**, 4144–4151.
Poland, D. C. & Scheraga, H. A. 1965 *Biopolymers* **3**, 379–399.
Stone, S. R. & Morrison, J. F. 1982 *Biochemistry* **21**, 3757–3765.
Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelson, J. N. & Kraut, J. 1983 *Science, Wash.* **222**, 782–788.
Villafranca, J. E., Howell, E. E., Oatley, S. J., Xuong, N.-H. & Kraut, J. 1986 *Biochemistry* (Submitted.)
Ziegler, D. A. 1985 *A. Rev. Biochem.* **54**, 305–329.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES