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Insights into Enzymic Catalysis from Studies on Dihydrofolate Reductases

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Summary

The role of DHFR in the maintenance of cellular DNA has sparked wide interest in the structure and dynamics of this enzyme. Kinetic studies of specific amino acid replacements on the enzyme isolated from *E. coli* has proved useful in the detailing of hydrophobic and ionic interactions both proximal and distal to the site of chemical transformation (e. g. Phe-31, Leu-54 and Arg-44). Despite the low sequence homology shared by the *E. coli* and *L. casei* enzymes, the free energy profiles are surprisingly comparable. This probably is the result of the high degree of structural similarity of the active site surfaces, but the deleterious effects of subtle replacements (e. g. Leu-54-Ile) at strictly conserved amino acids underscore the latter's unique role in attaining the required catalytic efficiency for the enzyme.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP⁺ oxidoreductase, EC 1.5.1.3) catalyses the NADPH-dependent reduction of 7,8-dihydrofolate (H₂F) to 5,6,7,8-tetrahydrofolate (H₄F). The enzyme is essential for both eucaryotes and procaryotes to avoid depletion of H₄F owing to its oxidation by thymidylate synthetase and thus directly influences pyrimidine biosynthesis. It has long been the target enzyme for chemotherapeutic agents and its inhibition by methotrexate (MTX) is mainly responsible for the mechanism of action of this drug (1). Because of its biological and pharmacological importance, dihydrofolate reductase (DHFR) has been the subject of

extensive structural and mechanistic investigations. In particular the X-ray crystallography has provided the structures of the MTX and MTX · NADPH enzyme complexes for the *E. coli* and *L. casei* species respectively (2–5). Stereochemistry has traced the course of the reduction and established that H₂F must be bound in a conformation in which the pteridine ring is rotated 180° relative to the C6–C9 bond of the side chain in MTX (6). With the development of the protocols for site specific mutagenesis, it has become possible to pursue several novel lines of inquiry: i) to evaluate the role of hydrophobic residues in catalysis and in substrate and drug binding; ii) to ascertain the importance of proximal and distal residues on the acidity and function of the unique active site carboxylate; and iii) to determine the influence of multiple amino acid changes on the thermodynamic and kinetic characteristics of the active site by comparing the *E. coli* and *L. casei* enzymes.

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Abbreviations: DHFR, dihydrofolate reductase; H₂F, 7,8-dihydrofolate; H₄F, 5,6,7,8-tetrahydrofolate; MTX, methotrexate. BIOGRAF* is an interactive molecular simulation 3-dimensional graphics program from BIODESIGN, Inc., Pasadena, CA.

Structural and Kinetic Features of Native DHFR

The active site structure of the *E. coli* DHFR · methotrexate · NADPH complex along with several conserved amino acid residues that have been altered by mutagenesis is depicted in figure 1. The NADPH has been positioned based on analogy to its observed locus in the *L. casei* complex. The active site is a cavity some 15 Å deep lined by hydrophobic side chains with the only acidic function present being the carboxyl of Asp-27. NADPH binds in an extended conformation with the nicotinamide moiety inserted through the entrance of the cavity and the pyrophosphoryl linkage making strong ionic and hydrogen bonding contacts

Figure 1. Carbon backbone structure of *E. coli* DHFR · MTX · NADPH and the side chains for key amino acids depicted using BIOGRAF*.

(Arg-44, His-45). MTX, and by inference H₂F, lies towards the back of the site where the pteridine and benzoyl rings are surrounded by hydrophobic residues (e.g. Ile-5, Phe-31, Ile-50, and Leu-54). The Asp-27 interacts with the N2 and N3 nitrogens of the pyrimidine ring and does not make a direct hydrogen bonded contact with N5, despite the fact that protonation of N5 is required to complete the reduction of the N5–C6 imine upon hydride transfer to C6.

The kinetic sequence for *E. coli* DHFR that predicts accurately the observed steady-state kinetic parameters and full-time course kinetics under a variety of substrate concentrations and as a function of pH was established by measuring the various ligand association and dissociation rate constants and pre-steady-state reaction transients employing stopped-flow fluorescence and absorbance spectroscopy (Fig. 2) (7). The enzyme has an intrinsic fluorescence that is quenched by the binding of ligands, particularly MTX. Independent measurements of the equilibrium binding for the various ligand combinations to form the binary and ternary reactant and product complexes gave dissociation constants in satisfactory agreement with those calculated from kinetic measurements of individual rate constants implying that the kinetic scheme is self consistent. Several features of the kinetic sequence germane to our considerations are: i) steady-state turnover is limited by H₄F release; ii) the nonequilibrium binding of H₂F and NADPH becomes ordered owing to the fact that the rate constant for H₄F dissociation is greatest from the mixed ternary E · NADPH · H₄F complex rather than either of the two possible product complexes; and iii) the overall reaction strongly favors H₄F formation ($K_{ov} \sim 10^{11}$) that is partially reflected in the internal equilibrium, $E \cdot NADPH \cdot H_2F \rightleftharpoons E \cdot NADP^+ \cdot H_4F$ ($K_{int} \sim 10^3$).

Figure 2. Kinetic scheme for DHFR at 25 °C, MTEN buffer, N, NADP; NH, NADPH; H₂F, dihydrofolate; H₄F, tetrahydrofolate.

The Active Site Asp-27

Reasoning from chemical precedent, the mechanism of imine reduction is expected to favor preprotonation at N5 followed by hydride transfer. However the absence of a proximal acid function calls into question this presumption and raises issues concerning the identity of the acidic residue and the rates of proton transfer steps to H₂F and medium.

Examination of the pH dependence of the steady-state turnover showed that the rate limiting step is H₄F release at pH < 7.5 which changes to the chemical step of hydride transfer at pH > 8.5. The pH-*V_M* transition is controlled by the ionization of an acidic residue with an apparent *pK_a* ~ 8.4 (Fig. 3). The rate constant for the isolated hydride transfer step is dependent on an acidic active site group with a *pK_a* ~ 6.5, a value that is the same for all forms of the enzyme: free, binary and ternary complexes. Although binding of H₂F/H₄F is pH independent, the

Figure 3. Observed rate constants for hydride transfer (---), H₄F dissociation (---) and *V_M* (—) as a function of pH.

rate of dissociation of H₄F is significantly slower than the rate of hydride transfer up to pH 8.4 increasing the *pK_a* observed in *V_M* by ca. 1.9 *pK_a* units. The identity of the acidic active site residue was clearly shown to be the remote Asp-27 by its replacement with Asn or Ser resulting in two mutant enzyme forms that require preprotonated H₂F for activity (8).

Several lines of evidence from wild-type and mutant enzymes suggest that despite its cloistered location at the back of the active site cavity, the proton on Asp-27 readily exchanges with solvent namely: deuterium kinetic isotope effects on *V_M*/*K_M* (H₂F) and *V_M* that at high pH converge to the same value of ca. 3 (9); and the *V_M*/*K_M* (H₂F) pH-rate profile for the Tyr-31 mutant that exhibits a "hollow" in the vicinity of the apparent *pK_a* (10). Similarly there is no sign that the proton transfer component of the chemical step becomes rate-limiting since i) the amplitude of the hydride transfer step in native DHFR, which is stoichiometric with enzyme, is not attenuated at a pH where the ternary complex exists equally in both protonated and nonprotonated forms and ii) the perturbed *pK_a* ($\Delta pK_a \sim 0-2$) of Asp-27 in various mutants does not correlate with the rate of hydride transfer. Thus, despite its location the carboxyl of Asp-27 can effectively function to donate its proton to N5 probably through a specific water channel or via the periphery of the pterin ring (N3 → O5 → N5) (11). There is also evidence from mutants of DHFR that the carboxyl can be repositioned, e.g. by substitution of an Asp for Thr-113 and a neutral replacement at position-27. However, the lower catalytic efficiency [*V_M*/*K_M* (H₂F)] cannot be as yet unequivocally attributed to a decreased rate of proton transfer (12).

Pressing our line of inquiry further, we questioned whether the *pK_a* of Asp-27, and hence its interaction with H₂F and H₄F, might be coupled to events in the NADPH binding site in order to rationalize the

NADPH assistance of H_4F dissociation. Mutation of Arg-44 to Leu-44 (13) effectively deletes the ionic interaction of this side chain with the 2'-phosphate and pyrophosphate moiety of the nicotinamide cofactor in the binary complex as well as to neighboring residues in the free enzyme (e.g. Pro-66 and Ser-63) and has a profound unexpected influence on the pK_a of Asp-27 in the free, binary, and ternary enzyme complexes. In all cases the pK_a is increased by ca. 2 pK_a units to 8.4 (14). Figure 4 traces the amino acid backbone from Arg-44 in the N terminal direction to Asp-27 and in the C-terminal direction to Arg-52 and Arg-57 — the latter residues form salt bridges to the two glutamate carboxylate residues of MTX. The distance from Arg-44 to Asp-27 is ca. 25 Å, suggesting subtle movement of the peptide chain is responsible for the pK_a change rather than an environmental effect. If the effect were purely electrostatic, a simple coulombic calculation ($\Delta pK_a = 244/Dr$ where D is the effective dielectric constant and r is the charge separation distance in Å) would require a dielectric constant of 5 (heptane ~ 4). This is far less than the range of 50–100 reported for the influence of mutations on the pK_a of His-64 of subtilisin (15). The Leu-44 mutant is also characterized by an accelerating effect by NADPH on the rate of H_4F dissociation ($> 45\text{ s}^{-1}$ vs. 12 s^{-1}) and a reduced rate for hydride transfer (50 s^{-1} and 950 s^{-1}), although the deuterium kinetic isotope effect (NADPD) on the hydride step remains the same (14). Thus we conjecture that the molecular origin of the NADPH effect commences at or near Arg-44 and extends to either or both Asp-27 and Arg-52/57 thereby weakening ionic/hydrogen bonding as well as hydrophobic interactions with H_4F .

Figure 4. Amino acid structure of *E. coli* from Arg-44 to Arg-57 (C-terminal direction) and Asp-27 (N-terminal direction) depicted using BIOGRAF*.

The reduced rate of hydride transfer in this mutant, however, is not a result of an inadequate rate for proton transfer but is probably a consequence of alternate conformations for bound NADPH (14).

Hydrophobic Effects

In the study of designed mutant proteins whose purpose is to evaluate specific interactions, i.e. hydrogen bonds, ionic pairs or hydrophobic effects, a recurring question is their structural integrity versus the wild-type. Crystallographic studies on DHFR *E. coli* mutants (Asn-27 and Ser-27) showed that neither the MTX binding geometry nor the detailed three-dimensional topography of the enzyme was altered within ± 0.2 Å (8). However a substitution, be it a conservative deletion or replacement, will lead to conformational changes that may be localized or subtly propagated through the protein. Since in estimating the value of a side chain contribution to the free energy of binding the most relevant comparison is its deletion to a glycine, which because of its reduced volume, most certainly will be solvated differently at the active site. Thus in general $\Delta\Delta G_{obs}$ may not be equated to the binding energy of the moiety in question, although this term may be the predominant contribution (16). With that caveat one can still assess the apparent binding energy and the relative contribution of a given residue to the active site ensemble.

It has been particularly instructive to examine the effect of a mutation (Phe-31 to Val-31) on the binding of the drug MTX. MTX is an example of a slow binding inhibitor where the bimolecular association proceeds in two stages via an initial encounter complex that isomerizes to the observed structure (17, 18). On the presumption that the free energies of the wild-type and mutant enzymes are equal, a free energy diagram has been constructed for the binding of MTX to the wild-type DHFR plus two mutants at Phe-31 (19). Several salient results emerged: i) replacement of Phe-31 by Val-31 alters the accessibility of MTX to its binding pocket by reducing the bimolecular combination rate a hundred fold; ii) the value of edge to face aromatic-aromatic interactions estimated from the binding of MTX and 6,7-dimethylpteridine are ca. 12.6 kJ mol^{-1} . Since the salt bridge formed between N-1 and N-2 of MTX and Asp-27 may contribute ca. 7.5 kJ/mol^{-1} to the drugs binding affinity (8), hydrophobic interactions are at least as important determinants in the tight binding of MTX.

The more complex question of the relationship between substrate/product binding and enzymic catalysis can also be scrutinized by mutagenesis. For at

least five mutants including those at Phe-31, Leu-54, and Thr-113 there is a linear free energy relationship between the rate constant for hydride transfer and the dissociation constant for H_2F plotted logarithmically (20). In essence there is *ca.* a 8.4 kJ mol^{-1} increase in the free energy of activation for the chemical step for every 4.2 kJ mol^{-1} decrease in the free energy of H_2F dissociation. In short the weaker ground state binding of the substrate translates into a slower rate of hydride transfer, an example of a loss in ground state as well as a greater loss in transition state stabilization (21). The weaker binding of H_2F is also paralleled in weaker H_4F binding so that the dissociation rate constant for this product no longer may be rate limiting.

Particularly informative is the Leu-54 to Gly-54 mutation, *ca.* $18 \text{ \AA}$ away from the locus of the hydride transfer, that decreases the rate of the chemical step from 950 s^{-1} to 14 s^{-1} and increases $K_D(\text{H}_2\text{F})$ from $0.21 \text{ }\mu\text{mol/l}$ to $140 \text{ }\mu\text{mol/l}$ (*ca.* 16.7 kJ/mol^{-1}) (22). If the Gly substituent is viewed as too extreme a deletion, replacement of Leu-54 with Ile surprisingly produces an analogous change in the rate of the hydride transfer step (30 s^{-1}) (23). The exquisite sensitivity of the locus to a single methyl group in the active site cannot at present be definitively rationalized; however an attractive hypothesis views bound H_2F as distributing between two conformers, one of which may be an unproductive complex akin to bound MTX whose equilibrium position is tuned by various residues. Moreover *ab initio* calculations suggest the pathway for hydride transfer to be exquisitely sensitive to the distance and angle between the participating atoms, i. e. changes on the order of less than $1 \text{ \AA}$ can decrease rates by factors of $10^2 - 10^3$ (24).

Comparison of Multiple Active Site Changes (*E. coli* and *L. casei* DHFR)

The detailed kinetic description of the *E. coli* DHFR catalyzed reaction provided an opportunity to evaluate the efficiency of this process. Given the definition of the kinetic sequence under physiological conditions (at concentrations for NADPH/NADP⁺ and $\text{H}_2\text{F}/\text{H}_4\text{F}$ approximating those of the *E. coli* cell) the turnover at steady state and its reciprocal the reaction flux were calculated (25). A maximum hypothetical catalytic turnover also can be computed after imposing H_2F diffusion to the $\text{E} \cdot \text{NADPH}$ complex (the predominant form of the enzyme in the cell) as the only rate-limiting step. At physiological conditions the *E. coli* DHFR would be operating at *ca.* 15% efficiency, suggesting that the free energy cost of the various steps has been well optimized (20). Yet com-

puter simulation revealed that there were a large number of pathways that efficiently solve the energetics of the reduction within the thermodynamic constraints imposed by the overall reaction equilibrium and the diffusional barrier of the enzyme/substrate combination. For example, the internal equilibrium constant ($\text{E} \cdot \text{NADPH} \cdot \text{H}_2\text{F} : \text{E} \cdot \text{NADP}^+ \cdot \text{H}_4\text{F}$) can be varied by 10^3 with less than a two-fold change in turnover maintaining the uniform binding constraint (26). Two related questions are prompted by the above calculations: i) is the catalytic process exhibited by the *E. coli* DHFR also employed by DHFRs from other species; ii) how many and what kinds of active site changes can be tolerated while still maintaining high catalytic efficiency?

The x-ray crystallographic structure of the active site amino acids of the *E. coli* and *L. casei* DHFRs were overlaid using MTX as a center for alignment resulting in a base ensemble of 40 amino acids within $5 \text{ \AA}$ of the drug. The active site equivalence was computed to be 55%; those amino acids that were either identical or have backbone interactions only were deemed equivalent. Despite these differences comparison of the total solvent accessible surfaces of the two proteins within this $5 \text{ \AA}$ radius showed them to be within 7% of each other and the protein surfaces to be remarkably congruent. However, several prominent changes (*E. coli*: *L. casei*) Ala-29: His-20; Leu-36: Val-35; Ile-50: Phe-49; and Arg-52: Lys-51 result in discernible differences in the positioning of the glutamate and benzoyl residues. A similar overlay was constructed for the NADPH binding site after positioning the NADPH in the *E. coli* site by analogy to its known position in the *L. casei*. In this case an active site ensemble of 58 amino acids $7 \text{ \AA}$ from the cofactor were counted with a 72% equivalence based on the above criteria. The close similarity in this section of the active site surface between the two species is shown in figure 5 despite the amino acid differences and the fact that the NADPH was modeled into the *E. coli* structure without changes in side chain positions.

Elucidation of the kinetic sequence for the *L. casei* DHFR using the stopped-flow methods described earlier revealed the same features as reported for the *E. coli* (20, 27). Most importantly when the individual rate constants are expressed in terms of free energy the differences for each step (*E. coli*: *L. casei*) are within 4 kJ mol^{-1} equivalent to that of the *E. coli* enzyme. These free energy differences for the two enzymes are depicted for the bound species in figure 6.

It is apparent that an active site with multiple amino acid changes can result in similar kinetic sequences and catalytic efficiencies. Since single mutations can

Figure 5. Superpositioning of the active sites of the *E. coli* and *L. casei* DHFRs with NADPH as a center for alignment. All residues within 7 Å were included. Some key residues that are visible in the left hand panel (adenine portion) are (*E. coli*: *L. casei*); Gln-65: Gln-65 (top); Arg-44: Arg-43 (middle right); Arg-98: Gln-101 (bottom); and Ser-64: His-64 (middle left). In the right hand panel (nicotinamide portion) the key residues are (*E. coli*: *L. casei*); Ile-94: Ala-97 (upper right); Phe-31: Phe-30 (middle right); Asp-27: Asp-26 (lower right); and Ile-50: Phe-29 (upper left). These residues are depicted using BIOGRAF*.

Figure 6. Plot of $\Delta\Delta G$ (*E. coli* minus *L. casei*) calculated from equilibrium constants vs. various bound species that are identified in figure 2. $E[TS]^*$ represents the transition state for the chemical hydride transfer step calculated from the forward rate constant. All free energy values are pH independent and are measured using standard state concentrations of 1 mol/l NADPH and H_2F ; $t = 25^\circ C$; 1 kcal = 4.1868 kJ.

result in marked loss in catalytic activity, i.e. Leu-54 to Ile-54, it is reasonable to presume that some of these changes must be compensatory; others are probably neutral. One may hypothesize that the active site residues act as an ensemble to create a unique surface with the observed novel kinetic properties. The answer to the rhetorical question why this surface and not others may lie not only in the need for catalytic

efficiency within the cell milieu but also in the stereochemical requirements of binding these substrates and in curtailing inhibition by others and the demands for stably folding a given primary sequence into this cavity shape. One may also speculate that residues strictly conserved during evolution may not be changed because no compensatory mutation exists that satisfies all these three requirements within the cell.

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