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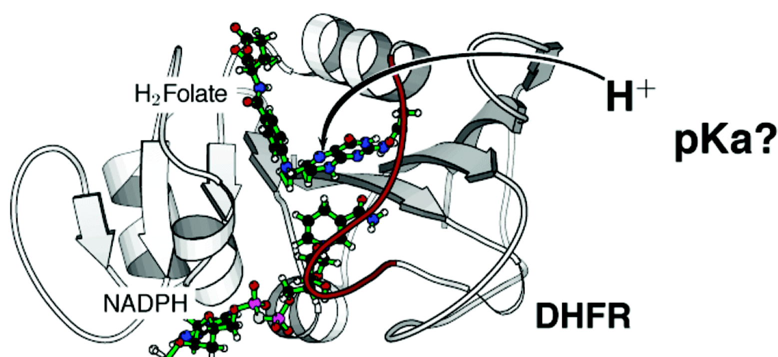

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How Dihydrofolate Reductase Facilitates Protonation of Dihydrofolate

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The enzyme dihydrofolate reductase (DHFR) catalyzes the cofactor-dependent reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H4F): a key step in the biosynthesis of purines, thymidylate, and several amino acids. Although the enzyme has been studied extensively, key aspects of the catalytic cycle are still debated. The questions we address in this communication are if and how the enzyme facilitates protonation of H_2F.

During the catalytic cycle the M20 loop (residues 14−24) of the enzyme is believed to cycle through different conformations.[1] At least three such conformations are known from crystallography, namely the closed, open, and occluded conformations. The closed and occluded conformations are depicted in Figure 1. The occluded conformation is characterized by an occluding Met16 residue that prevents the nicotinamide ring of the cofactor from residing in the active site. Instead the cofactor, if present, is dangling outside the enzyme as in Figure 1. Conversely, in the closed conformation the cofactor is aligned properly for donating a hydride to H_2F, (see Figure 1), and the M20 loop encloses the active site for the chemical transformation step.

The chemical transformation step progresses through a protonation of the N5 atom, and a hydride transfer from the cofactor NADPH to the C6 atom in H_2F, cf. Figure 1. The chemical transformation step is pH dependent, with a pK_a value of 6.5 for DHFR from *Escherichia coli.*[2] The order of the events (protonation vs hydride transfer), the source of the observed pK_a, and the source for the protonation are the subjects of ongoing debate. Originally, it was believed that the Asp27 residue exhibited the observed pK_a value and was the proton donor to H_2F.[3] These suggestions were based on studies of DHFR mutants, where the Asp27 residue was replaced by, e.g., Ser, resulting in severe loss of catalytic activity at neutral pH but not at low pH.[3a,c] However, studies by Callender and co-workers suggest that the N5 atom is responsible for the pH dependency of the reaction and that the Asp27 residue is deprotonated during the catalytic cycle.[4] This would imply that DHFR increases the pK_a value by ~4 pK_a units (the pK_a value for H_2F in solution is 2.6[5]), corresponding to an increase of the rate constant for the chemical transformation step at pH 7 by a factor of 6000. However, recent computational studies dispute the above suggestions.[6]

Despite the ongoing debate, attempts to probe the role of the enzyme in the protonation of H_2F by computational means do not exist. In this communication we explore if and how the enzyme facilitates protonation of the N5 atom by means of pK_a calculations for the key enzyme conformations, those involving the closed and occluded configurations for the M20 loop.[1]

The pK_a is related to the proton affinity, ΔG, by p$K_a = \Delta G/(k_BT \ln 10)$. By means of our employed force fields we cannot calculate ΔG directly, partly because it involves the solvation energy of a proton and partly because the employed force fields lack the description of (intrinsic) proton affinities. On the other hand, the force fields are capable of describing changes due to altered electrostatic interactions with the environment. Assuming that the shift in the proton affinity, $\Delta\Delta G$, upon binding of H_2F to DHFR·NADPH is due solely to altered electrostatic interactions, we can calculate $\Delta\Delta G$ by $\Delta\Delta G = \Delta G_{E\cdot S}^{calc} - \Delta G_S^{calc}$, where $\Delta G_{E\cdot S}^{calc}$ is the calculated free energy change associated with converting H_2F to H_3F^+ when H_2F is bound to DHFR·NADPH in either the closed or occluded conformation, and ΔG_S^{calc} is the change for converting H_2F to H_3F^+ in solution.

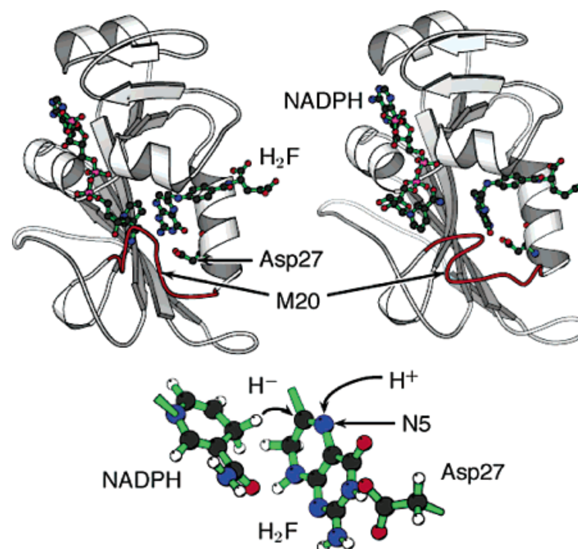

Figure 1. Structures illustrating the closed (upper left) and occluded (upper right) conformations of *ec*-DHFR. The lower panel is a close up of the molecules involved in the chemical transformation step and the Asp27 side chain for the closed conformation. Arrows illustrate the actions of hydride transfer and protonation. The structures are centroids of simulations of the ternary DHFR·NADPH·H_2F complex.[7]

Our calculations of ΔG_X^{calc} ($X = E\cdot S$, S) are based on 10−12.5 ns molecular dynamics simulations of DHFR·NADPH·H_2F in the closed and occluded conformations and of H_2F in solution. The free energy changes are calculated by using a free energy perturbation approach: $\Delta G_X^{calc} = -k_BT \ln \langle \exp(\Delta U_X/k_BT)\rangle$,[8] where ΔU_X is the energy change associated with $X \rightarrow XH^+$. $\langle ... \rangle$ denotes that the quantity $\exp(\Delta U_X/k_BT)$ is averaged over all snapshots taken from the simulations. For a given snapshot ΔU_X is calculated by first removing all water molecules and then recalculating the energy with and without a proton added to H_2F using a generalized Born implicit solvent model to describe the solvent.[9,10] Absolute pK_a values are obtained by adding the experimental value of 2.6[5] to the calculated pK_a shifts.

Within the accuracy of the force fields and implicit solvent model, our procedure should yield reasonable estimates of the pK_a shift, provided that reorganization of the enzyme due to the entrance of a proton is small, i.e. that the protonated structure samples the overlapping regions of configuration space as the deprotonated

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Table 1. Calculated pK_a Values for the N5 Atom in H_2F in *E. coli*-DHFR·NADPH·H_2F

conformation	full DHFR[a]	Z(D27) = 0[b]	no M20[c]	H_2F only[d]
closed	7.1(0.2)	−2.2(0.2)	5.7(0.2)	5.2(0.2)
occluded	7.7(0.2)	−0.1(0.3)	6.2(0.3)	4.5(0.3)

[a] No manipulation was performed. Experimental value is 6.5. [b] The charge of the Asp27 side chain was neutralized. [c] The M20 loop was removed. [d] All residues and NADPH have been removed.

structure. Fortunately, this seems to be a fair approximation for protonation of H_2F in DHFR, since as it turns out, the N5 atom is completely solvent-exposed and the residues in the proximity of the N5 atom are hydrophobic.[11] We expand upon this point further below.

To probe the role of the Asp27 residue and the M20 loop, we performed pK_a calculations where the charge of the Asp27 side chain was neutralized and where the M20 loop was removed for every snapshot. The role of the conformation imposed on H_2F by DHFR was explored by calculating the pK_a values also for the situation where everything is removed but H_2F, i.e., in aqueous solution, but with the conformational population sampled in the enzyme.

Before we discuss our calculated pK_a values, we note that in agreement with another study[6a] we find that the N5 atom is exposed to solvent. On the basis of our simulations we find that the N5 atom is in contact with a water molecule 50% of the time in the closed conformation, increasing to 80% in the occluded conformation. These numbers should be compared to 60% for H_2F in solution.[12] Hence, if the pK_a value increases, the N5 atom might be protonated directly from the solvent.

Our calculated pK_a values are listed in Table 1 together with the calculated standard errors. We find pK_a values of 7.1 and 7.7 for the closed and occluded conformation, respectively. These values suggest that, indeed, the enzyme facilitates protonation of the N5 atom in H_2F and indicate that the N5 atom in H_2F is responsible for the pH dependency with a pK_a value of 6.5. We note that the pK_a value is likely to be dominated by the closed conformation[1b,13] and also that movements at longer time scales than sampled here have been observed for both the closed and the occluded conformations.[1b]

It is well-known that enzymes can change the pK_a of ionizable groups relative to the corresponding solvated systems.[14] The basis for the increased pK_a in DHFR is the construction of the active site that, although accessible to water, is hydrophobic. The only hydrophilic and ionizable group in the active site is the Asp27 residue, which is deprotonated in our simulations in accordance with experiment.[4c] Calculations performed by us suggest, indeed, that the Asp27 has a pK_a value lower than that for the N5 atom.[15] Since a hydrophobic environment is hard to polarize and therefore poorly screens charge−charge interactions, it will enhance the positive electrostatic interactions between the negatively charged Asp27 side chain and a (positively charged) proton. In this regard the M20 loop plays an important role in screening the solvent, and accordingly, we observe that removal of the loop decreases the pK_a values to about 6, cf. Table 1. The pK_a value is affected even more severely if the charge of the Asp27 side chain is neutralized and, in our calculations, even drops to a value below that of free and solvated H_2F. This is not unlikely since it now costs energy to bring a proton from the polarizable solvent with moderate positive interactions to the hydrophobic active site with weak positive and negative interactions. This in turn may explain why the catalytic activity is reduced at neutral pH upon mutation of Asp27 to neutral residues, but not at low pH, since a mutation of the Asp27 residue will shift the pK_a to lower values.

Finally, another way that DHFR seems to facilitate protonation is to impose a conformation on H_2F that is favorable for protonation, with a calculated p$K_a \approx 5$, cf. Table 1. On the basis of average energies we find such conformations of H_2F to be unstable by about 4 kcal/mol, relative to the conformational population sampled by H_2F in solution. The closed conformation significantly restrains the movement of H_2F, which may explain why the pK_a drops mostly for the occluded conformation when DHFR and NADPH are removed. In this regard the Asp27 side chain plays an important role in restraining the conformational flexibility of H_2F since it forms hydrogen bonds to H_2F, cf. Figure 1. The M20 loop might play an important role by sterically maintaining the position of H_2F.

In conclusion, our calculations suggest that the N5 atom in H_2F is protonated directly from the solvent and is responsible for the pH dependency of the chemical transformation step. DHFR promotes protonation by enclosing the N5 atom in a hydrophobic pocket together with the negatively charged Asp27 residue. The mechanism behind the facilitation of the protonation provides an explanation for why, e.g., the D27S mutant is found to be less active. Finally, our results suggest that DHFR imposes a conformation on H_2F that is favorable for protonation.

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Supporting Information Available: Computational details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) See Supporting Information for more details.
(11) See also Supporting Information.
(12) The water molecule that the N5 atom interacts with at a given time is generally hydrogen-bonded to the oxygen atom of the H_2F pterine ring.
(13) We find, on the basis of averages energies, that the closed conformation is more stable by ∼20 kcal/mol. This is a large number, but generally speaking we cannot expect to get accurate conformational energy differences by this method.
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(15) Using a slightly different method, we find the pK_a value for the Asp27 residue to be 4.9 for the closed conformation. We do, however, find this result less reliable than the results for the N5 atom since the Asp27 side chain hydrogen-bonds to Thr113 and H_2F. The enzyme is therefore likely to sample other parts of configuration space when Asp27 is protonated.

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