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ARTICLE *in* CHEMBIOCHEM · NOVEMBER 2009

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The Role of Arginine 28 in Catalysis by Dihydrofolate Reductase from the Hyperthermophile *Thermotoga maritima*

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Dihydrofolate reductase (DHFR) catalyses the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) by using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. In the reaction, hydride is transferred from NADPH to C6 of the pterin ring of the substrate and N5 of the substrate is protonated. H_4F acts as a carrier of one-carbon units in various oxidation states and is required for the synthesis of thymidylate, purines, several amino acids and, in prokaryotes, pantothenate.[1] DHFR from the hyperthermophile *Thermotoga maritima* (TmDHFR) is the only chromosomal DHFR that exists as a dimer,[2] although its tertiary structure is very similar to that of the monomeric DHFRs such as that from *E. coli* (EcDHFR).[3] The rates of the TmDHFR-catalysed reaction are considerably lower than those of EcDHFR[4,5] and this had been proposed to be a consequence of the loss of flexibility in TmDHFR as a result of dimerisation.[2,4]

With a melting temperature approximately 30 °C above that of EcDHFR,[4] TmDHFR is the most thermostable DHFR isolated to date.[5–7] We have recently shown that the dimeric structure of TmDHFR is critical for its high thermostability. However, it does not significantly affect its catalytic activity; a monomeric single residue mutant of TmDHFR had catalytic properties that were similar to those of the wild-type enzyme.[8] Alternative explanations for the lower rates of TmDHFR are hence required and include the presence of arginine 28 in the substrate-binding region of the active site (Figure 1),[4] the more solvent-exposed active site of TmDHFR,[2] and the observation that TmDHFR and EcDHFR follow subtly different mechanisms for reduction of the substrate.[9]

A number of arginine residues are generally present around the active site to bind the glutamate "tail" of the substrate (Figure 1), but the presence of Arg28 in close proximity to N5 of the substrate is unusual in bacterial DHFRs. This position is normally occupied by a leucine or phenylalanine residue. The most likely mechanism by which Arg28 could reduce the rate of the TmDHFR-catalysed reaction is by disfavouring protonation of the substrate due to its positive charge. Both hydride transfer and steady-state turnover in TmDHFR show sigmoidal pH dependences with an apparent pK_a of around 6.0.[4] The apparent pK_a of the EcDHFR-catalysed hydride transfer on the other hand is 6.5,[10] which coincides both with the pK_a of bound dihydrofolate[11] and with a conformational change in the enzyme.[12] It is generally accepted that the apparent pK_a of the reaction is related to the population of protonated substrate.[13–15] The proximity of the guanidinium group of Arg28 could lead to an unfavourable electrostatic repulsion with the protonated substrate, and thereby depress its pK_a relative to that observed for the EcDHFR catalysed reaction and hence reduce the likelihood of reaction at physiological pH. Conversely, the proximity of the guanidinium group of Arg28 to the protonation site might indicate that this residue acts as the proton donor and therefore that Arg28 in fact plays a positive role in TmDHFR catalysis. While extensive research has shown that the proton source in the EcDHFR-catalysed reaction is most likely the solvent itself (see refs. [9], [15], [16], and references therein) this question has not been addressed in TmDHFR.

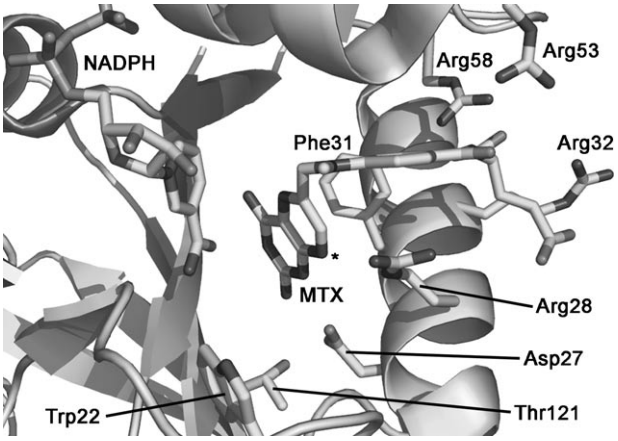

Figure 1. The active site of TmDHFR (PDB ID: 1D1G[2]) with bound NADPH and methotrexate (MTX). The enzyme is shown as a cartoon representation with ligands and important residues shown as sticks. In the equivalent EcDHFR complexes, folate binds with its pterin ring "flipped" relative to that of MTX, with its N5 atom in the position occupied by N8 (*) of MTX. Protonation of N5 is required for reaction to take place (note that the phenyl ring of MTX, seen here edge-on, at 90° to the plane of the page, is coplanar with the guanidinium group of Arg28).

In order to test the various proposals concerning the role of Arg28 in TmDHFR catalysis, we generated site-specific mutants of TmDHFR, in which this residue was converted to lysine and glutamine. The R28K mutation was chosen because of the more localised charge of the lysine side chain and therefore lower pK_a of its protonated form, while R28Q was chosen because the glutamine side chain has no charge, yet is polar and capable of forming hydrogen bonds. Circular dichroism (CD) spectra of the variants showed only small differences to that of the wild-type enzyme (see the Supporting Information). Mean

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900465.

residue ellipticities (MRE) at 222 nm of the mutant proteins were only slightly increased and melting temperatures (T_m) were essentially unchanged (Table 1); this demonstrates that the mutations had no measurable effect on the secondary structure or thermostability of TmDHFR.

Table 1. Comparison of wild-type TmDHFR and its R28K and R28Q variants.

Parameter	TmDHFR	TmDHFR-R28K	TmDHFR-R28Q
MRE[a,b] [deg cm^2 dmol^{-1}]	-10730 ± 150	-9630 ± 520	-9740 ± 420
T_m [°C]	83.0 ± 1.0	80.1 ± 0.2	81.0 ± 0.1
k_H[c] [s^{-1}]	0.61 ± 0.06	0.23 ± 0.02	0.18 ± 0.01
k_{cat}[c] [s^{-1}]	0.31 ± 0.04	0.12 ± 0.02	0.08 ± 0.03
K_M (NADPH)[c] [μM]	$\ll 0.5$	$\ll 0.5$	$\ll 0.5$
K_M (H_2F)[c] [μM]	$\ll 0.5$	$\ll 0.5$	1.88 ± 0.09
K_d (NADPH)[b] [nM]	23.5 ± 2.4	16.6 ± 2.8	24.3 ± 1.1
K_d (H_2F)[b] [nM]	10.4 ± 1.7	17.4 ± 3.0	261.6 ± 27.2
pK_a[c,d]	6.1 ± 0.1	5.9 ± 0.2	5.7 ± 0.2
E_A[d] [kJ mol^{-1}]	51.9 ± 0.6	52.9 ± 2.5	56.5 ± 2.0

[a] At 222 nm; [b] at 25 °C; [c] at 40 °C; [d] steady state.

Steady-state turnover numbers and rate constants for hydride transfer were determined at 40 °C (Table 1). Both TmDHFR variants showed a roughly threefold reduction in both parameters relative to the wild-type enzyme; this indicates that the presence of Arg28 in the active site is not responsible for the lower rates of the TmDHFR-catalysed reaction. Indeed, these results might suggest that Arg28 is beneficial for catalysis. Since hydride transfer remained partially rate limiting for both mutants and since above 25 °C k_{cat} and k_H show similar dependence on temperature and pH,[4] the Arrhenius plots of k_{cat} for both mutants were determined in the steady state (see the Supporting Information). The activation energies for the reaction were not significantly altered in either variant (Table 1); this suggests that the catalytic mechanism is not altered. In addition, the apparent pK_a of the reaction was determined in the steady state at 40 °C (see the Supporting Information) and only small differences in the pK_a were seen in either variant (Table 1).

The only notable difference between the three TmDHFR variants is the elevated K_M (H_2F) observed for the R28Q variant (Table 1). As accurate K_M values could not be obtained in all other cases due to the very tight binding of ligands to TmDHFR and the difficulty in measuring rates at low substrate concentrations as a result of the large amounts of enzyme needed to give good data, K_d values were obtained by observing the quenching of tryptophan fluorescence (Table 1). Although these K_d values are for the binary enzyme–ligand complexes and therefore approximations to the "true" K_d (formation of the Michaelis complex leads to immediate reaction, hence preventing measurement of binding data) they mirrored the behaviour observed for K_M. The K_d for NADPH was unchanged in both variants, while the K_d for dihydrofolate was increased by nearly 30-fold in the R28Q but not in the R28K variant; this strongly suggests a role for Arg28 in substrate binding.

These experiments show that Arg28 is not responsible for reducing the rate of the TmDHFR-catalysed reaction relative to monomeric EcDHFR and that Arg28 does not suppress the pK_a of the substrate in the TmDHFR active site. The alternative possibility that the reduced rates are due to a difference in mechanism between TmDHFR and monomeric DHFRs[9] is unlikely because the rates of the TmDHFR-catalysed reaction are low even at acidic pH,[4] when protonation of the substrate would be much more favourable. The same argument applies against the depressed pK_a itself being the cause of the reduced rates. The reduced rates and pK_a for the TmDHFR-catalysed reaction are therefore most likely due to the more solvent-exposed active site of TmDHFR compared to EcDHFR as is evident from a comparison of the X-ray crystal structures.[2,3] A more solvent accessible active site in TmDHFR should increase the dielectric response within the active site. It has previously been argued that the rate constant for hydride transfer in EcDHFR is inversely proportional to the dielectric response within the active site.[17] In addition, replacement of the central four residues (Met16-Ala19) of the M20 loop of EcDHFR with a single glycine, which should greatly increase the solvent accessibility of the active site, results in a 500-fold decrease in the rate constant for hydride transfer.[18] The rates of the reaction catalysed by this EcDHFR loop deletion mutant (0.7 s^{-1} at pH 6.0 and 25 °C[18]) are similar to those of the TmDHFR-catalysed reaction and hydride transfer is partially rate limiting in the steady state as has been observed for TmDHFR.[4] Conversely, we have shown recently that the TmDHFR-catalysed reaction rates decrease as the dielectric constant of the medium decreases.[19] The data presented here shows that the rate of the TmDHFR-catalysed reaction also decreases as the dielectric response of the active site decreases in contrast to the earlier work on the dielectric response of the active site of EcDHFR.[17]

Our study also rules out the possibility that the guanidinium group of Arg28 acts as an active-site acid to protonate the substrate. If this were the case, then the R28K variant might have been expected to increase the apparent pK_a of the reaction, as a protonated amine is a stronger acid than a protonated guanidinium group, while R28Q would be expected to reduce the apparent pK_a of the reaction. No change in pK_a was observed in either case (Table 1). Exclusion of Arg28 as the proton source makes it highly likely that water is also the proton source in TmDHFR catalysed reduction of dihydrofolate, particularly given the more solvent-exposed nature of the TmDHFR active site compared to that of EcDHFR.

The loss of affinity for dihydrofolate in the R28Q mutant suggests, however, that Arg28 plays an important role in the relatively tight binding of the substrate compared to other DHFRs. Like many cofactors, dihydrofolate is a thermally rather unstable molecule and, at the elevated temperatures at which *T. maritima* thrives, binding to proteins is likely to play an important role in preventing its nonenzymatic breakdown. The guanidinium group of Arg28 is positioned approximately 4.5 Å from and coplanar to the phenyl ring of methotrexate in the crystal structure (Figure 1). This suggests that it could form a

stabilising cation–π interaction with the substrate.[20] At elevated temperatures, the dielectric constant of water is reduced[21] and so the entropic cost of burying a charged residue in the hydrophobic active site is lower. On the other hand, creation of a cation–π interaction increases the binding enthalpy for the substrate, which might be necessary to counteract the less favourable entropy of binding at higher temperatures. The R28K mutant, which also has a positively charged group that might be used to form such an interaction, also has a high affinity for dihydrofolate (Table 1 and the Supporting Information). As mentioned above, position 28 is typically occupied by a leucine or phenylalanine residue in bacterial DHFRs including those from moderate thermophiles. A notable exception is the DHFR from the (nonthermophilic) halophilic archaeon *Haloferax volcanii* (HvDHFR), in which a lysine residue is found at position 28. Mutation of this residue to leucine in HvDHFR has been shown to increase the K_M approximately twofold, although the k_{cat} was also increased in this case.[22] Increasing the salt concentration also increased the affinity of both wild-type and mutant HvDHFR for dihydrofolate.[22]

In contrast to the results presented here, it has recently been reported that the F31R variant of human DHFR (HsDHFR), in which F31 occupies the same position as R28 in TmDHFR, displays an increased K_M for dihydrofolate.[23] In the crystal structure of the F31R/Q35E variant of HsDHFR (PDB ID: 1EIG), F31R has multiple conformations,[23] none of which is in a position to form a strong cation–π interaction with the phenyl ring of methotrexate. Therefore, the F31R mutation removes a stabilising π–π interaction, which is replaced by a van der Waals interaction involving the hydrophobic portion of the arginine side chain.

Whether the presence of an arginine residue at position 28 and subsequent enhanced affinity for dihydrofolate is generally required for activity at the extreme temperatures that hyperthermophiles have adapted to, is difficult to test at present. A number of hyperthermophilic species, particularly archaea, do not possess a gene encoding dihydrofolate reductase,[24] often because they use alternative C1 carriers, such as methanopterin.[25] Indeed many hyperthermophilic species have been shown not to contain folates at all,[26] whereas others, such as *Thermus thermophilus*, use the related enzyme dihydropteridine reductase to reduce dihydrofolate.[27] Both *Thermotoga neapolitana*[28] and *Thermotoga petrophila*[29] possess dihydrofolate reductases with arginine at position 28, although this could be due to their close relationship to *T. maritima* rather than being a specific adaptation to high temperatures. More hyperthermophilic DHFR sequences are required before firm conclusions can be made.

Another important point to consider is the fate of the product tetrahydrofolate. This too is unstable and liable to degrade at elevated temperatures. Furthermore, the thymidylate synthase of *T. maritima* is of the ThyX-type and hence uses FAD as a reducing agent and regenerates tetrahydrofolate, rather than the ThyA-type,[30] which form dihydrofolate.[31] ThyX-containing organisms require only very low DHFR activity, whereas ThyA-containing organisms are nonviable without more rapid dihydrofolate production.[32] *T. maritima* therefore has a reduced requirement for tetrahydrofolate production, and overproduction would be wasteful. The low k_{cat} for the TmDHFR-catalysed reaction is therefore likely to be a consequence of the need to regulate tetrahydrofolate production to meet these demands.

In summary, we have shown that the presence of Arg28 in the active site of TmDHFR does not influence protonation of the substrate either by preventing protonation due to unfavourable electrostatic interactions or by acting as the active site acid. Our results eliminate Arg28 as a possible cause of the low rates of the TmDHFR-catalysed reaction. It is likely that the reduced catalytic activity is a consequence of the more solvent-exposed active site of TmDHFR, which results from the restricted movements in the dimer interface of TmDHFR of loop regions that serve to close the active site in Michaelis complex of monomeric DHFRs.[2,3,9] Arg28 nevertheless appears to be important for TmDHFR catalysis in the context of the wider organism, as binding of the substrate to TmDHFR is enhanced by this residue, a role that could be essential for an enzyme required to function at extreme temperatures.

Experimental Section

TmDHFR variants were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions in the previously described pET-11c based expression vector harbouring the gene encoding TmDHFR[4] as the template. Mutagenic primers were: 5′-GCT GGA GCT CAT TTG AGG ACA AAA AAA ATT TCA GAA AAA TCA CC-3′ (R28K) and 5′-GCT GGA GCT CAT TTG AGG ACC AAA AAA ATT TCA GAA AAA TCA CC-3′ (R28Q; changes underlined). TmDHFR and its variants were purified as described previously.[4] NADPH was purchased from Melford. H_2F was synthesised by dithionite reduction of folate.[33]

CD experiments were performed on a JASCO J810 spectrometer. Unfolding of the protein (1.2 μM) in potassium phosphate buffer (10 mM, pH 7) was followed by monitoring the CD signal at 222 nm between 20 and 95 °C and applying a gradient of 0.5 °C min^{-1}.

Steady-state turnover rates were measured spectrophotometrically by following the decrease in absorbance at 340 nm during the oxidation of NADPH. Enzyme (0.1–1 μM) was preincubated with NADPH (0.5–100 μM) in MTEN buffer (25 mM Tris/25 mM ethanolamine/50 mM MES/100 mM NaCl) to avoid hysteresis, and H_2F (0.5–100 μM final concentration) in the same buffer was added to start the reaction. In all cases [S] was $\geq$5[E]. Due to the high enzyme concentration required to give an adequate signal-to-noise ratio, the cofactor and substrate concentrations were not reduced further. When no change in rate was seen at any substrate or cofactor concentration (i.e., zero-order kinetics were observed) the K_M are given as $\ll$0.5 μM. In the case of K_M (H_2F) for the R28Q variant, fitting to equations that take into account the fact that the Briggs–Haldane approximation is not satisfied did not yield satisfactory results. The data were therefore fit to the Michaelis–Menten equation by using SigmaPlot 10. The temperature was varied from 20 to 70 °C at pH 7 and the pH was varied between 4 and 9 at 40 °C by using cofactor and substrate concentrations of 100 μM.

Presteady-state kinetics experiments were performed on an Applied Photophysics stopped-flow spectrophotometer as described previously.[4,34] Hydride transfer rates were measured following the fluorescence energy transfer from the protein to reduced NADPH.

The sample was excited at 290 nm and the emission was measured by using a 400 nm output filter. Enzyme (20 μM final concentration) was preincubated with NADPH (10 μM final concentration) in MTEN buffer at 40 °C and rapidly mixed with H_2F (100 μM final concentration) in the same buffer. The data were fit to a single exponential expression.

Equilibrium binding constants for the binary enzyme–ligand complexes were determined at 25 °C by using a Perkin–Elmer LS55 luminescence spectrometer (excitation at 280 nm; slit widths 5 nm for excitation and 15 nm for emission). All measurements were performed in potassium phosphate buffer (100 mM, pH 7.0) containing NaCl (100 mM). Spectra of the protein (10 nM) in the presence of defined concentrations of ligands were recorded between 300 and 550 nm. The intensity at 350 nm was normalised and the resulting values were fitted to a standard one-site binding curve by using SigmaPlot 10 to obtain K_d.

Acknowledgements

This work was supported by the U.K. Biotechnology and Biological Sciences Research Council (grant E/BB008380/1 to R.K.A.), Cardiff University (R.K.A.) and the University of Birmingham (studentship to G.M.).

Keywords: dihydrofolate reductase · enzyme catalysis · enzymes · mutagenesis · *Thermotoga maritima*

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Received: July 27, 2009
Published online on October 8, 2009

