Hydride transfer during catalysis by dihydrofolate reductase from Thermotoga maritima

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INTRODUCTION

Enzymes are generally believed to adopt structures of marginal stability and high sensitivity to environmental changes, such as elevated temperatures. However, thermophilic organisms have evolved to cope with extreme conditions. Their enzymes resist heat denaturation at high temperatures and their optimal activity is reached well above 70 °C. Because the enzymes of thermophilic and mesophilic organisms are often homologous and most of their physicochemical properties are similar, Arrhenius theory predicts that thermophilic enzymes should be as active as their mesophilic counterparts at low temperatures. However, they often show only little activity at low temperatures, an observation that cannot be explained by cold denaturation as these enzymes do not unfold at low temperatures [1]. Many thermophilic and mesophilic enzymes appear to display optimal activity at the edge of their stability ranges. The reason for this correlation of stability and activity is not well understood but may lie in the dynamic nature of the molecular events associated with enzyme catalysis. The increased activity of thermophilic enzymes at elevated temperatures would therefore result from their increased dynamic flexibility [2–5].

A wealth of kinetic and computational data is available for DHFRs (dihydrofolate reductases) from mesophilic organisms [6]. DHFR is necessary for cellular metabolism in both prokaryotes and eukaryotes. It catalyses the reduction of 7,8-dihydrofolate (H2F) using NADPH as a cofactor. Several antineoplastic and antimicrobial drugs such as methotrexate and pyrimethamine act by inhibiting DHFR. Complete kinetic schemes for the enzymes from Escherichia coli [7] and human [8] have been determined. At neutral pH the steady-state kinetic turnover of DHFR is limited by 5,6,7,8-tetrahydrofolate (H4F) release from the mixed ternary complex DHFR–NADPH–H4F. At pH > 9 the hydride transfer becomes rate limiting [7].

Several lines of evidence have indicated the importance of dynamic properties of EcDHFR (E. coli DHFR) for its functional activity. The angle and the distance between hydride donor and acceptor have been shown to be critical determinants of the rate of hydride transfer both from the inspection of crystal structure data and from ab initio calculations [9–11]. The NMR-derived order parameters and molecular dynamics simulations for the complex of DHFR with folate or methotrexate identified three main regions of motion within three surface loops, namely the M20 loop (residues 14–24), the FG loop (residues 116–125) and the CD loop (residues 64–71) [12,13]. A series of site-directed-mutagenesis studies performed on residues in these loop regions showed that long-range effects influence the rate of hydride transfer. In particular, mutations in the FG loop, which is approx. 17 Å away from the active site, caused a significant decrease in the rate of hydride transfer suggesting that these distant residues change the global dynamics of the protein [14]. Classical molecular dynamics simulations identified strongly coupled motions of amino acid residues in the Michaelis complex of EcDHFR [15]. These correlated motions, which involved the mobile loops identified by NMR, disappeared in the product complex, implying that they might be linked to catalysis. A network of promoting motions has recently been identified which ranges from Asp-122 in the FG loop through Gly-15 and Ile-14 in the Met-20 loop to Phe-31 and involves both the hydride donor and acceptor [16].

The recent characterization of the X-ray structure of TmDHFR (DHFR from the hyperthermophilic Thermotoga maritima; Figure 1) revealed that its tertiary structure was similar to that of the E. coli enzyme despite only 27% sequence identity between the two enzymes [17]. However, unlike its mesophilic counterparts, TmDHFR forms stable homodimers. No isolated structured monomers could be detected either in equilibrium or during unfolding [18].
MATERIALS AND METHODS

Substrates and cofactors

H₂F was prepared by dithionite reduction of folate (Sigma) as described previously [19]. NADPH was purchased from Sigma. (4R)-[^3H]NADPH (NADPD) was prepared by reduction of NADP⁺ (Sigma) using NADP⁺-dependent alcohol dehydrogenase from Thermoanaerobium brokii (Sigma) [20] and purified by anion-exchange chromatography on Mono Q HR 5/5 (Amersham Biosciences) [21]. NADPH and NADPD concentrations were determined spectrophotometrically using an extinction coefficient of 6200 cm⁻¹·M⁻¹ at 339 nm [7]. Similarly the concentration of H₂F was measured assuming an extinction coefficient of 28 000 cm⁻¹·M⁻¹ at 282 nm for pH 7.4 [22].

Protein purification

A plasmid containing the cDNA for TmDHFR was a gift from Dr Nicolas Glansdorff (Research Institute, CERIA-COOVI, Brussels, Belgium) [23]. The cDNA was inserted between the Ndel and BamHl restriction sites of pET11c and used for protein production in E. coli Codon Plus BL21(DE3) RP™ cells (Stratagene). Cells were grown at 37 °C to an attenuation at 600 nm of 0.6 in LB medium containing 0.27 mM ampicillin. Expression was induced by adding isopropyl D-β-thiogalactoside to a final concentration of 0.4 mM. Cells were harvested by centrifugation and resuspended in 50 mM Tris, pH 7/1 mM EDTA (100 ml/10 g of cells). The suspension was sonicated for 5 min, incubated with DNase (20 µg/ml), RNase (20 µg/ml) and MgSO₄ (20 mM) for 30 min and centrifuged. The protein was purified as described previously [24]. In short, the supernatant was diluted 4-fold and incubated at 78 °C for 20 min. The extract was cooled on ice for 5 min and centrifuged. The soluble fraction was applied to a HiPrep™ 16/10 SP XL cation-exchange column (Amersham Biosciences) and eluted with a gradient of 1 M NaCl in 50 mM Tris, pH 7/1 mM EDTA over 70 min. TmDHFR eluted around 200 mM NaCl and the buffer was exchanged to 10 mM phosphate buffer, pH 7. The protein was essentially pure as judged by SDS gel electrophoresis (Figure 2A). The mass of 19237 Da determined by electrospray ionization MS corresponded well to the calculated mass of 19 236 Da. The protein concentration was measured spectrophotometrically (ε₂80 = 22 880 cm⁻¹·M⁻¹) [24].

EcDHFR was produced and purified according to published procedures [14].

CD spectroscopy

All CD experiments were performed on a JASCO J810 spectrophotometer at a protein concentration of 1.2 µM in 10 mM K₂PO₄ (pH 7) and 200 mM KF. Protein unfolding was followed by monitoring the CD signal at 222 nm between 20 and 95 °C, applying a temperature gradient of 0.5 °C/min.

At a given temperature the fraction Φ of unfolded was calculated from the CD signal [Θ]₂₂₂:


where [Θ]₁₂₂ and [Θ]₀₂₂ are the CD signals obtained from the baseline extrapolation before and after the unfolding transition.

Steady-state kinetic measurements

Turnover rates were measured spectrophotometrically by following the decrease in absorbance at 340 nm during the reaction [ε₂₅₅ (NADPH + DHF) = 13 200 M⁻¹·cm⁻¹] [25]. The temperature was varied from 25 to 75 °C at pH 7. In a typical experiment the enzyme (10 µM) was preincubated with NADPH (50 µM) to avoid hysteresis. Enzyme–NADPH solution (10 µl) was added to 970 µl of MTEN buffer (25 mM Tris/25 mM ethanolamine/50 mM Mes/100 mM NaCl). Then 10 µl of NADPH (100 µM final concentration) was added to the solution and the reaction was started by adding 10 µl of H₂F (100 µM final concentration). The temperature of the reaction was controlled carefully by pre-warming the buffer and utilizing a temperature-controlled cuvette holder. The extinction coefficient of NADPH is temperature-dependent and a correction factor of −0.13%/1 °C rise in temperature was applied for ε₂₅₅ [26].

For pH experiments MTEN buffer was used between pH 3 and 9. The reactions were run at 40 °C due to the low stability of substrate and cofactor at low pH and high temperature. All enzymic reaction rates were corrected for the non-enzymic decay of NADPH and H₂F.

Pre-steady-state kinetic measurements

Pre-steady-state kinetics experiments were performed on an Applied Photophysics stopped-flow spectrophotometer. Hydride-transfer rates were measured following the fluorescence energy...
incubated with NADPH or NADPD (10 \mu M) in MTEN buffer and rapidly mixed with H2F (200 \mu M) in the same buffer. A typical experiment is shown in Figure 4 (see below). These data fit well to a single exponential expression.

RESULTS AND DISCUSSION

Production of TmDHFR

Attempts to produce TmDHFR in E. coli BL21(DE3) cells led to the isolation of only small amounts of protein. A total of 11 Arg residues are encoded by nine AGA and two AGG codons in the cDNA for TmDHFR, while one CCA and two CCC codons are used for Pro [23]. The corresponding tRNAs are of low abundance in E. coli [27] and TmDHFR was therefore produced in E. coli Codon Plus BL21(DE3) RP™ cells, which contain extra copies of the argU and proL tRNA genes allowing the production of 20 mg of pure protein from 1 l of culture.

Rather than a distinct band for a protein of the molecular mass of TmDHFR, a poorly focused region was detected by gel electrophoresis (Figure 2A). The crude protein mixture was heated to 78 \degree C for 20 min followed by rapid cooling on ice. This led to the precipitation of the majority of E. coli proteins whereas TmDHFR remained soluble due to its high temperature stability. PAGE of the soluble fraction revealed two bands at approx. 19 and 31 kDa. It had been reported previously that TmDHFR was resistant to denaturation by SDS [28]. The two bands most likely corresponded to the monomer and dimer of the enzyme since denaturation of the fully purified protein in 8 M urea led to a single band of molecular mass 19.5 kDa on SDS/PAGE (Figure 2A).

Temperature-induced unfolding

The CD spectrum of recombinant TmDHFR showed a minimum at 221 nm and a maximum at 196 nm (Figure 2B). In good agreement with the values published previously [18,24], the mean residue ellipticities at 222 and 196 nm were −11000 and 26100 deg · cm² · dmol⁻¹ respectively, suggesting that the recombinant protein adopted the proper secondary and tertiary structures. The thermal stability of TmDHFR was measured by monitoring the CD spectrum as a function of temperature. The thermal unfolding profiles exhibited small linear variations of the signal between 20 and 77 \degree C corresponding to native baselines, a very sharp co-operative unfolding reaction and unfolded baseline regions at temperatures greater than 85 \degree C (Figure 2C). The midpoint of the transition was at 83 \degree C, which is approx. 30 \degree C higher than the melting temperature for EcDHFR [29] and the highest melting point for any DHFR studied so far. Interestingly, the thermal denaturation was not reversible due to precipitation of the denatured protein.

Steady-state kinetics

The steady-state rate of the reduction of H2F by NADPH catalysed by TmDHFR at pH 7 increased in a sigmoidal fashion with temperature (Figure 3A and Table 1). The turnover rate was maximal at 75 \degree C (4.05 s⁻¹), which corresponded to the maximal experimental temperature. This temperature was only slightly below the optimal growth temperature of T. maritima [30]. At the respective optimal growth temperatures of 37 and 80 \degree C the steady-state rate of TmDHFR (approx. 4.8 s⁻¹) was approx. eight times slower than that of the E. coli enzyme (approx. 37 s⁻¹) [7].

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Figure 3 Steady-state kinetics of the TmDHFR-catalysed reduction of dihydrofolate by NADPH

Effect of temperature (A) and pH (B) on the specific activity of TmDHFR. Reactions were initiated by adding the enzyme (100 nM) previously pre-incubated with NADPH (500 nM), NADPH (100 µM) and H₂F (100 µM) in this order. The buffer was MTEN buffer (see text). The temperature was varied from 20 to 75 °C (pH 7) and the pH from 3 to 10 (40 °C).

Table 1 Temperature dependence of the reaction rates for hydride transfer and steady-state turnover during catalysis by TmDHFR in MTEN buffer at pH 7.0

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>TmDHFR</th>
<th>EcDHFR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydride-transfer rate (s⁻¹)</td>
<td>Steady-state rate (s⁻¹)</td>
</tr>
<tr>
<td>15</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>25</td>
<td>0.14 ± 0.01</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>0.20 ± 0.02</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>35</td>
<td>0.33 ± 0.03</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>40</td>
<td>0.52 ± 0.03</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>45</td>
<td>0.84 ± 0.13</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>50</td>
<td>1.38 ± 0.19</td>
<td>1.17 ± 0.07</td>
</tr>
<tr>
<td>55</td>
<td>2.07 ± 0.16</td>
<td>1.53 ± 0.09</td>
</tr>
<tr>
<td>60</td>
<td>3.11 ± 0.21</td>
<td>2.00 ± 0.16</td>
</tr>
<tr>
<td>65</td>
<td>4.50 ± 0.41</td>
<td>2.70 ± 0.14</td>
</tr>
<tr>
<td>70</td>
<td>≈ 6.9*</td>
<td>3.40 ± 0.28</td>
</tr>
<tr>
<td>75</td>
<td>≈ 10.5*</td>
<td>4.05 ± 0.39</td>
</tr>
<tr>
<td>80</td>
<td>≈ 15.5*</td>
<td>≈ 4.8*</td>
</tr>
</tbody>
</table>

* Extrapolated data.

When NADPD was used as a cofactor, an isotope effect of 3.5 was measured for the steady-state rate. This value was similar to that reported for EcDHFR above pH 9 [31], where hydride transfer was rate limiting. The increase in the steady-state reaction rate for EcDHFR was also found to be sigmoidal up to approx. 45 °C (results not shown). Above this temperature the reaction rate decreased due to the reversible denaturation of the enzyme.

The steady-state rates for TmDHFR catalysis were found to depend on the pH of the solution in a sigmoidal fashion at 40 °C (Figure 3B and Table 2). The temperature of 40 °C was chosen for the pH studies as a compromise between the instabilities of the cofactor and the substrate [t₁/₂ (NADPH) ≈ 2 min at pH 3 and 40 °C] and the optimal working temperature of the enzyme. While the observed rates were slow above pH 7.5 (0.024 s⁻¹ at pH 9), a rapid increase was observed below that value and the maximal rate of 3.16 s⁻¹ was reached for pH values below 4. Changing the pH therefore led to more than a 100-fold variation in rate.

Table 2 pH dependence of the reaction rates for hydride transfer and steady-state turnover during catalysis by TmDHFR in MTEN buffer at 40 °C

Steady-state rates are for the dimer of TmDHFR. nd, not determined.

<table>
<thead>
<tr>
<th>pH</th>
<th>Hydride-transfer rate (s⁻¹)</th>
<th>Steady-state rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>0.023 ± 0.002</td>
<td>0.024 ± 0.015</td>
</tr>
<tr>
<td>8.5</td>
<td>0.059 ± 0.003</td>
<td>0.058 ± 0.015</td>
</tr>
<tr>
<td>8.0</td>
<td>0.15 ± 0.002</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>7.5</td>
<td>0.31 ± 0.05</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>7.0</td>
<td>0.52 ± 0.03</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>6.5</td>
<td>1.57 ± 0.14</td>
<td>1.09 ± 0.02</td>
</tr>
<tr>
<td>6.0</td>
<td>3.06 ± 0.04</td>
<td>1.29 ± 0.13</td>
</tr>
<tr>
<td>5.5</td>
<td>3.89 ± 0.21</td>
<td>1.55 ± 0.03</td>
</tr>
<tr>
<td>5.0</td>
<td>6.22 ± 0.28</td>
<td>2.29 ± 0.26</td>
</tr>
<tr>
<td>4.5</td>
<td>nd</td>
<td>2.27 ± 0.05</td>
</tr>
<tr>
<td>4.0</td>
<td>nd</td>
<td>3.05 ± 0.38</td>
</tr>
<tr>
<td>3.5</td>
<td>nd</td>
<td>3.15 ± 0.1</td>
</tr>
<tr>
<td>3.0</td>
<td>nd</td>
<td>3.16 ± 0.2</td>
</tr>
</tbody>
</table>

* Extrapolated data.
The enzyme (40 μM) was preincubated with NADPH or NADPD (10 μM) in MTEN buffer and the reaction was initiated by the addition of an equal volume of H2F (200 μM). The buffer used was MTEN (pH 7) and the reaction temperature was 40 °C.

The inversion point was approx. 6, similar to that observed for the hydride-transfer rate (see below). The protonation state appeared therefore to be important for the reaction.

Pre-steady-state experiments

Due to the overlap of the emission spectrum of TmDHFR at 340 nm with the excitation maximum of the reduced cofactor, stopped-flow fluorescence could be used to measure the rate of the hydride-transfer step for DHFRs from *T. maritima* and *E. coli*. In a typical experiment 40 μM DHFR was incubated with 10 μM NADPH and then mixed with a large excess of substrate (200 μM). The change in fluorescence was followed as a function of time and fit to a single exponential expression for both enzymes (Figure 4). The reaction catalysed by TmDHFR occurred at a rate of 0.2 s⁻¹ at neutral pH and 30 °C, which was three orders of magnitude slower than the corresponding rate for the *E. coli* enzyme at the same temperature (Table 1). The rate of the hydride-transfer step was found to be strongly dependent on the temperature (Figure 5A and Table 1). Because of the limitations of the stopped-flow apparatus used, the maximal reaction temperature studied was 65 °C. The hydride transfer rates for TmDHFR increased exponentially with the temperature in the experimentally accessible range. Extrapolation by fitting an exponential graph to the data resulted in an estimated value of approx. 15.5 s⁻¹ for the hydride transfer at 80 °C (Figure 4 and Table 1). For the *E. coli* enzyme the rate of the hydride transfer step increased linearly up to physiological temperature (Table 1). The rate measured for the hydride-transfer at 25 °C (222.8 s⁻¹) corresponded well with the rate published previously [7]. At higher temperatures a sharp decrease in the rate of the chemical step was observed as a consequence of the unfolding of the protein (Table 1).

The hydride-transfer rates for TmDHFR at the lower end of the temperature range were similar to the steady-state turnover rates (Table 1). The hydride-transfer step was therefore at least partly rate limiting in the catalytic cycle of TmDHFR below 50 °C. This behaviour was in sharp contrast to the *E. coli* enzyme where the hydride-transfer step at pH 7 was more than one order of magnitude faster than the steady-state rate [7]. For EcDHFR product release has been observed to be rate determining at physiological pH. Above 50 °C the hydride-transfer rate for TmDHFR was significantly higher than the steady-state rate, indicating that hydride transfer was no longer rate determining.

The logarithm of the hydride-transfer rates for TmDHFR depended linearly on the inverse of the absolute temperature, indicating that the hydride-transfer reaction followed an Arrhenius-type temperature profile. From the Arrhenius plot, values of 75.6 ± 0.4 kJ·mol⁻¹ and (2.64 ± 0.05) × 10¹² s⁻¹ were obtained for the activation energy and the pre-exponential factor, respectively. As expected, the activation energy for the hydride transfer within the active site of TmDHFR was more than twice the value measured for the catalytically more efficient EcDHFR (28.2 ± 0.9 kJ·mol⁻¹; Table 1).

When the pre-steady-state experiments were repeated using 10 μM NADPD rather than NADPH (Figure 4) a temperature-independent kinetic isotope effect for the hydride transfer of 3.35 ± 0.23 was measured for TmDHFR, which was similar to the kinetic isotope effect reported for the *E. coli* enzyme [7].

Hydride transfer showed a strong dependence on pH. The hydride-transfer rate decreased with increasing pH (Figure 5B and Table 2). Comparison of the hydride-transfer rates with the steady-state transfer rates of TmDHFR showed that hydride transfer was rate limiting at high pH similar to the *E. coli* enzyme, where the rate of hydride transfer rapidly decreased with increasing pH, making it the rate-determining step above pH 9 [7]. The dependence of the rate constant for the hydride transfer...
of TmDHFR on pH was well described by a single pH-dependent step with an apparent $pK_a$ of approx. 6.0 (Figure 5B). The maximal rate at low pH (<5) was 6.22 s$^{-1}$ at 40 °C (Table 2). At this pH the E. coli enzyme was inactive due to pH-induced denaturation [32]. The $pK_a$ observed in the pre-steady-state measurements of TmDHFR catalysis most likely represents the true $pK_a$ of the ternary enzyme complex (TmDHFR-H$_2$F:NADPH). In the E. coli enzyme a $pK_a$ for the reaction of 6.5 has been defined [6,7]. The $pK_a$ of 6.5 has been attributed previously to Asp-27 [6], which is over 5 Å from N5 of H$_2$F and forms hydrogen bonds with the 2-amino and 3-NH groups of the pterin ring of the substrate in all DHFRs studied so far (Figure 1B). However, for the DHFRs from Lactobacillus casei [33], human [34] and cow [35] Asp-27 has been shown to remain unprotonated. The $pK_a$ of 6.5 is therefore most likely due to the protonation of N5 of H$_2$F. The lower $pK_a$ observed during TmDHFR catalysis as compared with EcDHFR might be a consequence of the proximity of Arg-28 in the active site of the thermophilic enzyme (Figure 1B). The positively charged guanidinium group should depress the $pK_a$ of the protonated substrate relative to the E. coli enzyme where the equivalent residue is a leucine. In addition, the active site of TmDHFR appears from the X-ray structure [17] to be more solvent exposed than was observed in the E. coli enzyme [11] leading to a depression of the $pK_a$ of protonated dihydrofolate towards its solution $pK_a$ of 2.6 [36].

In summary, the catalytic properties of TmDHFR depended on the physical conditions of the reaction. At pH 7 hydride transfer was at least partially rate limiting for temperatures below 50 °C, whereas for higher temperatures hydride transfer was faster than the steady-state rate. At its physiological temperature the overall rate of TmDHFR catalysis was not determined by hydride transfer and it is plausible that at these high temperatures product release may be the slow step in the catalytic cycle, as was observed for EcDHFR [7]. Hydride-transfer and steady-state rates during TmDHFR catalysis were relatively slow when compared with mesophilic DHFRs at their respective physiological temperatures (EcDHFR was 20 times faster for hydride transfer, but only approx. 8 times faster in the steady state). Whereas there were differences in detail, the overall kinetic behaviour of TmDHFR was similar to that of the E. coli enzyme except for the higher rate accelerations observed with the E. coli enzyme. Catalysis by both enzymes depended on a single $pK_a$ of approx. 6. The catalytic efficiency increased with decreasing pH and with increasing temperature. The maximal activity was reached around the respective physiological temperatures, which could be a consequence of the different dynamic properties of the two enzymes. TmDHFR has evolved to maintain a stable three-dimensional structure at temperatures well above the melting temperature of the E. coli enzyme. The basis of this extraordinary stability appears to be a large extent the formation of homodimers between TmDHFR subunits [17,18]. A consequence of this increased stability is enhanced rigidity, which in turn might be the basis of the reduced activity of TmDHFR at mesophilic temperatures. Experiments are currently underway to determine the temperature dependence of the vibrational flexibilities of DHFRs from E. coli and T. maritima.

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