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Solvent Effects on Catalysis by *Escherichia coli* Dihydrofolate Reductase

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Abstract: Hydride transfer catalyzed by dihydrofolate reductase (DHFR) has been described previously within an environmentally coupled model of hydrogen tunneling, where protein motions control binding of substrate and cofactor to generate a tunneling ready conformation and modulate the width of the activation barrier and hence the reaction rate. Changes to the composition of the reaction medium are known to perturb protein motions. We have measured kinetic parameters of the reaction catalyzed by DHFR from *Escherichia coli* in the presence of various cosolvents and cosolutes and show that the dielectric constant, but not the viscosity, of the reaction medium affects the rate of reaction. Neither the primary kinetic isotope effect on the reaction nor its temperature dependence were affected by changes to the bulk solvent properties. These results are in agreement with our previous report on the effect of solvent composition on catalysis by DHFR from the hyperthermophile *Thermotoga maritima*. However, the effect of solvent on the temperature dependence of the kinetic isotope effect on hydride transfer catalyzed by *E. coli* DHFR is difficult to explain within a model, in which long-range motions couple to the chemical step of the reaction, but may indicate the existence of a short-range promoting vibration or the presence of multiple nearly isoenergetic conformational substates of enzymes with similar but distinct catalytic properties.

Introduction

Enzymes can achieve rate enhancements of up to 21 orders of magnitude relative to uncatalyzed reactions. To explain this enormous catalytic power, Linus Pauling proposed the concept of transition-state stabilization, in which the role of the enzyme is to reduce the height of the potential-energy barrier that must be overcome for the reaction to occur.[1] However, in recent years attention has turned to the role of quantum-mechanical tunneling through the potential-energy barrier, at least for enzymes that transfer particles of relatively low mass such as electrons and hydrogen atoms or ions, implicating barrier width to be as important as barrier height for these reactions.

Initially, hydrogen tunneling (H^+, $H^\cdot$, H^-) was treated by a correction to transition-state theory (the Bell correction).[2] However, subsequent discoveries of enzymes displaying greatly inflated kinetic isotope effects (KIEs),[3,4] unusual temperature dependences of their KIEs,[5,6] or ratios of Arrhenius prefactors outside the semiclassical limits[3] led to a collapse of the Bell model for hydrogen tunneling and new theoretical frameworks arose to account for the experimental observations. A number of models were developed that proposed a role for protein dynamics in driving hydrogen tunneling, under a variety of names such as promoting vibrations,[7] environmentally coupled tunneling,[8−11] vibrationally enhanced ground-state tunneling,[12] or multidimensional tunneling.[13] Although it is now generally accepted that all hydrogen transfer reactions occur with a significant contribution from quantum mechanical tunneling, the role played by protein motions to promote tunneling remains hotly debated.[11,14−18] Within the environmentally coupled tunneling model, adapted by Klinman and co-workers for enzyme-catalyzed reactions from the formalism proposed by Kuznetsov and Ulstrup,[8] enzyme motions that drive tunneling

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are divided into two classes: slow motions that preorganize the active site into a tunneling-ready conformation from which the reaction can occur, and fast gating motions that modulate the width of the potential-energy barrier and so alter the tunneling probability.[9] When an enzyme relies on gating dynamics, temperature-dependent KIEs are observed, whereas enzyme reactions that occur from tunneling-ready conformations without additional barrier compression are characterized by temperature-independent KIEs. In an attempt to clarify the terminology used to describe the various types of motion that have been proposed to promote tunneling, we use "promoting vibration" to describe a short-range, localized motion that contributes only to the modulation of the barrier width and "promoting motion" to describe a longer-range motion involving two or more amino acid residues that may contribute either to modulating the barrier width or to formation of a tunneling-ready configuration. Promoting motions are a subset of "correlated motions", which are larger-range motions involving two or more amino acid residues but which do not necessarily promote the tunneling reaction.

Dihydrofolate reductase (DHFR) has served as a model system to study the relationship between enzyme structure, dynamics, and catalysis[14,19−24] and results have often been interpreted within the environmentally coupled tunneling model.[9,10,25,26] DHFR catalyzes the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) by hydride transfer from C4 of NADPH and protonation of N5 of H_2F. The primary KIE for hydride transfer by DHFR from *E. coli* (EcDHFR) is temperature independent at high pH but temperature dependent at pH 7.[19] Within the environmentally coupled tunneling model these observations suggest that, at physiological pH, DHFR dynamics promote the compression of the potential-energy barrier leading to an increase of the reaction rate, whereas at elevated pH the reaction occurs from a tunneling-ready conformation without barrier compression. In contrast, DHFR from the hyperthermophile *Thermotoga maritima* (TmDHFR), the only chromosomal DHFR known to have a dimeric structure[27] (Figure 1), shows biphasic kinetics at pH 7 in that the primary KIE is temperature independent above 25 °C but temperature dependent below this temperature.[6]

A number of studies have suggested that the βFG loop of EcDHFR (Figure 1) is important for promoting hydride transfer. A network of interactions has been postulated that spans the whole protein and includes the βFG loop.[14,23,28,29] Mutations in this loop, which is remote from the active site, lead to an up

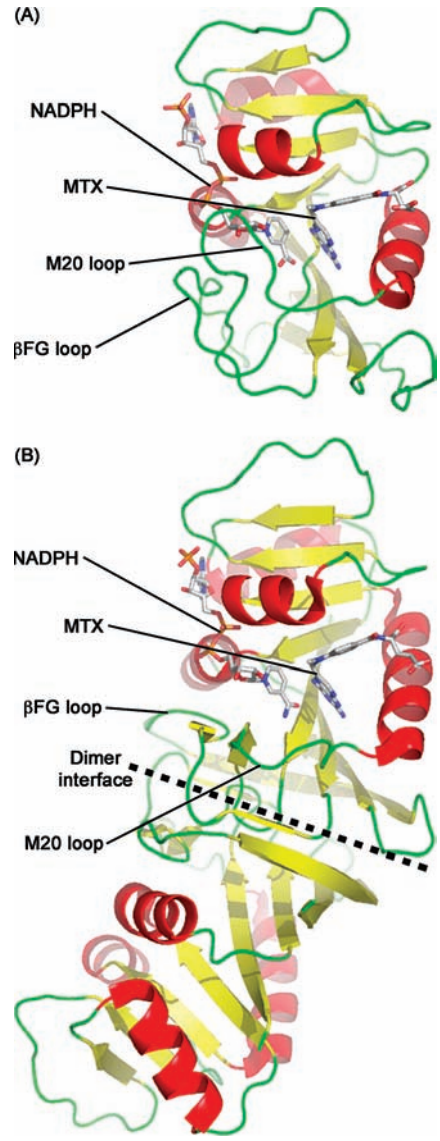

Figure 1. Cartoon of the structures of EcDHFR (A) (PDB 1DRE[23]) and TmDHFR (B) (PDB 1D1G[27]) in complex with NADPH and methotrexate (MTX). Ligands are shown as sticks, and the βFG and M20 loops are indicated (main text for details).

to 100-fold decrease in the rate constant for hydride transfer.[30] In the Michaelis complex, motions of the βFG loop are coupled to those of the nearby M20 loop,[28] which forms contacts with the bound cofactor[23] and which is important for the physical steps of the DHFR catalytic cycle,[24] in that it switches between the closed and occluded conformations depending on the ligands bound.[23] Coupled motions between the M20 and βFG loops are not observed in the product complexes.[28] Similar coupling of the motions of the M20 and βFG loops is seen in the Michaelis complex of TmDHFR with additional correlated motions across the dimer interface.[31]

It has been proposed that enzymes in which tunneling is coupled to long-range protein motions should be affected by

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changes to the solvent composition.[32] Reducing the dielectric constant of the solvent strengthens H-bonding networks, making the protein more stable and less flexible.[33] We have recently reported the effect of solvent composition on catalysis by TmDHFR and found that the rate constant for hydride transfer in the TmDHFR-catalyzed reaction determined in presteady-state kinetic measurements was indeed affected by the dielectric constant of the medium.[34] Interestingly, the viscosity of the medium did not affect the rate constant for hydride transfer, whereas the primary KIE was affected by neither parameter.[34] TmDHFR is known to be less flexible than EcDHFR[27] and therefore the absence of a viscosity dependence of the rates was interpreted to be due to the lower amplitude of the rate-promoting motions in the hyperthemophilic enzyme.[34] In contrast, catalysis by EcDHFR might be expected to depend on solvent viscosity due to the higher flexibility of this mesophilic enzyme.

We report here that the EcDHFR-catalyzed reaction is sensitive to changes in the solvent composition. A dependence of the rate constant for the hydride transfer step on the dielectric constant and the percentage of cosolvent/cosolute, but not on viscosity, was observed. These results exclude major contributions to tunneling from long-range motions and suggest either that local motions only contribute to catalysis of hydride transfer in EcDHFR or that populations of conformationally and kinetically distinct substates characterize EcDHFR catalysis.

Experimental Section

NADPH was purchased from Melford. EcDHFR, TmDHFR, NADPD, and H_2F were prepared as described previously.[21,35] In brief, EcDHFR was purified by methotrexate affinity chromatography followed by anion exchange chromatography on DEAE resin, whereas TmDHFR was purified by heating the crude cellular lysate of TmDHFR-containing *E. coli* cells to 75 °C for 30 min to precipitate native proteins followed by cation exchange chromatography on SP-sepharose resin. NADPD was prepared by enzymatic reduction of $NADP^+$ using the alcohol dehydrogenase from *Thermoanaerobacter brockii* with perdeuterated isopropanol as the deuteride source. Dihydrofolate was synthesized by dithionite reduction of folate.

Circular dichroism (CD) experiments were performed on an Applied Photophysics Chirascan spectrometer at a protein concentration of 10 μM in 5 mM potassium phosphate (pH 7.0) containing 50% cosolvent. Protein unfolding was followed by monitoring the CD signal at 222 nm between 5 and 90 °C with a temperature gradient of 0.2 °C min^{-1}.

All kinetic experiments at pH 7 were performed in 100 mM potassium phosphate buffer containing 100 mM NaCl. Kinetic experiments at pH 9.5 and activity versus pH measurements were performed in MTEN buffer (50 mM MES, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl). Cosolvent concentrations of 17%, 33%, and 50% (volume cosolvent per final solution volume) were used. As the rate of the DHFR-catalyzed reaction is pH sensitive,[21,36] the pH was adjusted after the addition of cosolvent to ensure consistency. Details of dielectric constants and viscosities of solvent mixtures are given in ref 34.

Presteady-state kinetic experiments were performed using an Applied Photophysics stopped flow instrument with 2.5 mL drive syringes. DHFR (40 μM) was preincubated with NADPH or NADPD (16 μM) for at least five minutes and the reaction was started by rapid mixing with an equal volume of dihydrofolate (200 μM). Reactions were monitored by fluorescence energy transfer with excitation at 292 nm and detection using a 400 nm cutoff filter. For the reductive half-reaction of morphinone reductase, organic cosolvents impaired binding of the cofactor.[32,37] In the case of EcDHFR (and TmDHFR), increasing the concentrations of substrate and cofactor did not lead to a change in the observed rate constant for all solvent compositions (Supporting Information).

Steady-state rates at 20 °C were measured spectrophotometrically, by following the decrease in absorbance at 340 nm during the reaction. EcDHFR (20 nM for pH 7 measurements, 200 nM for pH 9.5) was preincubated with NADPH (0.5−100 μM) for one minute to avoid hysteresis and the reaction was then started by addition of dihydrofolate (100 μM final concentration). Accurate K_m values could not be determined in all cases due to the relatively high error on the measured rates at low cofactor concentrations in certain cosolvents. All values for k_{cat} reported here were measured under saturating conditions.

Results and Discussion

Effect of Cosolvent on EcDHFR Catalysis. The steady-state rate constants (k_{cat}) at pH 7 and pH 9.5 and the rate constants for hydride transfer (k_H) at pH 7 for the EcDHFR-catalyzed reaction were measured in the presence of organic cosolvents or cosolutes at 20 °C (Figure 2 and Supporting Information). Increasing amounts of cosolvent/cosolute led to a reduction of all three rate constants in a solvent specific manner. The rate constants decreased proportionally to the dielectric constant at pH 7 but were not reduced in a manner directly proportional to the viscosity of the medium. This is similar to the effect that has previously been observed for TmDHFR.[34] At pH 9.5, the rate of reaction increased slightly as the dielectric constant decreased from 80 to 70 but decreased as the dielectric constant was reduced further. At pH 7, the KIE on k_H was largely independent of the solvent composition (Figure 2), whereas at pH 9.5 the KIE on k_{cat} decreased as the dielectric constant decreased, suggesting a change in the rate-limiting step and demonstrating that solvent composition has a greater influence on the physical steps of the reaction than on the chemical step. The steady-state data did not indicate reversible inhibition and the values for k_{cat} were unaffected by increased incubation times of the enzyme with the solvents (data not shown). Hence, we conclude that the reduction in rates is not due to specific inhibition of the enzyme. The effect of solvent on TmDHFR catalysis has been shown previously not to result from electrostatic effects on the reaction itself[34] and this is likely also the case for EcDHFR. k_{cat} at pH 7 reports on a physical step of the reaction rather than hydride transfer[36] yet is also reduced as the dielectric constant decreases even though hydride transfer is not rate limiting. This may be due to a change in the flexibility of the enzyme related to the strength of the network of hydrogen bonds between the M20 and βFG loops, which must break and reform during the catalytic cycle.[23] K_m values could not be determined accurately in all cases due to high errors on the measured rate data at low cofactor concentrations in certain cosolvents, but in general the presence of

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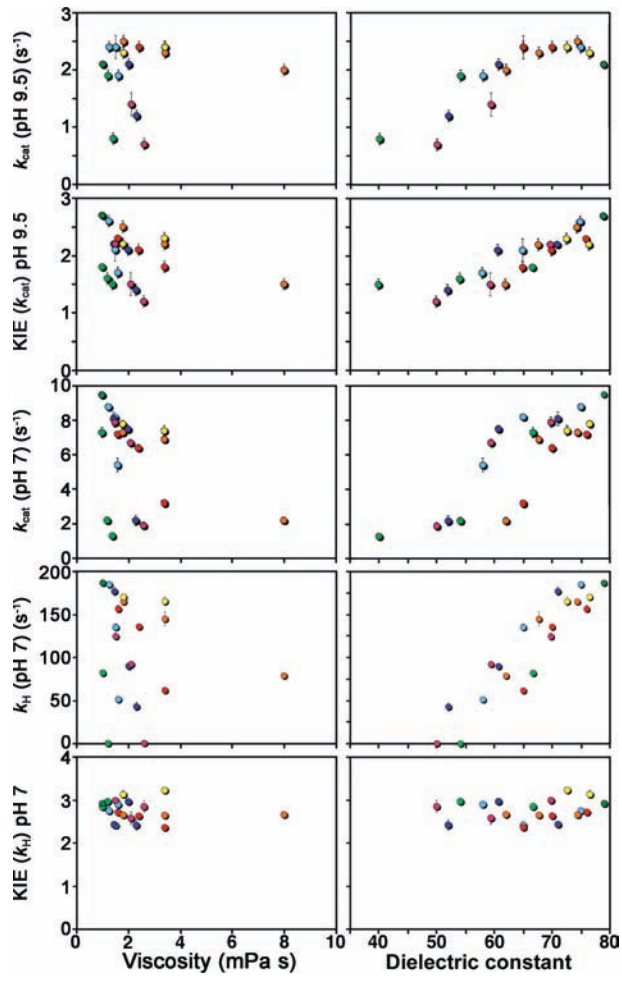

Figure 2. Plots of k_H and the KIEs on k_H at pH 7, k_{cat} at pH 7, and k_{cat} and the KIE on k_{cat} at pH 9.5 for the EcDHFR-catalyzed reaction against solution viscosity (left) and dielectric constant (right). Symbols represent the different cosolvents used, where dark green denotes no cosolvent, light blue = methanol, dark blue = ethanol, purple = isopropanol, red = ethylene glycol, orange = glycerol, yellow = sucrose, and light green = tetrahydrofuran.

cosolvent reduced the K_m, leading to an initial increase in k_{cat}/K_m followed by a decrease as the cosolvent concentration was increased further (Supporting Information).

Circular dichroism (CD) spectroscopy was used to investigate the effect of cosolvent on the secondary structure of EcDHFR. Although the enzyme showed little structural change in the presence of glycerol, sucrose, and methanol, some loss of structure was observed in the presence of other cosolvents, particularly tetrahydrofuran (THF) (Supporting Information). In these cases, it is likely that the reduction of the rate constants is partly due to a degree of solvent-induced denaturation. 50% glycerol and sucrose led to an increase in the melting temperature of EcDHFR from 51.2 ± 0.4 °C in the absence of cosolvent to 57.1 ± 1.1 and 56.4 ± 0.7 °C respectively, whereas 50% methanol caused a reduction in the melting temperature to 26.1 ± 0.2 °C (Supporting Information). We have previously shown that the melting temperature of EcDHFR is slightly increased (~2 °C) in the presence of 0.5 M (9% w/v) maltose.[38]

Because the enzyme is much larger than the solvent molecules, Ivković-Jensen and Kostić have shown that the effect

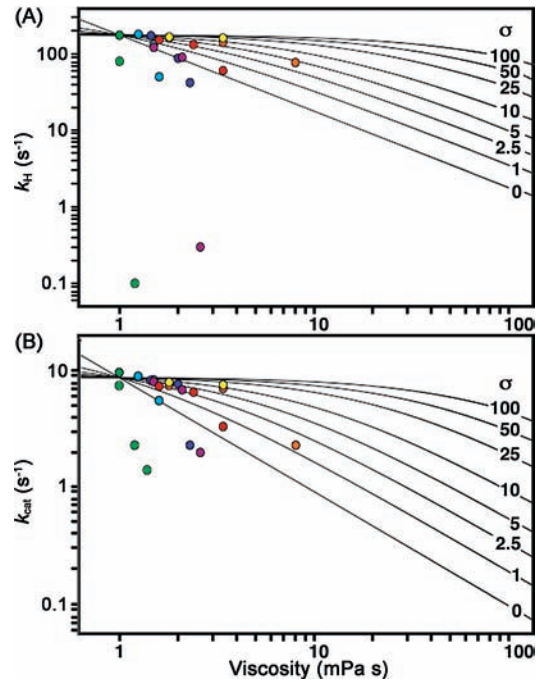

Figure 3. Rate constants k_H (A) and k_{cat} (B) for EcDHFR-catalyzed hydride transfer plotted against viscosity at pH 7. The rate-constant data are modeled (solid lines) according to the equation: $k_{obs} = (Tk_B/h)((1 + \sigma)/(\eta + \sigma))\exp(-\Delta G^{\dagger}/RT)$, which describes the contribution of the internal protein friction σ to the total friction of the system, where T is the absolute temperature, k_B the Boltzmann constant, h is Planck's constant, η is the solvent viscosity, $\Delta G^{\dagger}$ is the activation free energy for the reaction, and R is the gas constant.[40,41] $\Delta G^{\dagger}$ is assumed to be independent of viscosity.[41] Protein internal friction σ was varied between 0 (no internal friction) and 100 mPa·s. Values for $\Delta G°$ of 66.49 and 59.06 kJ mol^{-1} obtained from previous temperature-dependence measurements for k_{cat} and k_H were used.[21] The experimental data do not fit the model at any protein internal friction. Symbols represent the different cosolvents used, where dark green denotes no cosolvent, light blue = methanol, dark blue = ethanol, purple = isopropanol, red = ethylene glycol, orange = glycerol, yellow = sucrose, and light green = THF.

of viscosity on the rate constant can be expressed through a combination of the Eyring equation with the Eaton-Ansari extension of Kramers' theory.[39−41] As for TmDHFR,[34] the rate constants for the EcDHFR-catalyzed hydride transfer did not correlate with viscosity for any value of internal friction (Figure 3). By contrast, the values of k_{cat} at pH 7 fit reasonably well for an enzyme internal friction of approximately 2.5 mPa·s with the exception of tetrahydrofuran and high concentrations of methanol, ethanol, and isopropanol. These deviations may to some extent be due to solvent-induced partial denaturation (Supporting Information) but the dominant effect of dielectric constant over viscosity will also play a role. k_{cat} at pH 9.5, where the reaction rate is limited by hydride transfer in the absence of cosolvents, did not correlate well with viscosity (data not shown). In agreement with the results obtained using the extension of Kramers' method described above, plotting the normalized $1/k_{cat}$ for the EcDHFR catalyzed reaction against relative viscosity according to the method of Kirsch[42,43] gave an approximate linear fit with a slope of ~0.3 for pH 7

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(Supporting Information) indicating that diffusion plays a minor role in limiting the reaction rate, whereas similar plots for k_{cat}/K_m of EcDHFR, k_{cat} of TmDHFR, and k_H of both enzymes gave no correlation with viscosity (Supporting Information).

The absence of a viscosity effect for TmDHFR has previously been attributed to the presence of low amplitude motions only due to the increased rigidity necessary to support the structure of this hyperthermophilic enzyme at high temperatures.[34] Motions in EcDHFR would be expected to be of greater amplitude[27] and should therefore be affected by viscosity.[32] The proposal by Benkovic and Hammes-Schiffer that catalysis by EcDHFR is driven by a network of promoting motions that spans the entire protein[14,29] would also predict a viscosity dependence of the reaction. The reductive half-reaction of morphinone reductase has previously been shown to be unaffected by viscosity or dielectric constant, which was interpreted as indicating the absence of long-range promoting motions.[32,37] Computational studies detected localized promoting vibrations in the active sites of a related aromatic amine dehydrogenase[44] and of human purine nucleoside phosphorylase[45] as well as a more extended promoting motion in lactate dehydrogenase.[46] More recently, experimental evidence has been obtained that is in agreement with the existence of a localized promoting vibration in the active site of pentaerythritol tetranitrate reductase.[47]

In the case of TmDHFR, glycerol (and to a lesser extent sucrose) led to increased rate constants for hydride transfer.[34] This was not seen in EcDHFR, where all cosolvents caused a reduction in the rate constants for hydride transfer (Figure 2). To further investigate this, rate constants for hydride transfer in EcDHFR and TmDHFR were measured in 50% methanol, 50% glycerol, and 30% sucrose for varying values of pH (Figure 4) to determine the apparent pK_a of the reaction (Table 1). These experiments clearly show that the increase in activity seen for TmDHFR in the presence of glycerol is a direct consequence of a pK_a shift. A similar shift of the pK_a of the reaction was seen for EcDHFR (Table 1) but did not lead to an increase in the hydride transfer rate at pH 7. Methanol (50%) led to a pronounced increase in the pK_a for both enzymes but this too was counteracted by the overall decrease in the hydride transfer rate. Sucrose did not cause an apparent pK_a shift. Although these results suggest that the pK_a shift is caused by structure-specific effects rather than bulk solvent properties, the increase in hydride transfer rate in the presence of glycerol was most likely not due to interaction of the polyol with the TmDHFR dimer interface.[34] Further work is needed to explain this pK_a shift and the difference between the two enzymes.

Effect of Cosolvent on the Temperature Dependence of the KIE. The effects of cosolvents on the EcDHFR-catalyzed reaction were further analyzed by measuring the temperature dependence of the KIEs for the chemical step in the presence of methanol, glycerol and sucrose at pH 7 (Figure 5 and Supporting Information). Neither methanol nor glycerol had a significant effect in that the KIEs for hydride transfer remained

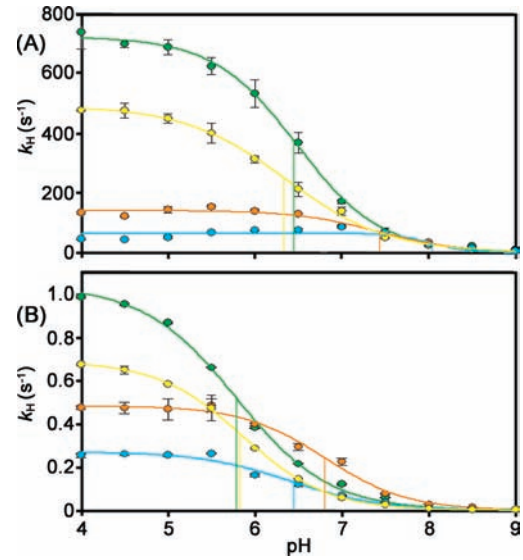

Figure 4. Plots of k_H against solution pH for EcDHFR (A) and TmDHFR (B) in MTEN buffer at 20 °C in the absence of cosolvents (dark green), and in the presence of 50% methanol (light blue), 50% glycerol (orange), and 30% sucrose (yellow). Vertical bars indicate the apparent pK_a of the reaction.

Table 1. Effect of Cosolvent on the Apparent pK_a of the Reactions Catalyzed by EcDHFR and TmDHFR

	pK_a	
cosolvent	EcDHFR	TmDHFR
none	6.48 ± 0.03	5.79 ± 0.04
50% methanol	8.03 ± 0.19	6.41 ± 0.10
50% glycerol	7.42 ± 0.10	6.81 ± 0.06
30% sucrose	6.36 ± 0.03	5.86 ± 0.01

temperature dependent between 15 and 35 °C (parts A and B of Figure 5). At lower temperatures, a reduction in the KIE indicates kinetic complexity from either H_2F binding or a conformational change preceding hydride transfer (as single-turnover conditions were used, any additional partially rate-limiting step must precede hydride transfer to have an effect on the observed rate). The addition of sucrose, on the other hand, led to largely temperature-independent KIEs (part C of Figure 5) as had been observed for TmDHFR.[34]

The behavior of the KIE for EcDHFR is not dominated by bulk solvent properties, as also observed for TmDHFR.[34] Equivalent glycerol and sucrose concentrations have similar viscosities but very different effects on the KIE. Methanol and glycerol concentrations with the same dielectric constants had comparable effects on the KIE. It is therefore not possible to comment directly on the effect of dielectric constant on the temperature dependence of the KIE, but clearly the dielectric constant had no effect on the magnitude of the KIE in either EcDHFR (vide supra) or TmDHFR.[34] In addition, the dielectric constant of the medium was not responsible for the effect on the temperature dependence of the KIE for TmDHFR.[34] Solvent-induced pK_a shifts are not responsible either, as sucrose has the largest effect on the temperature dependence of the KIE, yet does not affect the apparent pK_a of the reaction.

These observations are difficult to interpret within the environmentally coupled tunneling model. Our earlier studies on TmDHFR mainly dealt with temperature independent KIEs due to deleterious effects of methanol at low temperatures, which complicated the analysis. However, increasing glycerol

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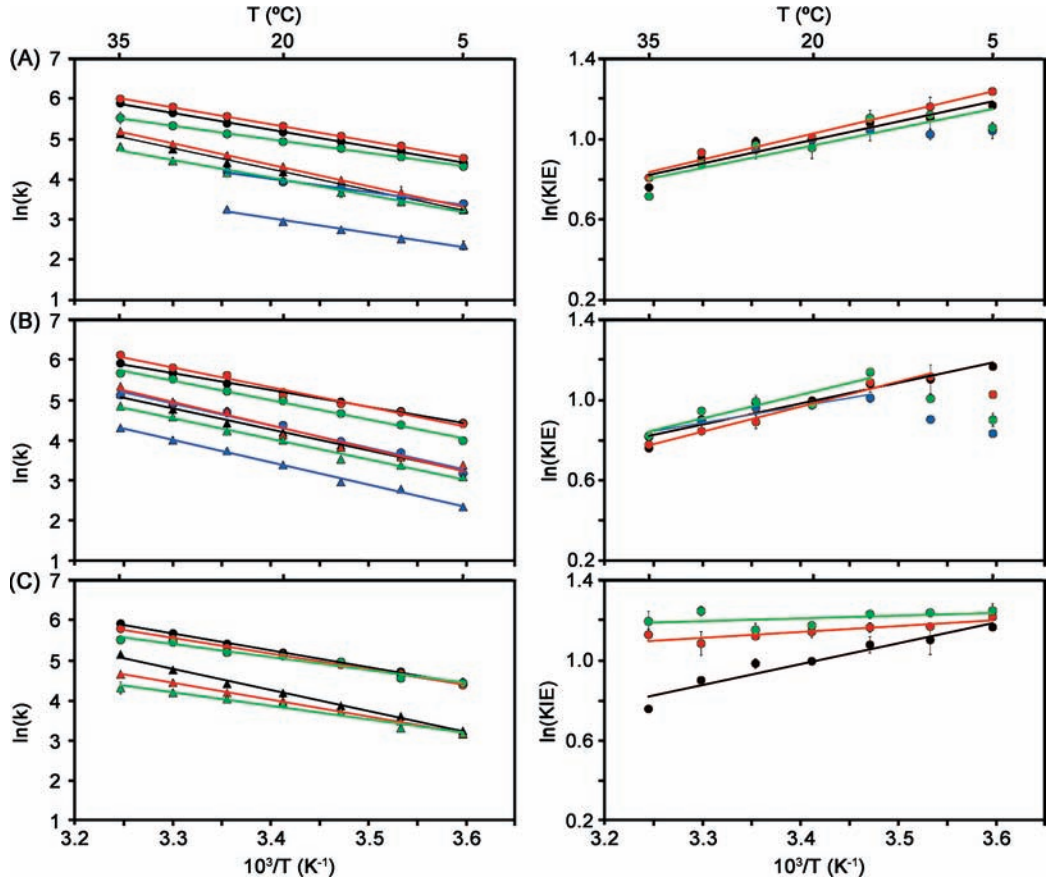

Figure 5. Arrhenius plots for EcDHFR-catalyzed hydride (circles) and deuteride (triangles) transfer (left) and the corresponding KIEs plotted on a logarithmic scale against the inverse temperature (right) at pH 7 in the presence of 0% (black), 17% (red), 33% (green; 30% in the case of sucrose), and 50% (blue) methanol (A), glycerol (B), or sucrose (C). Note that, for 50% methanol, rate constants could only be measured below 30 °C, consistent with CD measurements, which indicated thermal unfolding above 25 °C (vide supra).

concentrations led to an increase in the temperature dependence of the KIE below the kinetic breakpoint, whereas sucrose made the KIE fully temperature independent.[34] The results obtained for TmDHFR could be interpreted within the environmentally coupled tunneling model as demonstrating solvent effects on gating dynamics. However, the results reported here for EcDHFR do not bear this out. Neither methanol nor glycerol has any apparent effect on gating motions and only sucrose causes similar behavior in both EcDHFR and TmDHFR.

These results are consistent with environmentally coupled tunneling only if barrier compression in EcDHFR is not coupled to large-scale motions[14,25,29] but is instead restricted to the active site as a short-range promoting vibration. Long-range correlated motions between the M20 and βFG loops of EcDHFR in the Michaelis complex but not in the product complex were originally suggested as evidence that these motions are important for catalysis.[28] However, these loops are over 10 Å apart in the product complexes, whereas they form a hydrogen bonding network in the Michaelis complex.[23] The large geometric differences in these complexes provide a simpler explanation of their different catalytic properties. It is also worth noting here that under physiological conditions the chemical step in EcDHFR is 10-fold faster than the rate-limiting product release.[36] Hence, in the absence of selective pressure, it is not obvious why this enzyme should have evolved to optimize the chemical step through barrier compression.

Within an environmentally coupled tunneling model, the results presented here for EcDHFR and the published results for TmDHFR[34] suggest that the mechanism of coupling of protein motions to the reaction is unaffected by bulk solvent composition. Given the wide-ranging general effects of solvent composition on protein flexibility and motions,[33,48,49] this seems unlikely. The arguments presented above that the correlated motions seen in EcDHFR[28] and TmDHFR[31] do not necessarily have an effect on hydride transfer hold equally well here. Warshel and co-workers have argued that catalysis in DHFR is dominated by effects on the reorganization energy of the reaction.[50,51] Reorganization energies are likely to be perturbed by changes to the bulk solvent composition and this therefore represents a simple yet highly plausible explanation for the results presented here. Furthermore, several other experimental observations are difficult to interpret within an environmentally coupled model of hydrogen transfer and suggest an alternative explanation of the temperature dependence of the KIEs for DHFR. First, X-ray crystallography,[23] NMR analysis,[24] and single-molecule experiments[52] have indicated that EcDHFR exists in several well-defined conformations that control the physical steps of cofactor and substrate binding and release. These experiments point to the existence of free-energy landscapes for enzyme−substrate complexes with distinct

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reaction pathways.[53] In NMR relaxation studies, conformational substates of EcDHFR were identified that drive the reaction along the preferred kinetic path.[24] Equilibrium unfolding measurements performed in our laboratory indicated that the native form of EcDHFR may not exist as one single conformation but rather as an ensemble of active forms.[35] Some of these conformations have been shown in single-molecule and transient kinetic experiments to have distinct catalytic properties.[52] Our study of the thermal unfolding of EcDHFR and EcDHFR-G121V showed that the diminished catalytic activity of EcDHFR-G121V was mainly the consequence of nonlocal structural effects. Whereas this may be interpreted to rescue the proposal of a network of long-range promoting motions[14,29] as evidence for disruption of the proposed network, it may simply indicate that the bulky isopropyl group of EcDHFR-G121V is likely to prevent formation of the hydrogen bonding network between the M20 and βFG loops that usually exists in the closed conformation[23] and so may prevent the M20 loop from closing properly over the active site. Alternatively, insertion of an isopropyl group may affect the ensemble of enzyme conformations.[35]

In conclusion, the results presented here suggest strongly that a network of long-range promoting motions does not drive catalysis by EcDHFR and TmDHFR. However, they do not exclude the existence of local promoting vibrations in DHFRs as identified computationally in purine nucleoside phosphorylase[45] and aromatic amine dehydrogenase[44] or indeed of more extended motions of small amplitude as observed in lactate dehydrogenase.[46] The present study does not address the existence of such rapid promoting vibrations.

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Our results, which show that the average structure as determined by CD spectroscopy as well as the catalytic properties of TmDHFR and EcDHFR depend on the solvent but not on bulk solvent properties, could also be interpreted to suggest that the temperature dependence of the KIEs of EcDHFR is a consequence of a variation in the populations of the different conformational substates with varying catalytic activity. Each reactive enzyme conformation is capable of catalysis by way of the same chemistry but the microscopic rate constants for each reaction will differ potentially as a consequence of different low amplitude promoting vibrations. The overall reaction rate achieved by this ensemble of enzymes would then be the statistic average over all substates. This is an attractive and experimentally testable explanation of the solvent and temperature effects on catalysis by TmDHFR and EcDHFR. The perturbation of the thermal equilibrium between a large number of nearly isoenergetic substates, within which promoting vibrations may or may not contribute to catalysis, through alterations of the reaction conditions may then cause the observed effects on the reaction rates and the kinetic isotope effects for hydride transfer.

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Supporting Information Available: Circular dichroism spectra and melting curves; solvent dependence of k_{cat}/K_m; Kirsch plots of the rate data; tabulated data for k_H, KIE(k_H), k_{cat}, KIE(k_{cat}), K_m and k_{cat}/K_m at 20 °C, and for the pH and temperature dependences of k_H and KIE(k_H); activation energies and Arrhenius prefactors. This material is available free of charge via the Internet at http://pubs.acs.org.

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