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The Effect of Electrostatic Shielding on H Tunneling in R67 Dihydrofolate Reductase

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Dihydrofolate reductase (DHFR) catalyzes the hydride (H)-transfer reaction between nicotine adenine dinucleotide phosphate (NADPH) and dihydrofolate to produce tetrahydrofolate and $NADP^+$. R67 DHFR is a plasmid-encoded enzyme, and is considered to be a "primitive enzyme" due to its genomic, structural, and kinetic properties.[1,2] Interestingly, kinetic studies of R67 DHFR show an enhancement in H-transfer rate with increasing ionic strength.[3] To evaluate the source of this rate ancement, the temperature dependency of intrinsic kinetic isotope effects (KIEs) was measured, and the nature of the H-transfer step was evaluated at low and high ionic strengths. At high ionic strength, the KIEs were less temperature dependent than at lower ionic strength. These findings were evaluated by using a Marcus-like model, which suggests that, at higher ionic strength, the donor and acceptor of the hydride are better oriented for H tunneling than the same system at lower ionic strength. This comparison addresses the level of system preparation that brings the reaction coordinate into a tunneling-ready conformation. While the effect is small, it is statistically significant, as apparent from the comparative data and standard deviations presented in the Supporting Information (Table S2). These data demonstrate the high sensitivity of the methodology that was developed to study this system (see detailed methods in the Supporting Information). The differences in electrostatic potential surface between low and high ionic strengths were calculated, and the theoretical findings add a molecular perspective to the experimental data.

A kinetic isotope effect (KIE) is the ratio of rates of two reactants that only differ in their isotopic composition. The temperature dependence of intrinsic KIEs is sensitive to a reaction's potential surfaces and dynamics. It serves as an excellent probe for quantum-mechanical H tunneling and the organization of the H donor and acceptor at the enzyme's active site.[4,5] One way to assess the intrinsic KIEs involves measuring the KIEs for the three isotopes of hydrogen.[5–7] This methodology can evaluate the nature of the H-transfer reaction in enzyme catalysis by specifically focusing on the chemical step in the complex kinetic cascade of an enzymatic reaction. Because of the large mass ratio of the three different isotopes of hydrogen, large and distinct KIEs on the measurements give precious information on the reaction potential surface.[8] For instance, temperature-independent KIEs have been reported for several highly evolved enzymes under physiological conditions. This phenomenon was interpreted as an indication of precise organization of the reaction coordinate for efficient H tunneling. On the other hand, the same enzymes under nonphysiological conditions presented temperature-dependent KIEs, thus indicating a poorly organized reaction coordinate.[9–16] A primitive enzyme that was subjected to similar investigation also presented temperature-dependent KIEs.[17]

As discussed in more details in the above references, the temperature dependence of KIEs can be rationalized by using Marcus-like models. These models can be summarized in the following Equation (1):

$$k = Ce^{\frac{-(\Delta G^\circ+\lambda)^2}{4\lambda RT}} \int_{r_0}^{r_1} e^{F(m)} e^{\frac{-E_{F(m)}}{k_b T}} dDAD \qquad (1)$$

Here C is the fraction of reactive complexes, and the first exponential term is the traditional Marcus term, which is not very isotopically sensitive. ΔG° is the driving force for the reaction, λ is the reorganization energy, R is the gas constant, and T is the absolute temperature in Kelvin. The second exponential term is the Frank–Condon term representing the integrated tunneling probability of all of the relevant donor–acceptor distances as a function of the mass of the transferred particle ($F(m)$). It is isotopically sensitive, but temperature independent. The last exponential term describes the donor–acceptor distance (DAD) fluctuations, namely the conformational space sampled by the DAD at a given temperature, where $E_{F(m)}$ is the excitation energy for the gating motion between donor and acceptor, as a function of the mass of the transferring particle, and k_b is the Boltzmann constant. This last term is both isotopically and temperature sensitive.[18] Based on these models, a large temperature dependence of KIEs indicates poor organization of the reaction coordinate, whereas temperature-independent KIEs indicate that the enzyme has evolved to bring the donor and acceptor to an optimized hydrogen DAD for hydrogen tunneling (Figure 1).

This study focuses on a plasmid-encoded type of DHFR that is found in several strains of bacteria that have developed resistance to the antibiotic drug, trimethoprim (TMP). In contrast to chromosomal, *dfr*A- or *fol*A-coded DHFR (*c*DHFR), R67 DHFR is coded by the R-plasmid *dfrB*, and shares no sequence or structural similarities with *c*DHFR. Yet, both the chromosomal and plasmidal DHFRs catalyze the same reaction: reduction of

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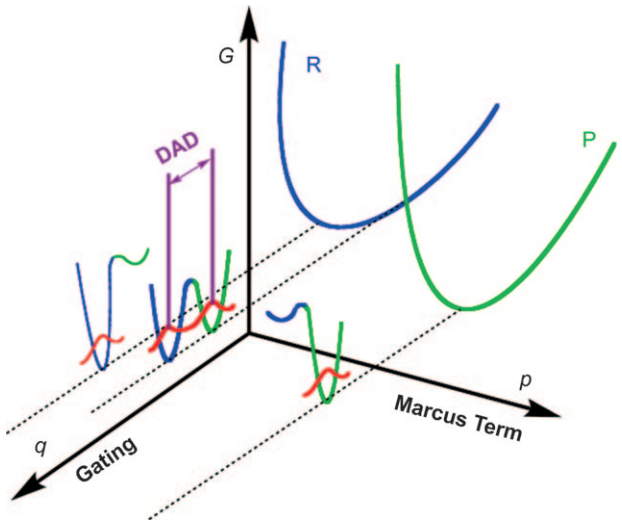

Figure 1. Three-dimensional illustration of Marcus-like models: energy surface of environmentally coupled H tunneling. Two orthogonal coordinates are presented: *p*, the environmental energy parabolas for the reaction state (R: blue) and the product state (P: green), and *q*, the gating coordinate, along which the red lines represent the hydrogen's probability wavefunction. Thermal fluctuations in the DAD along the *q* coordinate lead to the temperature dependence of the KIEs.[17]

7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) with the stereospecific hydride transfer from the pro-*R* C4 position of NADPH to the *S* C6 position of H_2F. In contrast to chromosomal DHFRs, R67 DHFR has a robust framework, and is considered to be a primitive enzyme, as described in detail in refs. [1] and [2]. R67 DHFR is a double-funnel-like homotetramer of 78 amino acid residues, and it forms a solvent-accessible large central pore as an active site. Not being sensitive to antibiotic inhibitors of chromosomal DHFRs, it allows antibiotic resistance. R67 DHFR has a catalytic efficiency two orders of magnitude lower than that of *c*DHFR, and makes a large entropic contribution to the activation parameters compared to the *c*DHFR.[19]

We previously reported that the nature of H transfer in the R67 DHFR-catalyzed reaction is very different from that of *c*DHFR. The chemical step (hydride transfer) is rate limiting, and the KIEs are temperature dependent, thus suggesting a poorly organized reaction coordinate and longer DAD.[17] Interestingly, we also found that the H transfer of R67 DHFR shows unusual salt dependence.[3] The Michaelis parameters, $K_{M(NADPH)}$ and k_{cat}, have the expected increase in K_M with higher ionic strength, but k_{cat} is also increased at higher salt concentrations (Table S1).[20] This phenomenon is not common and is not observed in the well-evolved cDHFRs, for which a much higher ionic strength than that of the cellular environment usually has a suppressing effect.[21] This is because high ionic strength increases the effective dielectric constant of the solvent, and shields the electrostatic effects at the active site. In this paper, we examine how the primitive R67 DHFR accelerates the H-transfer rate at elevated ionic strength.

To understand this unusual effect of ionic strength on R67 DHFR, we combined theoretical and experimental approaches. The differential electrostatic potential surface of the R67 DHFR–NADPH–H_2F ternary complex[22] between physiological ionic strength ($\mu = 0.15$ M) and high ionic strength ($\mu = 0.42$ M)[20] was calculated. The calculation suggested that, at a low ionic strength, the enzyme's active site is more positively charged, while the exterior of the protein is more negatively charged at the lower ionic strength (Figure 2 A and B). The calculations confirmed that, at high ionic strength, the effective charges in the whole system are reduced, and thus the electrostatic repulsion between the nicotinamide and pterin rings (hydrogen donor and acceptor) at the active site is also reduced (Figure 2 C). Based on these results, we hypothesized that the DAD might be reduced by this change, leading to a more organized reaction coordinate that is better poised for H tunneling.

To test this hypothesis, we compared the nature of the H-transfer at high and low ionic strengths. We used competitive KIE experiments with R67 DHFR at high ionic strengths ($\mu =$ 0.42 M) in a modification of a method we have previously

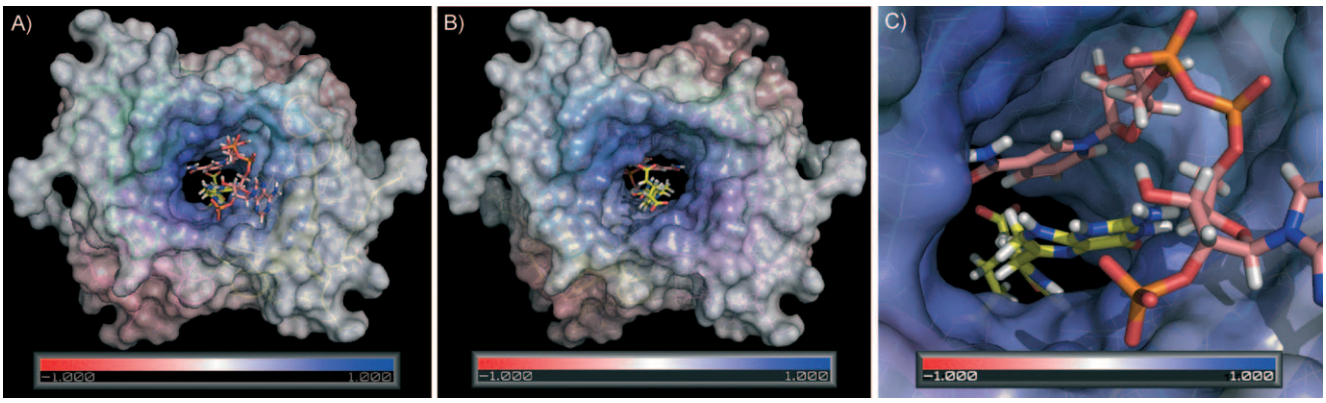

Figure 2. The change in the electrostatic potential of R67-DHFR at different salt concentrations. The electrostatic potential at $\mu = 0.15$ M minus the potential at $\mu = 0.42$ M is mapped onto the molecular surface of the protein, according to the color scale (kT/e). On this scale, blue surfaces indicate that the positive charge is higher at $\mu = 0.15$ M, while red surfaces indicate that the negative charge is higher at 0.42 M salt. The figure was produced using PyMol (DeLano, W.L., The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA (http://www.pymol.org)). A) NADPH binding domain, B) H_2F binding domain, C) the active site of the enzyme.

reported for R67 DHFR.[17,23] Both primary H/D and H/T KIEs on the second-order parameter (k_{cat}/K_M) were measured across a temperature range of 5–45 °C, as described in detail in the Supporting Information, under the Experimental Section.

The current determination of KIEs is more sensitive than previously reported due to the analysis of the larger H/D (0.87% deviation) rather than the smaller D/T KIE (1.2% deviation, see Table S2). A significant improvement in the methodology results from having all the radioactive labeling on the nicotinamide derivatives and none on the product H_4F or its derivatives. The relation between H/D and H/T KIEs clearly follows the semiclassical Swain–Schaad relationship,[24] and serves as a strong indication that the observed KIEs are the intrinsic KIEs across the temperature range. As found for low ionic strength,[17] the H-transfer step is the rate-limiting step at high ionic strength, and no kinetic-complexity is masking the reported data.

The intrinsic H/T KIEs were evaluated by using an Arrhenius plot, and the findings were compared to the same studies conducted at low ionic strength. The exponential fitting of the experimental data to the Arrhenius equation for KIE provides the isotope effects on the activation parameters for both ionic strengths (Figure 3). At low ionic strength, R67 DHFR KIEs showed steeper temperature dependence than at high ionic strength ($\Delta E_a = 0.87 \pm 0.03$ vs. $\Delta E_a = 0.80 \pm 0.03$ kcal mol^{-1} at low and high salt, respectively). The isotope effects on the pre-exponential factor were a little smaller at low ionic strength ($A_H/A_T = 1.36 \pm 0.07$ vs. $A_H/A_T = 1.49 \pm 0.08$ at low and high salt, respectively). These differences are at the limit of the detection of the improved methodology, but are statistically, and quantitatively significant (Table S3).[14–16]

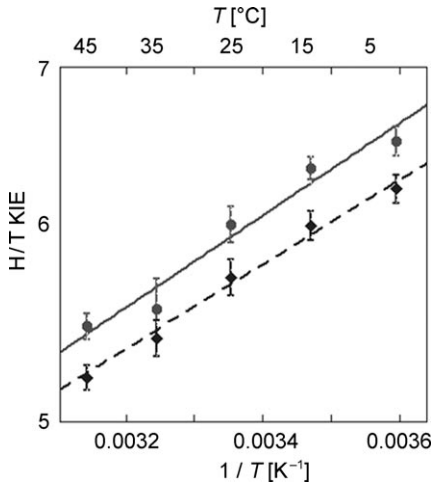

Figure 3. A comparison of the Arrhenius plots for the H/T KIEs (log scale) vs. the reciprocal of the absolute temperature at low (●) and high (◆) ionic strength.

Here, a small change (2.5-fold) in H-transfer rate at two different ionic strengths seems to be associated with a change in the temperature dependence of intrinsic KIEs. Additionally, at high ionic strength, the intrinsic KIEs became somewhat smaller. In accordance with Marcus-like models, a reduced temperature dependency in KIEs suggests that the average DAD at the tunneling-ready conformation at high salt is shorter and requires less "gating" than at low ionic strength. Together, these findings support the hypothesis suggested above, namely that the average DAD[25] is shorter at high ionic strength due to reduced electrostatic repulsion. Our findings indicate that, at the active site of R67 DHFR, the electrostatic shielding provided by the increased salt improves the orientation of the donor and acceptor at the tunneling-ready conformation and increases k_{cat}.

In summary, a modified analysis of the nature of H transfer in the reaction catalyzed by R67 DHFR improved sensitivity limits and exposed the effect of high ionic strength on the intrinsic properties of the catalyzed chemistry. The findings revealed a small but significant alteration of organization of the system and the DAD for H tunneling as result of altering salt concentration. The combination of calculations and kinetic studies suggests that, at high ionic strength, the reduced electrostatic repulsion leads to shorter DAD for H tunneling.

Experimental Section/Computational Methods

Kinetic isotope effect measurements: The synthesis of labeled substrates and the kinetic measurements followed the methods we have established in the past for the same enzyme.[17] Several improvements and modifications are deliberated in the Supporting Information.

Calculation of the difference in electrostatic potentials on R67 DHFR: The effect of the different salt concentrations on the electrostatic potential on the surface of the protein scaffold was calculated by using established methodologies (see details in the Supporting Information). The potential at high ionic strength was subtracted from that at low ionic strength, and the resulting image is presented in Figure 2. Since many possible errors in the calculations are similar in both calculations, the result presented in Figure 2 is more reliable than each individual calculation.

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