# Donor-Acceptor Distance Sampling Enhances the Performance of "better than Nature" Nicotinamide Coenzyme Biomimetics

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# Donor–Acceptor Distance Sampling Enhances the Performance of "Better than Nature" Nicotinamide Coenzyme Biomimetics

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Supporting Information

**ABSTRACT:** Understanding the mechanisms of enzymatic hydride transfer with nicotinamide coenzyme biomimetics (NCBs) is critical to enhancing the performance of nicotinamide coenzyme-dependent biocatalysts. Here the temperature dependence of kinetic isotope effects (KIEs) for hydride transfer between "better than nature" NCBs and several ene reductase biocatalysts is used to indicate transfer by quantum mechanical tunneling. A strong correlation between rate constants and temperature dependence of the KIE ($\Delta\Delta H^{\ddagger}$) for H/D transfer implies that faster reactions with NCBs are associated with enhanced donor–acceptor distance sampling. Our analysis provides the first mechanistic insight into how NCBs can outperform their natural counterparts and emphasizes the need to optimize donor–acceptor distance sampling to obtain high catalytic performance from H-transfer enzymes.

The search for synthetic biomimetics that can replace natural nicotinamide coenzymes has been driven by the instability and expense of NAD(P)H. This has prevented widespread use of natural coenzymes, for example, in biocatalytic manufacture of fine and specialty chemicals. The use of *in situ* regeneration systems to replenish natural coenzymes during catalytic turnover,[1−6] or through the use of hydrogen borrowing biocatalytic cascades,[7−11] is one solution; an alternative is to develop stable synthetic nicotinamide coenzyme biomimetics (NCBs) that can be regenerated and that have the potential to operate generally with biological oxidoreductases that normally function with NAD(P)H.[12] The development of such NCBs would be a game-changer in the green manufacture of chemicals. NCBs that satisfy these requirements are beginning to emerge. They offer hope for chemicals manufacture using oxidoreductases that catalyze a wide range of chemical transformations.[13]

NCBs support biocatalysis with many ene reductases (ERs),[12,14] which belong to the Old Yellow Enzyme (OYE) family (EC 1.3.1.31). These are broad specificity oxidoreductases that catalyze the asymmetric reduction of activated C=C bonds.[15] Their broad specificity and ability to introduce new stereogenic centers makes them attractive targets for industrial biocatalysis. Cognizant of these properties, we have reported crystal structures of selected ER-NCB complexes, a comprehensive analysis of reactions catalyzed by 12 ERs with 5 synthetic NCBs, and coenzyme analogue recycling to demonstrate the overall effectiveness of NCBs in ER-catalyzed biotransformations.[14] Some NCBs outperform natural coenzymes in the reduction of selected ERs.[14] The origin(s) of this enhanced catalytic performance is unknown. This knowledge is important for understanding of the physical basis of catalysis and also for the development of related biomimetics with high catalytic potential for other oxidoreductases.[16−19]

The ERs have been the subject of intensive study from the viewpoint of H-transfer,[20,21] aided in part by the availability of high-resolution crystal structures of several ERs and substrate/ligand complexes and the accessibility of the reaction cycle that comprises two half-reactions (Figure 1).[14,22−28] The kinetics of the reaction cycle have been derived using a number of ER–substrate combinations and, in selected cases, extended to obtain free energy profiles. Hydride transfer from natural nicotinamide coenzymes (NADH and/or NADPH) to the enzyme-bound flavin (flavin mononucleotide, FMN) involves significant quantum mechanical tunneling (QMT).[27] Temperature-dependent kinetic isotope effects (KIEs) on a range of enzymatic H-transfer reactions has led to the promoting vibrations hypothesis, where enzyme/substrate dynamics (conformational/distance sampling) are coupled to the H-transfer coordinate[29−32]—a model that has been developed extensively in relation to ER-catalyzed H-transfers.[27,33,34]

Here we set out to ascertain the origin of the enhanced performance of selected NCBs/ER combinations by studying the isotope dependence of reaction rate as a function of temperature.[20,21,28,35] The temperature dependence of KIEs is a key descriptor for distinguishing between semiclassical and QM mechanisms of transfer and for uncovering the relative and inferred importance of distance sampling/conformational coupling to the reaction coordinate.[29,32]

The temperature dependence of hydride transfer during the reductive half-reactions of the three ERs [PETNR, XenA, and thermophilic OYE (TOYE)] was measured with saturating concentrations of four coenzymes and NCBs [NADH, NADPH, 1-benzyl-1,4-dihydronicotinamide ($mNH_2$), and 1-butyl-1,4-dihydronicotinamide (mBu)] by stopped-flow spectroscopy that monitors the loss of oxidized FMN absorbance at 464 nm. Observed rate constants are given in Tables S1−S7, and Eyring plots of these data are shown in Figure 2 (and in expanded format in Figures S1−S3). The observed rate

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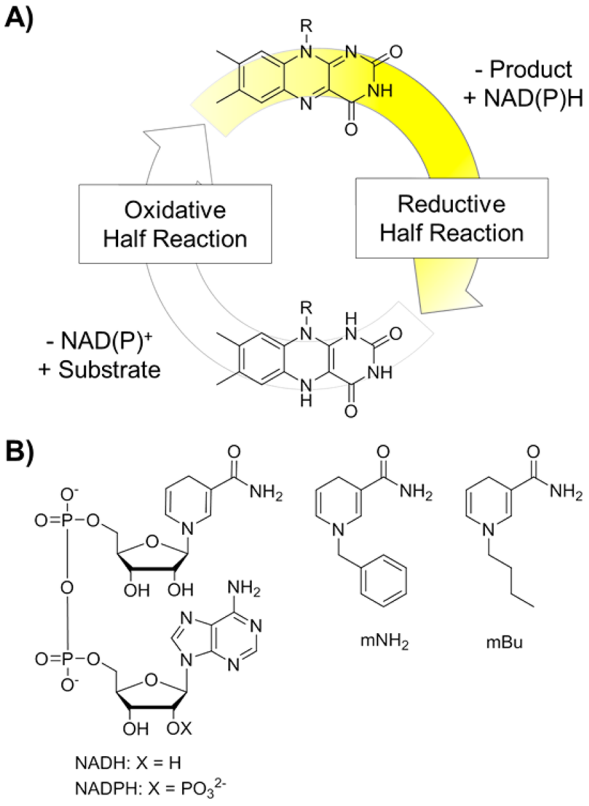


**Figure 1.** Catalytic cycle of PETNR and structures of the natural coenzymes and synthetic biomimetics. A) Reductive half-reaction, involving hydride transfer from the nicotinamide C4 pro-*R* position of NAD(P)H, or synthetic mimics, to the FMN N5 resulting in bleached flavin absorbance at 464 nm. The cycle culminates with hydride transfer from the dihydroFMN N5 and proton transfer from solvent to the oxidative substrate. B) The structure of natural coenzymes, NADPH and NADH, and the synthetic coenzyme mimics, 1-benzyl-1,4-dihydronicotinamide ($mNH_2$) and 1-butyl-1,4-dihydronicotinamide (mBu).

constants vary by 200-fold at 25 °C, from 2 $s^{-1}$ (PETNR:NADH) to 434 $s^{-1}$ (TOYE:mBu). In all 3 enzymes, NADH is the slowest substrate, and mBu is the fastest. The apparent activation enthalpy ($\Delta H^{\ddagger}$) varies by a factor of 2.5-fold from 22.8 ± 1.2 kJ $mol^{-1}$ (TOYE:mBu) to 58.5 ± 1.8 kJ $mol^{-1}$ (XenA:NADH) (Figure 3; Tables S7–S10). Some

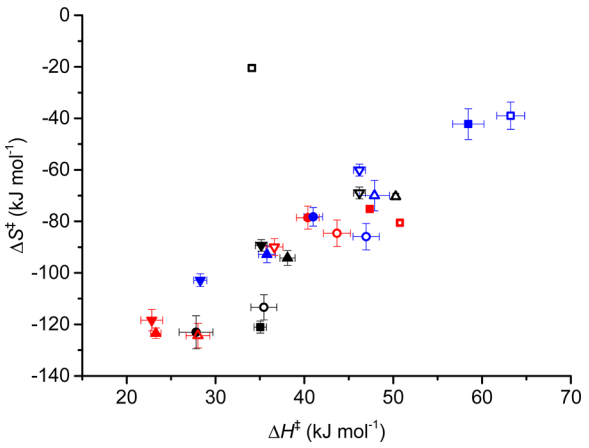


**Figure 3.** Relationship between apparent activation enthalpy and activation entropy determined from the Eyring plots in Figure 2. Black, red, and blue points correspond to those reactions with PETNR, TOYE, and XenA, respectively, using NADH (squares), NADPH (circles), $mNH_2$ (up-triangles), and mBu (down-triangles). Filled and hollow points correspond to those reactions with protiated and deuterated coenzymes, respectively.

enthalpy–entropy compensation is apparent, and the apparent activation entropy ($\Delta S^{\ddagger}$) varies from −124 ± 2 kJ $mol^{-1}$ $K^{-1}$ (TOYE:$mNH_2$) to −42 ± 6 kJ $mol^{-1}$ $K^{-1}$ (XenA:NADH).

In the TOYE and XenA reactions, there is some correlation between log $k_{obs}$ and $\Delta H^{\ddagger}$ for hydride (Figure 4) and deuteride (Figure S7) transfer, consistent with both transition state theory (TST) and models of enzymatic non-adiabatic H-transfer (i.e., by QMT). The PETNR reactions do not show

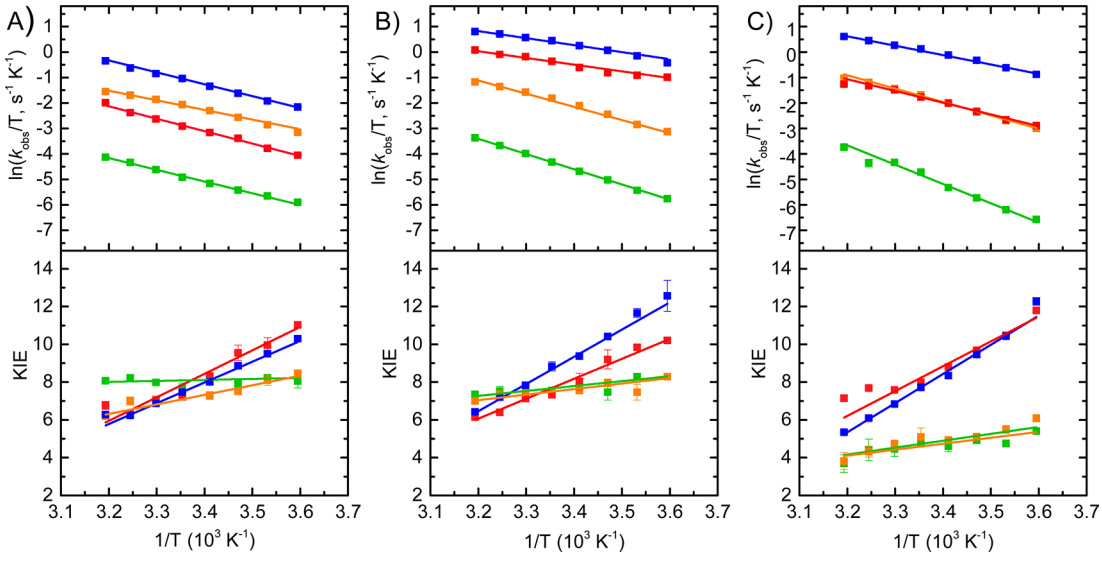


**Figure 2.** Eyring plots (top panels) and temperature dependence of the KIEs (bottom panels) on the reactions of PETNR (A), TOYE (B), and XenA (C) with natural coenzymes and synthetic coenzyme mimics NADH (green), NADPH (orange), $mNH_2$ (red), and mBu (blue). Data are fitted to the Eyring equation, with fitted parameters shown in Figure 3 and given in Tables S7–S10. Only protiated data are shown in the Eyring plots here; all data are shown in Figures S1–S3.



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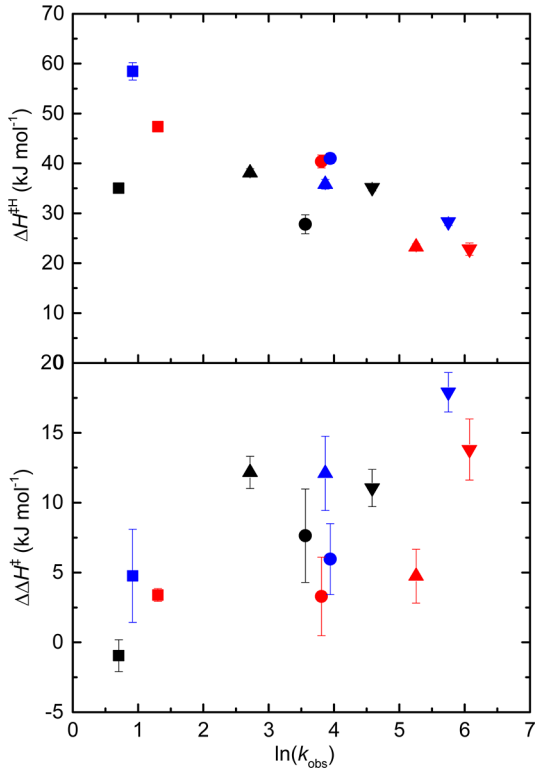


**Figure 4.** Relationship between $\Delta H^{\ddagger H}$ (upper panel; equivalent plot for $\Delta H^{\ddagger D}$ given in Figure S7) and $\Delta\Delta H^{\ddagger}$ ($\Delta\Delta H^{\ddagger} = \Delta H^{\ddagger D} - \Delta H^{\ddagger H}$; lower panel) and the observed rate constant for each hydride transfer reaction at 25 °C. Black, red, and blue points correspond to those reactions with PETNR, TOYE, and XenA, respectively, using NADH (squares), NADPH (circles), $mNH_2$ (up-triangles), and mBu (down-triangles).

this correlation, suggesting that the origin(s) of the reduction in activation enthalpy on the TOYE and XenA reactions must involve the enzyme and that the reactions do not solely originate from differing properties of the coenzymes/NCBs. In all cases, there is less correlation between log $k_{obs}$ and $\Delta S^{\ddagger}$ (Figure S6), suggesting that enthalpy effects dominate the rate of reaction.

The primary KIEs on these 12 reactions were measured using (*R*)-[4-$^2$H]-NADH, (*R*)-[4-$^2$H]-NADPH, [4-$^2H_2$]-$mNH_2$, and [4-$^2H_2$]-mBu (Figure 2). Dideuterated NCBs were synthesized by sequential dithionite reduction, chloranil oxidation, and re-reduction with dithionite in $D_2O$ as described (Supporting Information). NMR indicated the NCBs were 98% dideuterated at C4. There is relatively little variation in the magnitude of the observed KIE at 25 °C (Figure 2). All KIEs fall within the range of 7−9, except those measured with XenA and the natural coenzymes: KIE = 4.74 ± 0.13 and 5.09 ± 0.48 with NADH with NADPH, respectively. The $\alpha$-secondary deuterium KIEs have been previously measured on several OYE ERs including PETNR. The observed KIEs on the PETNR reactions with (*S*)-[4-$^2$H]-NADH and (*S*)-[4-$^2$H]-NADPH are 1.16−1.20 and are not significantly temperature dependent.[36] The $\alpha$-2° KIEs on the reactions of the ERs with $mNH_2$ or mBu are unlikely to be larger than 1.20. The observed KIEs measured with dideuterated $mNH_2$ and mBu are thus unlikely to exceed the primary KIE by more than 20%.

The temperature dependence of the KIEs varies widely from 0−18 kJ mol$^{-1}$ (PETNR:NADH and XenA:mBu, respectively; Figures 2,4, S4, and S5). Five combinations of enzyme/reductant (XenA:mBu, TOYE:mBu, PETNR:mBu, XenA:$mNH_2$, and PETNR:$mNH_2$) have reactions with $\Delta\Delta H^{\ddagger}$ > 10 kJ mol$^{-1}$. This is good evidence for H transfer by significant QMT, as such large values of $\Delta\Delta H^{\ddagger}$ are inconsistent with either TST or Bell-type models of H-transfer where QMT occurs near the transition state.[37,38]

In all three enzymes, there is some correlation between observed rate constant and $\Delta\Delta H^{\ddagger}$ (Figure 4), with the faster reactions typically having more temperature-dependent KIEs. This suggests that whatever gives rise to the temperature dependence of the KIEs—e.g., conformation/distance sampling within the promoting vibrations hypothesis—may also enhance the rate of H-transfer/FMN reduction in the three ERs investigated here. As the most temperature-dependent KIEs are observed on reactions involving NCBs, an alternative explanation is that the differences in kinetics arise through chemical differences between the coenzymes and NCBs.

Density functional theory models of NAD(P)H and the two NCBs show that, while the C4−H stretching frequencies and gas-phase bond dissociation energies (Figure S9) are very similar between NCBs and natural coenzymes, the methylbenzene and butyl ("tail") moieties of the NCBs have greater electron-withdrawing properties than the (2′-phospho)-adenosine diphosphate ribose moieties of NAD(P)H (Table S12). This is expected to facilitate oxidation of the NCBs due to stabilization of positive charge buildup in the oxidized species. The redox potentials of NAD(P)H, $mNH_2$, and the mBu analogue 1-propyl-1,4-dihydronicotinamide have been reported.[13,39] These are in qualitative agreement with our DFT calculations (Table S12). The likely order of reduction potentials are NADPH = NADH > $mNH_2$ > mBu, consistent with hydride self-exchange reactions in acetonitrile with the reactivity order mBu > $mNH_2$ > NAD(P)H. The reduced reduction potentials/increased driving force of NCBs relative to natural coenzymes may explain some of the observed rate enhancement of the ER-NCB reactions. However, the reduction potentials of NADH and NADPH are not significantly different, yet the reactions of all three ERs with NADPH are 10−20-fold faster than with NADH. Further, in both PETNR and XenA, NADPH reacts as fast as or faster than $mNH_2$, despite the likely larger driving force of the $mNH_2$ reaction.[40] Modest changes to the reaction driving force are also not expected to alter the magnitude of the KIE,[31,41] so the kinetic differences observed between natural and biomimetic coenzymes do not appear to solely arise through differing reaction driving forces.

ER active sites are evolutionarily constrained by the necessity to bind both oxidative and reductive substrates within the same active site region, thus the evolution of improved NAD(P)H binding may have been constrained to the binding of the coenzyme "tail" on the periphery of the active site, beyond the reach of oxidative substrates.[22−26] While the tail allows coenzyme capture, the $K_m$ and $K_S$ values are relatively large (cf. other flavoproteins where the coenzyme adenosyl ribose moiety is a key factor in binding).[42] We hypothesize that by removing/reducing the size of the "tail", NCBs can bind and react in a manner more similar to the oxidative substrates of ERs. These differences must be subtle as X-ray crystal structures of NCB-and coenzyme-bound ERs show the nicotinamide moieties to be essentially superimposed (Figures S10 and S11).

NCBs typically have lower activation enthalpies compared to their natural counterparts (Figure 3). However, this is at the


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expense of higher activation entropies, which suggests that the enzyme−NCB complexes are more disordered than the physiological enzyme−coenzyme complex. The temperature dependence of the primary KIE is often interpreted in terms of environmental coupling between the protein and reaction coordinate, e.g., via promoting vibrations.[32] Within this framework, thermally activated distance sampling of the donor−acceptor coordinate is reflected in the temperature dependence of the KIE. Others have shown with variant enzymes that the reaction is often slower and has a more strongly temperature-dependent KIE than with wild-type enzymes.[30,43,44] However, we have shown the opposite behavior in PETNR,[28] and this is now further corroborated in reactions with NCBs. Distance sampling may therefore play both compensatory (i.e., in variant enzymes) and promoting roles, and this is likely to be enzyme-specific.

In conclusion, we have shown a correlation between the rates of hydride transfer and the temperature dependence of KIEs, suggesting that donor−acceptor sampling is a factor in enhancing the performance of NCBs. Further efforts to optimize this performance with ERs and other oxidoreductases that take into account the importance of QMT and donor−acceptor sampling are underway.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05625.

Experimental details and characterization data, including Figures S1−S11 and Tables S1−S12 (PDF)

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### Notes

The authors declare no competing financial interest.

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