Published in final edited form as:

J Phys Org Chem. 2010 July 1; 23(7): 696–701. doi:10.1002/poc.1653.

Probing active site geometry using high pressure and secondary isotope effects in an enzyme-catalysed ‘deep’ H-tunnelling reaction

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Abstract

We report the first study of the effects of hydrostatic pressure on α -2° KIEs for an enzyme-catalysed H-transfer reaction that occurs by ‘deep’ tunnelling. High pressure causes a significant decrease in the observed α -2° KIE on the pre-steady-state hydride transfer from NADH to FMN in the flavoprotein morphinone reductase. We have recently shown that high pressure causes a reduction in macroscopic reaction barrier width for this reaction. Using DFT vibrational analysis of a simple active site model, we posit that the decrease in α -2° KIE with pressure may arise due to a decrease in the vibrational coupling between the NADH primary (transferred) and secondary hydrogens in the ‘tunnelling ready configuration’, which more closely resembles the reactant state than the transition state.

Keywords

flavoprotein; H-transfer; pressure; secondary isotope effects; tunnelling

INTRODUCTION

Enzymes are unparalleled catalysts yet the precise origin(s) of their catalytic power has remained unresolved after more than a century of research.[1-5] The most simple enzymic reactions involve hydrogen (H^+ , $H^\bullet$ or H^-) transfers and in many cases, these H-transfers occur in part, or in full, by quantum mechanical tunnelling.[3,5-9] The rate of a tunnelling reaction – which proceeds through part of the reaction barrier rather than over it – is governed by the overall shape, and particularly the width, of the barrier.[2,3,5-13] We are interested in the role of the enzyme in optimizing the shape of the reaction barrier for H transfer reactions.

We have recently shown that, during the reductive half-reaction (RHR) of the flavoprotein morphinone reductase (MR) with the coenzyme NADH, increasing hydrostatic pressure causes an increase in both the observed rate constant and the magnitude of the primary (1°) kinetic isotope effect (KIE) for hydride transfer from NADH to FMN.[14] We have

interpreted these data such that pressure causes a narrowing of the apparent reaction barrier to this reaction.[15] This H-transfer has been shown to occur by deep tunnelling (where H transfer occurs $\sim 25 \text{ kJ mol}^{-1}$ below the top of the $\sim 80 \text{ kJ mol}^{-1}$ reaction barrier, and $\sim 99\%$ transfer occurs by tunnelling)[16] and is thought to have a putative promoting vibration that dynamically compresses the reaction barrier width – leading to a depressed and highly temperature-dependent 1° KIE.[14,17-21] During the RHR of MR, hydride transfer from the NADH C4 to the FMN N5 (Fig. 1) is concomitant with FMN reduction and can be directly observed in a stopped-flow instrument. The RHR proceeds as described by Scheme 1. H-transfer is kinetically resolved from the preceding steps involving coenzyme binding and formation of the MR-NADH binary complex, which for simplicity are shown as a single step in Scheme 1:[14,22,23]

In the binary complex – i.e. the reactant state – the FMN isoalloxazine and NADH nicotinamide rings are roughly coplanar within the active site forming a charge-transfer (CT) complex[15,16,24] (Fig. 1). We have shown that high pressure appears to effectively ‘squeeze’ the isoalloxazine and nicotinamide rings together within the active site by restricting the conformation space available to these groups.[15] This ‘squeezing’ reduces the average NADH C4 (heavy atom donor) and FMN N5 (acceptor) separation, and appears to cause the significant H-transfer rate enhancement (Fig. 2 and References [14,21]).

α - 2° H/D KIEs arise when a hydrogen neighbouring the transferred (1°) hydrogen is deuterated (refer Fig. 1C) and these KIEs are thought to be a sensitive probe of the reactant state geometry and vibrational energy.[19] To further probe the effect of pressure on enzyme active site geometry, we have, in the current study, examined the effect of high pressure on the α - 2° KIE on the hydride transfer during the RHR in MR. These experiments are then interpreted using a DFT model of the MR active site.

EXPERIMENTAL AND COMPUTATIONAL METHODS

All materials were obtained from Sigma-Aldrich (St. Louis, MO), except NADH (Melford Laboratories, Cheltenham, UK) and 1- ^{2}H -glucose (Cambridge Isotope Laboratories, Andover, MA). MR was purified as described previously,[17,22] and the enzyme concentration was determined by $\epsilon(462 \text{ nm})=11.3 \text{ mM}^{-1}\text{cm}^{-1}$. (S)-[4- ^{2}H]-NADH was prepared through stereospecific reduction of NAD^+ with 1- ^{2}H -glucose using glucose dehydrogenase as described previously.[19,25] The isotopic purity of NAD^{2}H was determined using NMR to be $>99\%$ (Figure S1 in the Supporting Information). Coenzyme solutions were prepared fresh, and their concentrations were determined by $\epsilon(340 \text{ nm})=6.22 \text{ mM}^{-1}\text{cm}^{-1}$.

High-pressure rapid-reaction kinetic experiments were performed with a Hi-Tech Scientific HPSF-56 high-pressure stopped-flow spectrophotometer (TgK Scientific, Bradford on Avon, U.K.). All reactions were performed in 50 mM Tris-HCl, 2 mM 2-mercaptoethanol, pH 8.0 and 5–10 units ml^{-1} glucose oxidase and 50 mM glucose were added to make the samples anaerobic.[14] All stopped-flow experiments were performed with a saturating post-mixing concentration of 5 mM NADH to confer pseudo-first-order reaction kinetics. [14] Spectral changes accompanying FMN reduction were monitored at 466 nm, and rate constants were determined by fitting a single-exponential ($\sim 1.5 \text{ kbar}$) or double-exponential

(>1.5 kbar) function to each trace. Rate constants and errors were estimated by taking the standard deviation of typically 3–6 measurements at each condition. Unfortunately, we were only able to measure an accurate 2° KIE at low temperatures due to the relatively long (~10 ms) dead time of the high pressure stopped-flow instrument and the combination of rate enhancements caused by both higher temperatures and pressures.

Density functional theory (DFT) calculations were performed using Gaussian 03.[26] The MR active site model was built as follows: The FMN isoalloxazine and NADH nicotinamide X-ray crystal coordinates (pdb 2r14) were used as the starting structure. As the nicotinamide amide is H-bonded to His 186 and Asn 189 in the MR active site,[24] and not to the FMN, the isoalloxazine was pruned to remove any potential H-bond donors/acceptors – i.e. the C2 and C4 carbonyl oxygen. The C7 and C8 methyl groups were removed to reduce the size of the system and N3 and N10 were converted to sp² carbons to keep the ring planar and isoelectronic with the isoalloxazine starting structure. The model was optimised using BH&H/6-31G* in the gas phase as this method is fairly fast, and BH&H appears to be more accurate than B3LYP for modelling π -stacking interactions (refer References [26,27] and the SI). No restraints were applied, as the optimized structure is quite similar to the crystal structure coordinates (Figure S4 in the SI). The transition state was then found by calculating the potential energy surface (PES) for H transfer (Fig. 4), then optimising the approximate transition state structure using Opt=TS in Gaussian 03.[26] The C4–N5 separation of the MR active site model was then fixed (with values between 2.60 and 3.60 Å) and optimised with no additional restraints applied. Transition state structures of the restrained models were again found using PES scans followed by Opt=TS optimisation. A single imaginary frequency was found in each of the transition state structures. The CT absorbance (Figure S6) of the MR active site models was calculated semi-empirically using ZINDO-CI (implicitly solvated with water) implemented in Arguslab 4.0.1 (minimal basis set (STO-6G), TDA CI-type).[28] The CT absorbance was taken to be the lowest-energy transition and calculations of only the FMN portion of the model showed no equivalent transition.

RESULTS AND DISCUSSION

α -2° KIEs are difficult to measure as the magnitude of the KIE is often not much larger than the precision of the method of measurement. While competitive kinetic isotope effects[29] are usually used to measure 2° KIEs, we have previously demonstrated that it is also possible to measure non-competitive pre-steady-state α -2° KIEs in several enzymes using a conventional stopped-flow instrument.[18,19,30] In the current study, we have extended this approach to measure the α -2° KIE on the hydride transfer during the RHR of MR with NADH at 4 °C at varying pressures between 1 bar (1 bar=100 kPa, ~1 atmosphere) and 2 kbar. A large decrease in the magnitude of the 2° KIE with increasing pressure was observed (Fig. 2). As both the observed rate of H-transfer and magnitude of the 1° KIE increase with pressure (Fig. 2 and Reference [14]), it is very unlikely that the decrease in 2° KIE observed here is due to an increase in kinetic complexity – which would likely reduce the observed rate constant and also cause the observed 1° KIE to decrease. Above 1.5 kbar, the 2° KIE appears to become normal (less than the equilibrium isotope effect (EIE) at an atmospheric pressure of 1.13[31]) (Fig. 2). However, it became necessary to fit the stopped-flow traces measured at these pressures (>1.5 kbar) to a double as opposed to a single-exponential function (Figure S2) as a small but significant slow phase became evident. While this slow phase did not have a significant KIE, it may reflect an additional perturbation of the enzyme at pressures >1.5 kbar and, consequently, the additional decrease in 2° KIE from 1.14±0.02 at 1.5 kbar to 1.04±0.05 at 2 kbar may arise due to multiple contribution factors. Nevertheless, from 1 bar to 1.5 kbar, the observed 2° KIE significantly decreases from 1.22±0.04 to 1.14±0.02 (note that rate constants and KIEs are tabulated in Table S1).

For a classical reaction (with no tunnelling), the magnitude of the 2° KIE has been long used to report on the nature of the transition state,[32] with values falling between unity (reactant-like) and the EIE (TST-like).[33] Consequently, we could interpret the pressure dependence of the 2° KIE in MR as arising from the change in transition state geometry to become more reactant-like at elevated pressure. However, the MR 2° KIE is significantly larger than the EIE and the reaction is known to proceed predominantly by tunneling.[16] Further, computational studies have warned against such an interpretation of 2° KIEs.[34] As a result, we will look to other explanations for the pressure dependence of MR 2° KIE.

The pressure dependence of the 2° KIE is opposite to that previously observed for the 1° KIE, which increases with pressure[14] (also shown in Fig. 2). It would then appear, at least in MR, that the magnitudes of the 1° and 2° KIEs are anti-correlated. In order to begin to investigate the consequences of reaction barrier compression in MR on the H-transfer chemistry, DFT calculations on a simplified active site model were performed. We were not interested in reproducing absolute rate constants and/or KIEs but rather trends – particularly the anti-correlation of the magnitudes of the 1° and 2° KIEs.

Huskey and Schowen have suggested that exalted 2° KIEs (those larger than the EIE) may arise due to strong vibrational coupling of the 2° hydrogen to the reaction coordinate at the transition state.[35] To investigate the pressure dependence of the MR KIEs, we turned to vibrational analysis of an active site model based on the NADH nicotinamide and FMN isoalloxazine moieties (i.e. the active site of MR). The model is shown in Fig. 3 and can be compared to Fig. 1B (refer also Figures S3 and S4 in the SI). As mentioned earlier, we have previously shown that pressure appears to reduce the average distance between the NADH C4 and FMN N5 in the MR binary complex.[15] We chose to model the effect of pressure by systematically varying (i.e. restraining) the C4–N5 distance in the active site model. The C4–N5 separation varied from 3.27 Å in the reactant state to 2.64 Å in the transition state and 3.10 Å in the product state along the PES in Fig. 4. In the X-ray crystal structure of the MR binary complex, the (reactant state) C4–N5 separation is ~3.5 Å. Consequently, we determined both the reactant and transition states of the model at varying values of $d(\text{C4–N5})$ between 3.60 and 2.60 Å. It was not possible to reduce the separation further as a saddle point was formed and when $d(\text{C4–N5})=3.6$ Å, the nicotinamide and modified isoalloxazine rings are no longer coplanar due to a break down in π – π stacking. Note, we do not expect that pressure causes such a large compression in MR. Rather, we chose a range of C4–N5 distances that were computationally tractable with the aim to scan as large a change in distance as possible, in order to magnify any changes in the calculated rates and KIEs. As expected, the reaction barrier height (potential) increased with increasing $d(\text{C4–N5})$ (Figure S5), from 98 kJ mol⁻¹ when $d(\text{C4–N5})=2.6$ Å to 240 kJ mol⁻¹ when $d(\text{C4–N5})=3.6$ Å. The free energies of activation for H and D transfer were also calculated and show the same trend. The imaginary frequencies of the reaction coordinate at the transition state ($\nu^\ddagger$) also increased with increasing $d(\text{C4–N5})$. We have previously experimentally observed, with increasing pressure, an increase in the CT absorbance of the binary complex of MR with 1,4,5,6-tetrahydro-NADH.[15] In order to validate our approach to modelling pressure, the CT absorbance of the active site model was semi-empirically calculated using ZINDO-CI as a function of C4–N5 separation. While the spectral wavelengths were not particularly well reproduced, a progressive increase in the oscillator strength (absorbance) and red-shifting of the absorbance maxima was observed upon decreasing $d(\text{C4–N5})$ (Figure S5). As a result, it appears that it is possible to qualitatively model the effect of pressure in the MR active site model by reducing the C4–N5 separation.

We next calculated, using transition state theory (Eqn 2), H transfer rate constants from the activation free energies of the restrained models. Hydride transfer in MR occurs

predominantly by tunnelling,[16] and we qualitatively accounted for this using the simple Wigner tunnelling correction (Γ):[36]

$$\begin{aligned} k_{\text{obs}} &= \Gamma k_{\text{TST}} \\ k_{\text{TST}} &= (k_{\text{B}} T / h) \exp(-\Delta G^\ddagger / k_{\text{B}} T) \\ \Gamma &= 1 + (1/24) \left(h |v^\ddagger| / k_{\text{B}} T \right)^2 \end{aligned} \quad (2)$$

We chose to use this correction as it is the simplest of the transition state correction methods. The calculated rates are shown in Figure S7 and show a roughly exponential increase with decreasing $d(\text{C4-N5})$. Both the 1° and 2° KIEs were then calculated and these are shown in Fig. 5. Like experiment, the 1° and 2° KIEs are anti-correlated. However, they show the opposite trends to the experimental values, with the 1° and 2° KIEs decreasing and increasing, respectively with decreasing $d(\text{C4-N5})$ – which should be equivalent to increasing pressure. While more accurate calculations would be interesting, perhaps using newer density functionals such as M05-2X[37] in combination with QM/MM calculations of the entire enzyme (which are ongoing), we feel that the calculated KIEs in Fig. 4B are qualitatively useful – particularly if we simply consider the trends. It is also encouraging that the magnitude of the calculated 1° KIE of 4–10 agrees with the experimental value of ~5. [19,38] Note, however, that this model would predict, counter to experiment,[14,17,19] a very weak temperature dependence on the KIE.

Why do we observe trends in Fig. 5 that are opposite to the experiment trends? It is possible to numerically model an H tunnelling reaction using modifications to the electron transfer theory of Marcus[39] and we have previously used this approach to model the effect of pressure on the 1° KIE in MR.[14,21] We found that, if one assumes that pressure causes a reduction in the H-transfer distance – as we have since shown[15] – then pressure is expected to cause an increase in the rate of H transfer and a decrease in the 1° KIE – the trend observed in our calculations here (Fig. 5 and S7). This would argue against the possibility that our DFT calculations do not reproduce the experimental trends because the active site model is too simple or the tunnelling correction is not accurate enough. If the H transfer is coupled to a promoting vibration, as we argue is likely the case in MR,[14,17-21] then pressure can cause the 1° KIE to increase if the frequency of this promoting vibration also increases with pressure.[14,21] Unfortunately, it is not possible to model 2° KIEs using this method.

We next tested Huskey and Schowen's hypothesis[35] that the magnitude of the 2° KIE should be correlated with the vibrational coupling of the 2° H to the reaction coordinate at the transition state. Vibrational analyses of the NADH C4 pro-*R* (1°) and pro-*S* (2°) hydrogens in the reactant and transition states of the restrained active site model are shown in Fig. 6. As $d(\text{C4-N5})$ decreases in the transition state, there is a significant increase in the magnitude of the pro-*S* eigenvector along the reaction coordinate. This increase in vibrational coupling correlates with the increase in calculated 2° KIE with decreasing $d(\text{C4-N5})$ seen in Fig. 5 and is consistent with the ideas of Huskey and Schowen.[35] However, the opposite trend is observed in the reactant state. In this case, the frequency of the C4 pro-*R* (1°) hydrogen increases while that of the C4 pro-*S* (2°) hydrogen decreases as the C4–N5 separation decreases (Fig. 6C). As a consequence, there is a large decrease in the vibrational coupling between the 1° and 2° hydrogens as $d(\text{C4-N5})$ decreases (Fig. 6D). We have previously shown – using QM/MM molecular dynamics simulations and variational transition-state theory calculations on the entire protein – that the H transfer in this reaction occurs by a deep tunnelling mechanism with the hydrogen tunnelling ~25 kJ mol⁻¹ below the top of the reaction barrier (which is ~80 kJ mol⁻¹ high).[16] We have also shown that the magnitude of the 2° KIE on H transfer (at 1 bar) is identical in both MR and a

homologous enzyme[19] and have suggested that the magnitude of the 2° KIE reflects the active site geometry, which we have termed the ‘tunnelling ready configuration’.[19,30] Consequently, if the magnitude of the α -2° KIE is correlated with the vibrational coupling of the 1° and 2° H, then, in the case of MR, we should consider the vibrational coupling, not at the transition state, but at the tunnelling ready configuration. The tunnelling ready configuration may be thought of as the active site geometry at a point along the PES where the H transfer probability is highest. If there is a considerable degree of tunnelling, then this will be at an intermediate point between the reactant and transition states (at the representative tunnelling energy along the PES[40]). For reference, the vibrational coupling between the 1° and 2° hydrogens was calculated for snapshots along the PES between the reactant and transition states of the unrestrained model (Fig. 4) and this coupling varies considerably across the PES. However, if the tunnelling ready configuration is more reactant-like than transition state-like, then the vibrational coupling between the 1° and 2° hydrogens would be expected to decrease with decreasing C4–N5 separation (as seen in Fig. 6D) and thus the 2° KIE would decrease with increasing pressure – as seen experimentally in Fig. 2. Clearly more sophisticated calculations on the entire MR binary complex will be required to test this idea in the future.

As a final note, exalted 2° KIEs are often taken to be evidence of a tunnelling mechanism of H transfer. While we were unable to calculate an exalted 2° KIE using the MR active site model in Fig. 3, we did find an inverse relationship between the calculated 2° KIEs and the Wigner tunnelling correction – which is shown in Figure S7 and is related to the % tunnelling. The Wigner tunnelling correction[36] is known to be a rather crude method for accounting for H tunnelling but the trends here should be qualitatively correct. Consequently, we would like to stress that 2° KIEs are not a good probe of the tunnelling contribution to a reaction.

CONCLUSIONS

To the best of our knowledge, we have, in this study, measured the first pressure dependence of a 2° KIE, which significantly decreases with pressure (note that Northrop has previously described the pressure dependence of an enzymatic heavy atom isotope effect[41]). By combining experimental and computational methods, we have provided a possible and tentative explanation for the observed decrease in 2° KIE with pressure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC). NSS is a BBSRC Professorial Research Fellow.

Abbreviations

CT	charge transfer
DFT	density functional theory
EIE	equilibrium isotope effect
FMN	flavin mononucleotide
KIE	kinetic isotope effect

MR	morphinone reductase
NADH	nicotinamide adenine dinucleotide
PES	potential energy surface
RHR	reductive half reaction

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Figure 1.

(A) The X-ray crystal structure (pdb 2r14)[24] of 1,4,5,6-tetrahydro-NADH (green carbons) bound in the active site of MR. The FMN cofactor (yellow carbons) is also shown. (B) The relative orientation of the NADH nicotinamide (top) and FMN isoalloxazine rings with key atoms numbered. (C) Schematic showing the vibrational coupling of the pro-*R* 1° H (H_P) to the pro-*S* 2° H (H_S). The position of H_P in both the reactant and product states is shown in (C) to illustrate the expected direction of the reaction coordinate (dotted arrow). Refer to text for more details

Figure 2.

(A), The effect of pressure on the apparent rate constant for FMN reduction/hydride-transfer during the reductive half reaction of MR with NADH (open circles) and (*S*)-[4-²H]-NADH (filled squares) at 4 °C. The data are fit to: $k_{\text{obs}} = k_0 \exp(-\Delta V^\ddagger p/RT) \exp(\Delta\beta^\ddagger p^2/RT)$, with $\Delta V^\ddagger_{\text{H}}$ and $\Delta V^\ddagger_{\text{D}} = -23.0 \pm 1.2$ and $-22.4 \pm 0.4 \text{ cm}^3 \text{ mol}^{-1}$, respectively, and with $\Delta\beta^\ddagger_{\text{H}}$ and $\Delta\beta^\ddagger_{\text{D}} = -7.0 \pm 0.6$ and $-5.2 \pm 0.5 \text{ cm}^3 \text{ mol}^{-1} \text{ kbar}^{-1}$, respectively. The resulting observed α -² KIE (B, filled circles) shows a loss in magnitude of the KIE with increasing pressure – the opposite trend to the ¹ KIE – which is also shown in *Panel B* as open triangles (5 °C data from [14]). The ² KIE was also measured using a conventional anaerobic stopped-flow at 1 bar (open square), giving an equivalent value to those measured previously.[19,38] The arrow marks the EIE at 1 bar of 1.13.[31] Representative stopped-flow traces and tabulated rate constants are shown in Figure S2 and Table S1

Figure 3.

MR active site DFT modelling of (A) the simplified active site model used for calculations (compare with Fig. 1B and Figure S3 in the SI) and representative BH&H/6-31G*-minimized reactant (B) and transition state (C) structures of this model with a fixed C4–N5 separation of 3.0 Å. More details of the model are given in the Supporting Information

Figure 4.

MR active site DFT modelling. The unrestrained gas phase potential energy surface (filled circles) for H transfer in the MR active site model shown in Fig. 3. The reaction is endothermic as the hydride transfer leads to charge separation. The free energies of selected points from the reactant to transition state were determined from vibrational calculations (open circles). Also shown is the vibrational coupling of the pro-*R* (open triangles) and pro-*S* (filled triangles) hydrogens to the pro-*S* C—H stretching frequency. The ‘spike’ at $q \sim -1.1$ arises due to a ‘cross-over’ of the pro-*R* and pro-*S* stretching frequencies. Refer the main text for more details

Figure 5.

MR active site DFT modelling. 1° (squares) and 2° (circles) KIEs calculated as a function of restrained C4–N5 separation. The unrestrained optimised model has a C4–N5 separation of 3.27 Å in the reactant state and 2.64 Å in the transition state and the points marked with an * are the KIEs calculated from this model. The unfilled points are the semi-classical KIEs and the black points those with a Wigner tunnelling correction

Figure 6.

The effect of C4–N5 separation on the NADH C4–H stretching frequencies in the MR active site model in the transition state (A,B) and reactant state (C,D). (A), Frequencies of the pro-*S* (2°) C—H stretch (filled circles) and the imaginary frequency of the reaction coordinate (open circles) and (B), the eigenvector of the C- 2° H along the reaction coordinate. (C), Frequencies of the pro-*S* (filled circles) and pro-*R* (open circles) C—H stretches and (D), the eigenvectors of the C- 2° H along the C- 1° H stretch (open squares) and of the C- 1° H along the C- 2° H stretch (filled squares). *Note*: The eigenvectors of e.g. the C- 2° H along the C- 2° H stretch are ~ 1 . These values are tabulated in Tables S4 and S5