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Ab Initio QM/MM Modeling of the Rate-Limiting Proton Transfer Step in the Deamination of Tryptamine by Aromatic Amine Dehydrogenase

Kara E. Ranaghan^a, William G. Morris^a, Laura Masgrau^{b,c}, Kittusamy Senthilkumar^d, Linus O. Johannissen^e, Nigel S. Scrutton^e, Jeremy N. Harvey^f, Frederick R. Manby^a and Adrian J. Mulholland^{a*}

^a*Centre for Computational Chemistry, School of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, UK.*

^b*Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain*

^c*Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain*

^d*Department of Physics, Bharathiar University, Coimbatore, India.*

^e*Manchester Institute of Biotechnology, University of Manchester, Manchester, UK.*

^f*Department of Chemistry, KU Leuven, Celestijnenlaan 200F, B-3001 Heverlee, Belgium.*

**Corresponding author: Prof. Adrian Mulholland
(Adrian.Mulholland@bristol.ac.uk)*

Abstract

Aromatic amine dehydrogenase (AADH) and related enzymes are at the heart of debates on the roles of quantum tunneling and protein dynamics in catalysis. The reaction of tryptamine in AADH involves significant quantum tunneling in the rate-limiting proton transfer step, shown e.g. by large H/D primary kinetic isotope effects (KIEs), with unusual temperature dependence. We apply correlated ab initio combined quantum mechanics/molecular mechanics (QM/MM) methods, at levels up to local coupled cluster theory (LCCSD(T)/(*aug*)-cc-pVTZ), to calculate accurate potential energy surfaces for this reaction, which are necessary for quantitative analysis of tunneling contributions and reaction dynamics. Different levels of QM/MM treatment are tested. Multiple pathways are calculated with fully flexible transition state optimization by the climbing-image nudged elastic band method at the density functional QM/MM level. The average LCCSD(T) potential energy barriers to proton transfer are 16.7 kcal/mol and 14.0 kcal/mol for proton transfer to the two carboxylate atoms of the catalytic base, Asp128 β . The results show that two similar, but distinct pathways are energetically accessible. These two pathways have different barriers, exothermicity and curvature, and should be considered in analyses of the temperature dependence of reaction and KIEs in AADH and other enzymes. These results provide a benchmark for this prototypical enzyme reaction and will be useful for developing empirical models, and analysing experimental data, to distinguish between different conceptual models of enzyme catalysis.

Introduction

The tryptophan tryptophylquinone (TTQ)-dependent amine dehydrogenases methylamine dehydrogenase (MADH) and aromatic amine dehydrogenase (AADH) are central examples in intensive debates on the role of quantum tunneling and protein dynamics in enzyme catalysis. Resolving these debates, by developing molecular level understanding (e.g. through combined experimental and simulation analyses), requires accurate potential energy surfaces. Proton transfer reactions catalysed by AADH and MADH have been shown to involve significant quantum mechanical tunneling, with intriguing temperature dependence of kinetic isotope effects in some cases¹⁻³. AADH and MADH catalyse the oxidative conversion of primary amines (aromatic and aliphatic, respectively) to the corresponding aldehyde and ammonia (scheme 1).⁴⁻⁷ For AADH (from the organism *Alcaligenes faecalis*), the rate-limiting proton transfer step from the substrate C-H to the aspartate base (D128 β) has a primary H/D kinetic isotope effect (KIE) of 55 ± 6 (for substitution from perprotio to dideutero, at temperatures between 5 and 20 °C).⁶ This is among the highest reported primary KIEs for biological proton transfers, significantly in excess of the semiclassical limit of ~ 7 . This KIE also shows no measurable temperature dependence over this temperature range.⁶ Soybean lipoxygenase (SLO-1) also exhibits very high H/D KIEs for hydrogen transfer (proton-coupled electron transfer): ~ 80 for the wild type enzyme and in the range 500-700 for the L546A/L754A double mutant.⁸⁻⁹

The contribution of dynamics and quantum tunneling is at the heart of current debates on enzyme catalysis. The complex temperature dependence of the KIEs for enzyme-catalysed hydrogen transfer reactions involving

quantum tunneling have led some to suggest that protein and/or substrate dynamics play a role in catalysis by enhancing the tunneling probability.^{2, 10-14} On the other hand, Glowacki *et al.*¹⁵⁻¹⁶ have shown that a kinetic model (based on transition state theory (TST), with a temperature-dependent treatment of tunneling) involving only one or two conformations with different reactivity can reproduce the temperature-dependent KIEs of AADH, MADH; in the case of other enzymes such as SLO-1, and dihydrofolate reductase (DHFR), a single conformation is sufficient. This ‘two state model’ does not preclude the presence of “promoting motions”, each state could involve different promoting motions, with different associated temperature dependences, but rather indicates that effects beyond TST do not need to be invoked to account for the experimental observations. To understand, and to analyse which models and conceptual pictures actually describe enzyme reactivity and catalysis, a molecular level analysis is required, in which molecular simulations have a crucial role to play.¹⁷⁻¹⁸ Atomically detailed simulations (e.g. using combined quantum mechanics/molecular mechanics (QM/MM) methods) employing modern frameworks of TST that take into account ensemble averaging have been successful in reproducing the temperature dependence of reaction rates (and KIEs) when tunneling is involved.¹⁹⁻²⁵ However, to date, computational demands have limited such simulations to approximate levels of QM treatment, e.g. using semiempirical molecular orbital methods (see below). While reaction specific parameterization can reproduce reaction energetics and other details reasonably well, accurate first-principles predictions of enzyme reaction barriers and e.g. curvature that plays a vital role in determining quantum

tunneling, and accurate molecular simulations of reaction dynamics, require accurate potential energy surfaces, for which correlated ab initio electronic structure methods are essential, and have only recently begun to be applied to enzyme-catalysed reactions.

Insight into the role of protein dynamics in catalysis has come from studies of ‘heavy’ enzymes, in which all heavy atoms and non-exchangeable hydrogens are replaced by heavier isotopes.²⁶⁻³⁰ In most cases isotopically substituted proteins with an increased mass exhibit slightly lower rates, suggesting that protein dynamics have some effect on the chemical reaction rate. In the case of pentaerythritol tetranitrate reductase, a dramatic increase in the temperature-dependence of the KIE was also observed.²⁷ For DHFR, QM/MM simulations and separate analysis with the kinetic model of Glowacki et al. concur in indicating that the slight decrease in rate is due to differences in environmental coupling to the hydride transfer step between the heavy and light (wild-type) enzymes; they also agree in showing the contribution of quantum tunneling to the reaction rate is not affected by isotopic substitution of the whole enzyme, indicating that even large changes in protein dynamics do not affect tunneling in DHFR.²⁸

QM/MM simulations of the rate-limiting proton transfer step in the deamination of tryptamine in AADH, applying a well-established variational TST / multidimensional tunneling (VTST/MT) framework have provided an atomic-level description of the reaction, giving insight into factors governing the reaction and the contribution of quantum mechanical tunneling.³¹ The two carboxylate oxygen atoms of the aspartate base in AADH are distinguishable in the enzyme environment due to their different hydrogen bonding

environments: OD1 (O2) forms a hydrogen bond with T172 β whilst OD2 (O1) forms a hydrogen bond with W160 β , part of the TTQ co-factor.^{6, 31} (We use the nomenclature O2 and O1 for OD1/OD2 of D128 β to be consistent with previous modeling).^{6, 31} QM/MM simulations identified the possibility of proton abstraction by either O2 or O1 of the catalytic base D128 β .^{6, 31} These two pathways have different kinetic and thermodynamic properties (different barriers and reaction energies), and different tunneling contributions, according to semiempirical QM/MM calculations. The calculated primary H/D KIE (at 300 K) for proton transfer to O2 of D128 β was 30, compared to a value of 12 for proton transfer to O1.³¹ The presence of these two distinct pathways may contribute to, or account for, the complex temperature dependence of the KIEs as demonstrated by Glowacki *et al.*¹⁶ However, these earlier calculations applied semiempirical QM/MM methods, which provide at best an approximate prediction of reaction barriers and energetics. These previous QM/MM studies^{6, 31} were at the PM3/MM level: such semiempirical QM. The energetics of the reaction are not very accurately described by this method. Also, notably, the free energy barrier calculated at this level is significantly lower than experiment, and the reaction is calculated to be significantly exothermic, most likely due to the overestimation of the proton affinity of the aspartate base.^{6, 31-32} Reliable prediction of tunneling probabilities and reactivity requires accurate potential energy surfaces, which typically require high-level correlated ab initio calculations (e.g. with coupled cluster methods). Theoretical and methodological developments (e.g. using localized molecular orbitals) now make it possible to apply such highly accurate methods to enzyme-catalysed reactions, within the framework of

QM/MM calculations.³³⁻³⁷ While calculations at lower levels of QM/MM theory can provide very useful qualitative insight, even DFT energies are sometimes significantly in error^{35, 38}, which can lead to qualitatively incorrect mechanistic conclusions. Quantitative agreement with experiment is only possible when high levels of ab initio QM theory are used in first principles (as opposed to specifically parameterized) QM/MM calculations^{34, 38}. Here, we apply such high level correlated ab initio QM/MM methods (using localized molecular orbitals) to calculate potential energy profiles for the reaction catalysed by AADH.

Its importance as a paradigm, and the intriguing temperature dependence of its KIEs, has led to AADH being investigated by a variety of modeling and simulation techniques.^{6, 12, 39} QM/MM techniques have been used to calculate spectroscopic properties of the TTQ cofactor⁴⁰ and also to investigate the multiple steps in the reductive half-reaction of tryptamine with AADH, identifying several intermediates in the reaction.⁴¹ Ren et al.⁴² used DFT QM/MM techniques to calculate the potential energy surfaces and dipole moment surfaces for the motion of the reactive proton in the AADH/tryptamine system. Optimal control theory was then used to design a pulse to excite the proton from its lowest vibrational state to selected vibrationally excited states. Although such an experiment would face significant practical challenges, these calculations provide a proof of principle that laser pulses could be designed and used to promote reactivity and tunneling in an enzyme. These DFT QM/MM calculations are the highest level calculations on a reaction in AADH to date; correlated ab initio methods have not yet been used to investigate this important enzyme.

Scheme 1: The multi-step reaction mechanism for the oxidative deamination of tryptamine by AADH. The rate-limiting proton transfer step (III $\rightarrow$ IV) modelled here is highlighted in the boxed region.^{6, 31} Tryptamine is shown in purple, part of the TTK cofactor is shown in black, the catalytic base Asp128 β is shown in green and key water molecules are shown in cyan.

Much of the debate surrounding quantum tunneling in enzyme-catalysed reactions centres on the role of enzyme dynamics and possible 'promoting motions'.^{10-11, 43 44} No long-range coupled motions were identified in molecular dynamics simulations of AADH with tryptamine.⁶ KIEs in good agreement with experiment were obtained using VTST/MT calculations with a fixed protein environment, either using a single snapshot or an ensemble of protein configurations, with semiempirical QM/MM methods as described above.^{6, 31, 45} These results indicate that there is no need to invoke motion of the environment coupled to the reaction in order to explain these large KIEs.³¹ Multidimensional models of tunneling (e.g. using the small curvature tunneling approximation (SCT)⁴⁶) are essential in the calculation of such elevated KIEs. Such models include the coupling to the reaction coordinate of vibrational modes transverse to it, leading to shorter tunneling paths (corner-cutting), and thus enhanced tunneling probabilities. For AADH, it has been found that reaction is initiated by classical thermal activation until a point is reached where the proton is able to tunnel. At this point, a rapid (short-range, sub-ps) promoting vibration has been proposed to enhance the tunneling probability by modulating the distance between the donor and acceptor atoms, whose motion still dominates the reaction coordinate and couples to the C-H stretching mode.^{12, 31, 39}

Barrier shape is a critically important factor in determining the contribution of quantum tunneling: tunneling probabilities are highly sensitive to curvature.^{7, 10} Thus, an accurate PES is crucial for accurate calculations. However, due to the high computational cost involved, calculations have usually been limited to the semiempirical or DFT QM/MM level, which have

significant limitations and, not being systematically improvable, cannot be relied upon in general for accurate calculations of PESs.^{3, 21, 45, 47-50} Some of these calculations also applied approximate reaction coordinates, which may not correctly describe structural features of the reaction correctly. Here, we generate reaction pathways with flexible optimization using climbing image nudged elastic band methods using DFT, and apply high level correlated ab initio QM/MM methods up to the coupled cluster level of theory (with local approximations) for energy calculations to calculate multiple QM/MM potential energy profiles for the proton transfer to O2 or O1 of D128 β in AADH. Coupled cluster calculations are considered to be the ‘gold standard’ of ab initio methods in single determinant cases giving results for reactions to within chemical accuracy (i.e. barriers to within 1 kcal/mol of experiment), using sufficiently large basis sets.³³ We test different levels of QM/MM treatment (e.g. DFT functionals, basis sets, MP2, SCS-MP2, etc.), and the results will inform future investigations of AADH and related enzymes (such as MADH). We also model the reaction in solution, using continuum solvent models to compare the reaction in the enzyme environment with its counterpart in solution, which provides insight into the role of the enzyme environment in determining the energetics of the reaction. Crucially, these results demonstrate that two distinct pathways are energetically feasible, and, as they show different barriers and curvature. Both of these pathways should be considered in any analysis of the temperature dependence of reactivity and KIEs in AADH.

Methods

Model preparation and PM3/CHARMM22 simulations. The protocol for the setup of our model of the AADH-tryptamine system has been described in detail in previous work.^{6, 31} Briefly, the model of complex III (Scheme 1) is based on a high resolution (1.1 Å) X-ray crystal structure of the Schiff base intermediate **V** (PDB⁵¹ accession code 2AGY⁶).⁶ Protonation states were assigned to titratable residues and the system was solvated and truncated to a 25 Å radius sphere (centred on atom NT of tryptamine; see Figure 1 for atom names), a procedure that has been applied to investigate other enzymes successfully previously. Atoms were assigned atom types in accordance with the CHARMM22 MM forcefield.⁵² After initial equilibration and MM relaxation (with the CHARMM program⁵³), the QM/MM partition was defined with the QM region consisting of 48 atoms (including 3 HQ type link atoms⁵⁴; Figure 1) with an overall charge of zero (formal charges of $-1e$ of D128 β and $+1e$ of the bound tryptamine). A stochastic boundary approach⁵⁵ was then used first to optimize the entire system at the PM3/CHARMM22 QM/MM level and then to perform umbrella sampling molecular dynamics (MD) simulations to calculate the classical free energy profile for proton transfer to either O2 or O1 of D128 β .³¹ The reaction coordinates used for these simulations were defined as $Z_{O2} = [d(C1-H1) - d(O2-H1)]$ Å and $Z_{O1} = [d(C1-H1) - d(O1-H1)]$ Å. This definition of the reaction coordinate was chosen as it has been shown previously to model proton transfers well^{6, 35, 45}.

Figure 1: The active site of AADH with the iminoquinone (III) bound, showing the QM atoms as sticks with cyan carbon atoms and the MM region with green carbon atoms. The hydrogen bonds formed between O1 of D128 β and HN of W160 β and O2 and T172 β HG1 are indicated by dotted lines. Three 'link atoms' terminate the QM region, and are located where the colour changes from cyan to green.

QM/MM reaction pathway calculations. Transition state structures generated by PM3/CHARMM22 umbrella sampling MD simulations were used as the starting points for an adiabatic mapping procedure to model the proton transfer from the tryptamine-derived iminoquinone to either O2 or O1 of D128 β . Five starting structures for each proton transfer were taken at 5 ps intervals from a 30 ps simulation of the TS-sampling window at the PM3/CHARMM22 level ($Z_{O2} = 0.0$ Å and $Z_{O1} = 0.1$ Å).³¹ This is a relatively short simulation, but the reaction cycle in AADH does not involve any large-

scale conformational changes in the protein.⁶ Comparison of interactions in the active site of AADH with those obtained from longer (100 ns) simulations of the reactive complex III (at the MM level using the CHARMM22 forcefield, see Supporting Information (SI)) shows that the sampling is sufficient to provide representative structures for our higher level calculations (see Table S1).

We applied the QM/MM program QoMMMa,⁵⁶ which provides an interface between the QM packages JAGUAR,⁵⁷⁻⁵⁸ GAUSSIAN⁵⁹ or MOLPRO⁶⁰⁻⁶¹ and the TINKER⁶² MM program for the evaluation of MM terms (using the CHARMM27 all-atom force field⁶³). Note that there is no difference in the parameters for proteins between the CHARMM22 and CHARMM27 parameter sets, so the notation CHARMM22 or CHARMM27 is equivalent here and will be abbreviated to MM in the remainder of the text. QoMMMa creates input for both programs and automatically extracts the required information from output. QoMMMa⁵⁶ was used to optimize the system at the B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM levels using JAGUAR⁵⁷⁻⁵⁸ for the QM part of the calculations. Optimizations were carried out with a reaction coordinate restraint applied to drive the reaction from the TS towards the reactants and products in 0.1 Å steps. B3LYP⁶⁴⁻⁶⁶ is often used in QM/MM studies, but BH&HLYP^{65, 67-68} is known to give better results than B3LYP⁶⁴⁻⁶⁶ for some proton transfers⁶⁹ and also a better description of hydrogen bonding⁷⁰⁻⁷¹. The BH&HLYP method was found to give results in better agreement with ab initio methods than B3LYP, and so the discussion presented below focuses on the BH&HLYP results. The B3LYP results are provided in the SI for comparison. Comparison of ab initio single point

QM/MM profiles using structures generated with these different DFT/MM methods also provides a test of sensitivity of the energy profiles to the structures used.^{35, 72}

The resulting profiles were refined using nudged elastic band (NEB)⁷³ and climbing image NEB⁷⁴ techniques to optimize and characterize the reaction pathways without any imposed reaction coordinate. Harmonic vibrational frequencies were calculated for zero-point energy (ZPE) corrections and to verify that real transition state structures had been found. Only the sub-block of the full Hessian corresponding to the QM atoms was generated and diagonalized to compute the frequencies.⁷⁵ Single-point energy QM/MM calculations on the B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM optimized geometries were carried out at the (L)MP2/(aug)-cc-pVTZ/MM, SCS-(L)MP2/(aug)-cc-pVTZ/MM and L-CCSD(T)/(aug)-cc-pVTZ/MM levels of theory using QoMMMa to interface with MOLPRO⁶⁰⁻⁶¹. SCS indicates that the spin component scaled method developed by Grimme⁷⁶ for the MP2⁷⁷ calculations; this has shown to give results close to those from coupled cluster methods⁷⁸ for other enzyme-catalysed reactions.^{35, 38, 79} The (aug) in the notation of the (aug)-cc-pVTZ⁸⁰ basis set indicates that augmented functions were used for the oxygen atoms only. The L in these acronyms for the *ab initio* methods indicates that local approximations⁸¹⁻⁸³ are used in the calculations; these local approximations were tested by comparing localized and non-localized QM/MM calculations at both the MP2 and SCS-MP2 levels of theory, in order to test their accuracy and therefore justify their use at the coupled cluster level (see Figure S1). Note that the averaged barrier heights reported in the Results section below are the result of finding

the simple arithmetic mean of the 5 data points (for the 5 different starting structures). Boltzmann-weighted averaging of reaction barriers⁸⁴⁻⁸⁶ was also performed but results in the same value to the number of decimal places reported here, e.g. the B3LYP O1 barrier from simple averaging of the 5 barriers is 11.96083 kcal/mol, while the Boltzmann-weighted barrier is 11.96076 kcal/mol; we thus report the average barrier for this pathway as 11.96 kcal/mol; we quote energies to two decimal places for detailed comparison of different QM/MM treatments. The profiles calculated from different starting snapshots are very similar, as shown by the small variation in barriers, demonstrating that the energy profiles are not affected significantly by small conformational changes in the protein.

Solvation models were used to examine the effect of the environment on the equivalent ('reference') reaction in solution.⁸⁷ This provides an approximate insight into energetic contributions to catalysis, i.e. by comparing exactly the same reaction within different environments (in enzyme and in aqueous solution, respectively). Single-point energy calculations were carried out on the atoms of QM region from the NEB pathways (without any optimization of the geometry), with (aqueous) solvation treated by the polarized continuum model (PCM)⁸⁸ in Gaussian⁵⁹ or the SM8 solvation model⁸⁹ in JAGUAR⁵⁷⁻⁵⁸ for comparison, using the B3LYP and BH&HLYP methods with the 6-31G(d) and 6-311+G(d) basis sets to be consistent with the DFT results in the enzyme environment. This provides a direct comparison of the relative stabilization effects of the enzyme environment with that of aqueous solution.

Results

Reaction pathways from adiabatic mapping and NEB techniques. The potential energy profiles for proton transfer to O2 and O1 of D128 β generated using adiabatic mapping techniques at the BH&HLYP/6-31G(d)/MM and B3LYP/6-31G(d)/MM levels are shown in Figure S2. Table 1 shows the potential energy barriers and reaction energies for proton transfer to O2 and O1 of D128 β calculated with adiabatic mapping and the results of CINEB refinement of these pathways at the BH&HLYP/6-31G(d)/MM level of theory (the equivalent results for the B3LYP/6-31G(d)/MM level are given in Table S2). The average potential energy barrier for proton transfer to O2 of D128 β is 13.76 (± 0.36) kcal/mol after refinement with CINEB techniques. The average potential energy barrier for proton transfer to O1 is lower: 10.61 (± 0.54) kcal/mol. The transition state for proton transfer to O1 of D128 β at $Z_{O1} = 0.16$ Å is located slightly earlier on the reaction coordinate than the value of $Z_{O2} = 0.19$ Å obtained for proton transfer to O1. Note that no reaction coordinate is used in the generation of the CINEB paths, but the reaction coordinate value is a useful geometric descriptor for comparison of the pathways. The structures of these fully optimised TSs are in good agreement with the approximate TSs generated by adiabatic mapping. For proton transfer to O2, the approximate TS is located at $Z_{O2} = 0.20$ Å [$d(\text{C1-H1}) = 1.42$ (± 0.01) Å and $d(\text{H1-O2}) = 1.23$ (± 0.01) Å; $\angle \text{C1-H1-O2} = 171$ (± 2)°] and the TS from CINEB calculations is located at $Z_{O2} = 0.19$ Å. For proton transfer to O1, the approximate TS and the CINEB TS are located at the same reaction coordinate value of $Z_{O1} = 0.16$ Å.

Table 1: Reaction energetics (in kcal/mol, relative to the reactant) and reaction coordinate values ($Z/\text{\AA}$) from adiabatic mapping (E_{AM}) and CINEB (E_{CINEB}) calculations for proton transfer to O2 and O1 of D128 β at the BH&HLYP/6-31G(d)/MM level of theory.

		O2				O1			
		Z	E_{AM}	Z	E_{CINEB}	Z	E_{AM}	Z	E_{CINEB}
PATH 1	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.81	14.31	0.81	14.14	0.74	10.71	0.16	10.59
	P	1.10	3.12	1.10	3.12	1.01	1.94	1.01	1.94
PATH 2	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.82	13.92	0.82	13.72	0.72	10.24	0.15	10.16
	P	1.10	3.08	1.10	3.08	1.00	1.03	1.00	1.03
PATH 3	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.88	14.24	0.88	14.10	0.73	11.73	0.19	11.47
	P	1.10	3.77	1.10	3.77	1.01	2.50	1.01	2.50
PATH 4	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.78	13.49	0.78	13.28	0.71	12.04	0.17	10.16
	P	1.10	3.43	1.10	3.43	1.00	2.34	1.00	2.34
PATH 5	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.81	14.17	0.81	13.57	0.85	11.35	0.11	10.67
	P	1.10	5.74	1.10	5.74	1.01	1.17	1.01	1.17
AVE	R	-	0.00	-	0.00	-	0.00	-	0.00
	TS	0.82	14.02 (± 0.33)	0.82	13.76 (± 0.36)	0.75	11.21 (± 0.74)	0.16	10.61 (± 0.54)
	P	1.10	3.83 (± 1.10)	1.10	3.83 (± 1.10)	1.00	1.79 (± 0.67)	1.00	1.79 (± 0.67)

Both proton transfers are endothermic at the DFT/MM level. The average reaction energy for proton transfer to O2 of D128 β is 3.83 (± 1.10) kcal/mol (product at a reaction coordinate value of $Z_{\text{O2}} = 1.10 \text{ \AA}$ [$d(\text{C1-H1}) = 2.09 (\pm 0.01) \text{ \AA}$ and $d(\text{H1-O2}) = 0.99 (\pm 0.00) \text{ \AA}$]). The average reaction energy for proton transfer to O1 is 2.12 (± 0.46) kcal/mol, and the product lies at a reaction coordinate value of $Z_{\text{O1}} = 0.96 \text{ \AA}$ [$d(\text{C1-H1}) = 1.96 (\pm 0.03) \text{ \AA}$ and $d(\text{H1-O1}) = 1.00 (\pm 0.00) \text{ \AA}$].

The BH&HLYP/MM paths have (on average) higher energy barriers and are slightly less endothermic than those generated by B3LYP/MM. For example: $\Delta^\ddagger V_{O_2} = 10.46 (\pm 0.75)$ kcal/mol (B3LYP/6-31G(d)/MM) vs $\Delta^\ddagger V_{O_2} = 13.76 (\pm 0.36)$ kcal/mol (BH&HLYP/6-31G(d)/MM) and $\Delta_r V_{O_2} = 4.87 (\pm 1.10)$ kcal/mol (B3LYP/6-31G(d)/MM) vs $\Delta_r V_{O_2} = 3.83 (\pm 0.75)$ kcal/mol (BH&HLYP/6-31G(d)/MM). The structures generated with the two different functionals are very similar (see below).

The energy profiles from adiabatic mapping and CINEB techniques are very similar. The energies differ by only 0.1 – 0.6 kcal/mol, and the position of the TS is similar, showing that the reaction coordinate used for adiabatic mapping provides a good representation of the true reaction pathway. Figure S3 (a) shows a comparison of an adiabatic mapping path with NEB and CINEB optimized pathways. NEB optimization significantly underestimates the barrier (not surprisingly, as it does not optimize to a maximum on the path): refinement by the CINEB technique is necessary to locate the true TS. Figure S3 (b) shows a CINEB paths generated with 7 or 10 initial images. The CINEB pathways are very similar to each other, with barriers of 10.88 and 10.90 kcal/mol, respectively and the imaginary frequencies of the TSs are $1305i$ cm⁻¹ and $1325i$ cm⁻¹. As pathways with 7 images showed better convergence, all other paths were generated using 7 initial images.

Harmonic vibrational frequencies were calculated for the reactant, TS and product geometries from CINEB calculations to calculate ZPE corrections (see Table 3). For proton transfer to O2, the inclusion of ZPE reduces the barrier by an average of 2.75 kcal/mol (B3LYP) or 3.26 kcal/mol (BH&HLYP). ZPE reduces the barrier for proton transfer to O1 by a similar amount: (2.99

and 3.31 kcal/mol for B3LYP and BH&HLYP, respectively). The relative energy of the product is also reduced slightly when ZPE is included (by less than 1 kcal/mol).

Table 2: Average ZPE contributions (in kcal/mol, relative to the reactant) to the TS (ZPE_{TS}) and product (ZPE_P) energies from frequency calculations at the B3LYP/6-31G(d) and BH&HLYP/6-31G(d) levels of theory including the effects of the MM region as point charges (see text).

		O2	O1
ZPE_{TS}	B3LYP	-2.75	-2.99
	BH&HLYP	-3.26	-3.31
ZPE_P	B3LYP	-0.29	-0.34
	BH&HLYP	-1.00	-0.47

Figure 3: (a) A comparison of TS structures for the proton transfer from tryptamine to O2 of D128 β in AADH calculated at the B3LYP/6-31G(d)/MM (pink carbon atoms) and BH&HLYP/6-31G(d)/MM (green carbon atoms) levels of QM/MM theory. (b) A comparison of TS structures for proton transfer to O2 (cyan carbon atoms) and O1 (yellow carbon atomss) of D128 β calculated at the B3LYP/6-31G(d)/MM level of theory.

Hydrogen bonding along the CINEB reaction paths. Structures generated along the reaction pathway were examined for hydrogen bonds between the QM and MM regions along the reaction path. Table 3 shows important interactions and their variation along the path for proton transfer to either O2 or O1 of D128 β at the BH&HLYP/MM level of theory (the B3LYP/6-31G(d)/MM results are shown in Table S3). Figure 3 shows a comparison of the TS structures, comparing the results from the different DFT functionals in (a) and the TSs of the two proton transfer pathways in (b). Both DFT methods give very similar geometries along the reaction paths, in terms of both reaction coordinate distances and hydrogen bonding. One slight difference between the structures generated by the two DFT methods is that the hydrogen bond between O1 and W160 β HN is slightly shorter at all points on the reaction coordinate in the BH&HLYP structures compared to the B3LYP values [e.g. $d(\text{D128}\beta \text{ O1} - \text{W160}\beta \text{ HN}) = 1.92 \text{ \AA}$ at R in the B3LYP/6-31G(d)/MM and $d(\text{D128}\beta \text{ O1} - \text{W160}\beta \text{ HN}) = 1.86 \text{ \AA}$ at R in the BH&HLYP/6-31G(d)/MM].

The same hydrogen bonds between the enzyme and tryptamine/TTQ are present throughout the paths for proton transfer to O2 or O1 of D128 β (Table 3). Hydrogen bonds involving HNT of the TTQ cofactor are slightly longer in the O1 pathways [e.g. $d(\text{HNT-D84}\beta \text{ HN}) = 2.04 \text{ \AA}$ in R for the O2 pathway and $d(\text{HNT-D84}\beta \text{ HN}) = 2.12 \text{ \AA}$ in R for the O1 pathway at the BH&HLYP/MM level of theory]. Protonation of D128 β on either O2 or O1 affects the hydrogen bond between O1 and W160 β HN more significantly than the hydrogen bond between O2 and T172 β HG1. Protonation of O2 causes the O2-T172 β HG1 hydrogen bond to lengthen by 0.14 $\text{\AA}$ (with either DFT

method) and the O1-W160 β HN hydrogen bond to lengthen by 0.17 – 0.20 Å. Whereas, when O1 is protonated, the O2-T172 β HG1 hydrogen bond lengthens by 0.07 Å and the O1-W160 β HN hydrogen bond lengthens by ~0.4 Å (with either DFT method).

Table 3: Average interatomic distances and hydrogen bonds with residues in the active site along the reaction paths for proton transfer to either O2 or O1 of D128 β (BH&HLYP/6-31G(d)/MM). Distances are given in Å, upper number is the average and the number in parentheses is the standard deviation. See Figure 1 for atom labels.

Proton transfer to O2 of D128 β				Proton transfer to O1 of D128 β			
	R	TS	P		R	TS	P
C1-H1	1.10 (0.00)	1.41 (0.01)	2.09 (0.01)	C1-H1	1.11 (0.00)	1.39 (0.02)	1.99 (0.00)
O2-C1	2.96 (0.02)	2.64 (0.01)	3.04 (0.01)	O1-C1	2.88 (0.02)	2.59 (0.01)	2.94 (0.01)
O2-H1	1.92 (0.04)	1.23 (0.01)	0.99 (0.00)	O1-H1	1.86 (0.06)	1.23 (0.01)	0.98 (0.00)
HE1-O7	2.78 (0.01)	2.76 (0.01)	2.75 (0.01)	HE1-O7	2.78 (0.02)	2.76 (0.02)	2.74 (0.01)
HE1-A82β O	1.77 (0.03)	1.79 (0.03)	1.81 (0.03)	HE1-A82β O	1.76 (0.04)	1.77 (0.04)	1.80 (0.04)
HNT-O7	2.16 (0.02)	2.16 (0.02)	2.08 (0.03)	HNT-O7	2.23 (0.02)	2.25 (0.02)	2.16 (0.03)
HNT-D84β O	2.04 (0.07)	2.11 (0.06)	2.17 (0.08)	HNT-D84β O	2.12 (0.18)	2.25 (0.20)	2.26 (0.20)
O7-D84 HN	2.02 (0.06)	2.03 (0.06)	2.01 (0.06)	O7-D84β HN	1.98 (0.07)	1.98 (0.08)	1.93 (0.08)
O2-T172β HG1	1.77 (0.02)	1.86 (0.03)	1.91 (0.01)	O2-T172β HG1	1.81 (0.03)	1.80 (0.02)	1.88 (0.01)
O1-W160β HN	1.95 (0.05)	1.97 (0.05)	2.15 (0.09)	O1-W160β HN	1.86 (0.06)	2.12 (0.13)	2.25 (0.12)

As identified in previous modeling of the AADH/tryptamine system, the two hydrogen bonds formed by the sidechain of D128 β (with T172 β and W160 β) determine its reactivity.³¹ The contribution of these residues to the QM/MM electrostatic energy was calculated by setting the MM atomic charges of these residues to zero. The average contributions of these residues to the

QM/MM electrostatic energy at the reactant (R), TS and product (P) are shown in Table 4 (the B3LYP/6-31G(d)/MM results are shown in Table S4). W160 β provides ~1 kcal/mol more electrostatic stabilization than T172 β in all structures of R. The electrostatic stabilization provided by both residues is greatest in the reactant and decreases significantly along the reaction coordinate, as expected as the negative charge on the aspartate lessens during the reaction. For example: T172 β provides ~ 14 kcal/mol stabilization in R, ~ 11 kcal/mol at the TS and ~ 9 kcal/mol in P for the O2 pathway, BH&HLYP/6-31G(d)/MM. The stabilization provided by W160 drops more rapidly and T172 provides more stabilization to the TS and P [W160 β ~ 15 kcal/mol stabilization in R, ~ 9 kcal/mol at the TS and ~ 4 kcal/mol in P]. The stabilization of the products by these two residues is similar. In contrast, at the B3LYP/6-31G(d)/MM level, W160 β continues to provide more electrostatic stabilization than T172 β in the product of either pathway: T172 β provides ~6 kcal/mol and W160 β ~8 kcal/mol stabilization in P. There is more variation in the electrostatic stabilization energies for the B3LYP/6-31G(d)/MM pathway for proton transfer to O2 of D128 β than in the other paths, indicated by the larger standard deviation of the energies for this path (Table S4). However, hydrogen bonds involving these residues show only small deviations from the average (maximum 0.07Å) with both functionals.

Higher level energy corrections. Single point energy calculations were carried out on the B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM geometries using a larger basis set for the DFT method (6-311+G(d)) and then (L)MP2 and SCS-(L)MP2, and LCCSD(T) methods with the (aug)-cc-pVTZ basis set

for the QM region (Table 5). At the LCCSD(T)/(aug)-cc-pVTZ/MM level of theory, the average barrier for proton transfer to O2 of D128 β is 16.7 kcal/mol for the B3LYP/MM geometries and 16.9 kcal/mol for the BH&HLYP/MM geometries. For transfer to O1, the corresponding barriers are 14.2 and 14.0 kcal/mol for the B3LYP/6-31G(d)/ MM and BH&HLYP/6-31G(d)/MM geometries, respectively. The SCS-MP2/(aug)cc-pVTZ/MM results are in good agreement (1.5 - 2 kcal/mol) with the LCCSD(T)/(aug)cc-pVTZ/MM energies, whereas the MP2/(aug)cc-pVTZ/MM energies are significantly lower (~ 6 kcal/mol). This confirms previous findings for citrate synthase³⁵ that SCS-MP2 is a good choice for calculations on enzyme-catalysed reactions. Higher accuracy is obtained using the spin component scaled method than with standard MP2.

Table 4: Average contribution (in kcal/mol) of T172 β and W160 β to the QM/MM electrostatic energy (QM/MM_{el}) at different points along the reaction pathway for proton transfer to O2 or O1 of D128 β (BH&HLYP/6-31G(d)/MM). The standard deviation of the average energy is given in parentheses.

	Proton transfer to O2 of D128 β		Proton transfer to O1 of D128 β	
	QM/MM _{el}	QM/MM _{el}	QM/MM _{el}	QM/MM _{el}
	T172 β	W160 β	T172 β	W160 β
R	-14.24 (± 0.45)	-15.41 (± 0.71)	-14.81 (± 0.50)	-17.34 (± 0.68)
TS	-11.45 (± 0.38)	-8.49 (± 0.81)	-11.93 (± 0.37)	-9.60 (± 0.75)
P	-8.51 (± 0.20)	-4.11 (± 0.51)	-8.83 (± 0.17)	-3.98 (± 0.47)

Increasing the size of the basis set from 6-31G(d) to 6-311+G(d) has a significant effect on the DFT energetics. The reaction barrier is increased by ~ 4 kcal/mol for both functionals. The average reaction barriers are 14.4 and

17.6 kcal/mol for proton transfer to O2 of D128 β , and 12.0 and 14.6 kcal/mol for proton transfer to O1, with B3LYP/MM and BH&HLYP/MM, respectively. Empirical dispersion corrections to energy barriers can be important for DFT calculations of reaction barriers.⁹⁰⁻⁹² However, dispersion effects are likely to be relatively small for this reaction as there is very little heavy atom movement/structural change involved, thus the correction due to changes in dispersion energy along the path is expected to be small.

Local approximations reduce computational expense of ab initio methods, but should be tested, as we do here. Calculations at the MP2 and LMP2 levels of theory show that any errors introduced by the local approximations are very small for this system, with the largest difference being ~ 0.5 kcal/mol (see Figure S1). The very small errors introduced by the local approximations are likely to be similar at the LCCSD(T) level, showing that the reduction in computational expense does not lead to any compromise in accuracy and justifying the choice of the LCCSD(T) approach here.

Modeling the reaction in solution using continuum solvent models. As described in the Methods section, continuum solvent calculations were performed on structures of the QM region from modeling the reaction in the enzyme. In this way, the effect of modifying the electrostatic environment from the enzymic one to a water-solution one can be estimated; this is not intended to model an actual reactive process in solution (which would require calculation of the energy needed to bring the reactants together, for example). Representative potential energy profiles (B3LYP/6-31G(d) and B3LYP/6-311+G(d)) in the gas phase, in solution and in the enzyme are shown in

Figure S8. The barrier to the reaction in the gas phase is very small (~1 kcal/mol), effectively barrierless, with both the solution and enzyme environments raising the barrier to reaction significantly.

Table 5: Average potential energy barriers ($\Delta^\ddagger v$) and reaction energies ($\Delta_r v$) calculated with DFT/6-31G(d), DFT/6-311+G(d), (L)MP2/(aug)-cc-pVTZ, SCS-(L)MP2/(aug)-cc-pVTZ and LCCSD(T)/(aug)-cc-pVTZ QM/MM methods on B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM optimized geometries. Reaction coordinate values (Z) are in Å and energies are in kcal/mol. The L in these acronyms indicates that local approximations were used for the ab initio methods and (aug) indicates that only the basis functions for oxygen atoms were augmented.

Were assigned.

		Z	DFT	DFT Larger basis	MP2	LMP2	SCS-MP2	SCS-LMP2	LCCSD(T)
$\Delta^\ddagger V_{O_2}$	B3LYP	0.19	10.46	14.36	9.67	9.59	14.74	14.62	16.68
	BH&HLYP	0.19	13.76	17.59	10.66	10.77	15.36	15.41	16.90
$\Delta^\ddagger V_{O_1}$	B3LYP	0.14	8.06	11.96	7.64	7.10	12.27	11.71	14.22
	BH&HLYP	0.16	10.61	14.55	8.11	7.88	12.62	12.37	14.00
$\Delta_r V_{O_2}$	B3LYP	1.02	4.87	7.94	-0.09	0.13	4.93	5.16	6.32
	BH&HLYP	1.10	3.83	6.52	-0.08	0.39	4.63	5.10	4.70
$\Delta_r V_{O_1}$	B3LYP	0.96	2.12	5.77	-3.89	-4.39	1.14	0.69	2.45

BH&HLYP 1.00 1.79 5.22 -2.83 -2.52 2.18 2.49 2.67

Table 6: Average potential energy barriers ($\Delta^\ddagger V$) and reaction energies ($\Delta_r V$) in solution calculated with DFT/6-311+G(d) using the SM8 and PCM solvation models. The average potential energy barriers in the enzyme (ENZ) are included to aid comparison. The standard deviation of the average energy is given in parentheses.

		ENZ	O2 SM8	PCM	ENZ	O1 SM8	PCM
$\Delta^\ddagger V$ / kcal/mol	B3LYP	14.36 (± 0.83)	13.18 (± 0.95)	6.14 (± 0.77)	11.96 (± 0.67)	9.85 (± 0.13)	1.41 (± 0.38)
	BH&HLYP	17.59 (± 0.30)	16.31 (± 0.86)	13.54 (± 0.74)	14.55 (± 0.57)	12.87 (± 0.17)	8.59 (± 0.36)
	B3LYP	7.94 (± 0.82)	-4.71 (± 0.35)	-10.61 (± 0.36)	5.77 (± 0.39)	-4.21 (± 0.38)	-13.55 (± 0.38)
	BH&HLYP	6.52 (± 1.10)	-9.45 (± 0.70)	-11.67 (± 0.58)	5.22 (± 0.61)	-8.20 (± 0.26)	-13.22 (± 0.28)

The average energetics of the reaction predicted using solvent continuum models are given in Table 9 and plots of the average paths are shown in Figures S9-S12. Both the PCM⁸⁸ and SM8⁸⁹ continuum solvent models predict lower potential energy barriers for the proton transfers than obtained in the enzyme environment, with the PCM⁸⁸ model predicting lower barriers than the SM8⁸⁹ model e.g. 16.31 (± 0.86) kcal/mol BH&HLYP/6-311+G(d)/SM8⁸⁹, 13.54 (± 0.74) kcal/mol BH&HLYP/6-311+G(d)/PCM⁸⁸ and 17.59 (± 0.86) kcal/mol BH&HLYP/6-311+G(d)/MM. With the SM8⁸⁹ solvent model, there is ~3 kcal/mol difference in the energies predicted by B3LYP and BH&HLYP methods, similar to the difference observed in the enzyme. There is a much larger difference of ~7 kcal/mol between the energies predicted by the two functionals with the PCM⁸⁸ model. The values predicted by the SM8⁸⁹ model are 1-2 kcal/mol lower than the enzymic barriers with either QM method, whereas the PCM⁸⁸ results are 8 kcal/mol lower at the B3LYP level

and 4 kcal/mol lower in energy with the BH&HLYP method. The standard deviations of these average barriers are all less than 1 kcal/mol as in the enzymic paths. However, the spread in barrier heights predicted for proton transfer to O1 is smaller than obtained for the enzymic paths (0.13 kcal/mol vs 0.67 kcal/mol with B3LYP for SM8⁸⁹ and QM/MM paths, respectively) showing that the larger spread in the enzymic barriers is due to difference in the (MM) enzyme environment, not the configuration of the QM atoms.

The reaction energies are very different in the two different environments. Both DFT methods show that the reaction in the enzyme is endothermic but that the reaction in solution is exothermic. This reflects modulation of the pK_a of the catalytic aspartate within the enzyme active site. For proton transfer to O2 of D128 β , the SM8 model predicts average reaction energies of -4.71 ± 0.35 kcal/mol and -9.45 ± 0.35 kcal/mol with B3LYP/6-311+G(d) and BH&HLYP/6-311+G(d), respectively. The SM8 continuum model gives similar reaction energies for proton transfer to O1: -4.21 ± 0.38 kcal/mol and -8.20 ± 0.26 kcal/mol (B3LYP/6-311+G(d) and BH&HLYP/6-311+G(d), respectively). These reaction energies are more than ~ 6 kcal/mol more exothermic than those for the enzymic paths. The reaction energies with the PCM models are all ~ 15 kcal/mol more exothermic than their enzymic counterparts. The reaction energies predicted by the PCM model are less dependent on the QM method than those calculated using the SM8 model; average energies of -10.61 ± 0.36 kcal/mol and -11.67 ± 0.58 kcal/mol are predicted for proton transfer to O2 of D128 β (B3LYP/6-311+G(d) and BH&HLYP/6-311+G(d), respectively). The corresponding values for proton transfer to O1 of D128 β are: -13.55 ± 0.38 kcal/mol and -13.22 ± 0.28 kcal/mol

(B3LYP//6-311+G(d) and BH&HLYP/6-311+G(d,p), respectively). The main effect of the enzyme on the energetics of this reaction step is stabilization of the carboxylate anion, which is required to maintain its reactive form in the enzyme.

Discussion

While semiempirical QM methods such as PM3 are useful for sampling e.g. in QM/MM MD simulations, they do not provide an accurate description of the reaction energetics.^{6, 31} Accuracy can be improved by using specific reaction parameters (SRP) but this cannot overcome fundamental limitations of these approximate QM methods.^{47, 93} DFT/MM MD simulations of enzymes are now possible,⁹⁴⁻⁹⁵ but require significant computer time. Interpretations of DFT/MM results should bear in mind the limitations of DFT methods for the reaction energetics that are revealed by the results here. Quantitative conclusions should not be drawn from DFT or DFT/MM calculations, although they can certainly provide qualitative and mechanistically useful insight for many systems. DFT results should be tested against correlated ab initio (CCSD(T) or SCS-MP2 calculations. Developments e.g. in QM codes for GPU technologies⁹⁶⁻⁹⁷ and other methodological, computational and algorithmic advances are making DFT calculations feasible for large systems⁹⁸ and, increasingly, for MD simulations, and thus of course DFT will remain the workhorse of computational chemistry for the foreseeable future. DFT is a popular choice for QM/MM (and e.g. QM only 'cluster') calculations due to the favourable compromise between accuracy and computational expense it offers. However, the lack of systematic improvability of current DFT methods (rather than DFT per se) means that caution should be used in drawing

quantitative conclusions, and where possible, DFT results should always be tested against ab initio calculations; where this is not possible, alternative functionals should be tested to investigate the sensitivity (and uncertainty) of DFT calculations.⁹⁹

The validity of the reaction coordinate used in adiabatic mapping was tested here by refining the pathways with NEB⁷³ and CINEB⁷⁴ methods. Pathways were initiated from the TS sampling window of QM/MM umbrella sampling MD simulations at the PM3/MM level of theory.³¹ Structures were generated along the adiabatic mapping paths by moving towards the reactants and products, respectively, in 0.1 Å intervals along a defined reaction coordinate. Structures from these pathways were further refined with NEB⁷³. The use of seven images for the pathway optimisation gave a good balance between convergence and accuracy; there was only a small difference in the imaginary frequency of the TS ($\sim 20 \text{ cm}^{-1}$) for pathways generated with 10 and 7 initial images. The pathways found using CINEB and adiabatic mapping agree well for barrier height and TS location, confirming that the reaction coordinate used here provides a good description of these (and similar enzyme-catalysed) reactions.

The most accurate results calculated here are those obtained at the LCCSD(T)/(aug)-cc-pVTZ level (barrier heights of $\sim 16.8/14.1$ (O2/O1) kcal/mol and reaction energies of $\sim 5.5/2.6$ (O2/O1) kcal/mol), and we use these as a reference for comparisons below. The average reaction barrier for proton transfer to D128 β O2 is 10.46 (± 0.75) kcal/mol at the B3LYP/6-31G(d)/MM level and 13.76 (± 0.36) kcal/mol at the BH&HLYP/6-31G(d)/MM level of theory. For proton transfer to D128 β O1 the barriers are 8.06 (± 0.70)

kcal/mol and 10.61 (± 0.54) kcal/mol with B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM, respectively. The inclusion of ZPEs and tunneling contributions would reduce these barrier heights by more than ~ 3 kcal/mol (see below). An apparent activation energy of ~ 12.7 kcal/mol is deduced from the Eyring plot of the data obtained from stopped-flow kinetics experiments over a range of temperatures ($T=5-20^\circ\text{C}$).⁶ Thus, while the barriers here are potential energy barriers that cannot be compared directly to free energy barriers (which include the effects of entropy, tunneling, etc), it is apparent that DFT calculations with small basis sets give barriers for proton transfer that are too low. In particular, B3LYP/6-31G(d)/MM gives the lowest barrier heights (by ~ 3 kcal/mol with the same basis set), which is consistent with B3LYP reaction barriers being too low in many (but certainly not all) cases.³⁴ BH&HLYP has previously been shown to give better results for proton transfers⁷⁰ and hydrogen bonding⁷². The difference in reaction energy predicted by the two different DFT methods is smaller at ~ 1 kcal/mol. B3LYP and BH&HLYP structures here are very similar, indicating that the structural results are not sensitive to the choice of functional.

Increasing the basis set from 6-31G(d) to 6-311+G(d) significantly improved the reaction energetics, giving barriers within ~ 0.7 kcal/mol of the LCCSD(T)/(aug)-cc-pVTZ results. This emphasises the importance of using reasonably large basis sets in DFT calculations;³² diffuse functions should generally be used for anions. SCS-MP2/(aug)-cc-pVTZ/MM and SCS-LMP2/(aug)-cc-pVTZ/MM methods also give good energy barriers [e.g. $\Delta^\ddagger V_{\text{O}_2} = 17.59$ kcal/mol, 15.36 kcal/mol, 16.90 kcal/mol for BH&HLYP/MM, SCS-MP2/MM and LCCSD(T)/MM, respectively]. These results show that

BH&HLYP QM/MM with a large basis set provides reasonably good accuracy for this reaction.

The apparent experimental barrier to reaction for AADH with tryptamine is ~ 12.7 kcal/mol (at 300 K).⁶ In terms of Transition State Theory, this is a free energy barrier and contains entropic, tunneling and ZPE contributions. Although the purpose of this work is not to compute accurate rate constants or kinetic isotope effects, but to compare the different QM methods tested against the LCCSD(T)/(aug)cc-pVTZ energy description of the reaction, we can estimate phenomenological free energy barriers by combining the current ab initio QM/MM results with findings from previous work. ZPE is important in the transfer of light particles such as protons. ZPE corrections (B3LYP/6-31G(d)/MM and BH&HLYP/6-31G(d)/MM, Table 3) are similar for both pathways (~ -3 kcal/mol), similar to values reported for this kind of reaction²³. These ZPE corrections would result in BH&HLYP/6-311+G(d) barrier heights of 14.33 and 11.24 kcal/mol for O2 and O1. Table S5 shows the data in Table 5 corrected for ZPE. Calculating multidimensional tunneling contributions (e.g. via small curvature approaches⁹³) is computationally expensive, even at the semiempirical level of QM/MM theory, so was not possible with the higher level methods used here. Tunneling contributions of -3.1 (O2) and -2.4 (O1) kcal/mol calculated previously at the PM3-SRP/MM level of theory do provide a useful indication of the magnitude of the tunneling contribution.³¹ This would lead to effective energy barriers (with no thermal effects) of 11.5 and 8.84 kcal/mol for O2 and O1, respectively (BH&HLYP/6-311+G(d)). The equivalent LCCSD(T)/(aug)cc-pVTZ values are 10.6 kcal/mol and 8.3 kcal/mol for O2 and O1, respectively. The contribution ($-T\Delta S$) of entropy to the free should

also be considered; it is likely to increase the barrier by 0.5-4 kcal/mol at 300K^{34, 100} (see also Supporting Information). Adding this to the ZPE and tunneling corrections indicates the barriers for both pathways are consistent with the apparent experimental value of ~12.7 kcal/mol (these corrections can be applied to all the potential energy barriers calculated here for comparison with experiment). The results here show that the rate-limiting proton transfer may take place by both pathways; both are kinetically and thermodynamically accessible. These two pathways are distinct, with different tunneling contributions and different temperature dependence. Both pathways should be considered in AADH, and in (the many) other enzymes in which a carboxylate group acts as a base and the two carboxylate oxygen atoms are distinguished by different environments.

The reaction is endothermic for both proton transfers. Transfer to O2 is more endothermic (4.70 (± 1.09) kcal/mol) than transfer to O1 (2.67 (± 0.78) kcal/mol LCCSD(T)//BH&HLYP/6-31G(d)/MM), i.e. transfer to O1 is predicted to be thermodynamically favoured by around 2 kcal/mol. The representative tunneling energy (RTE) of the system indicates the energy at which tunneling dominates the proton transfer (e.g. see Figure 5(a) in our previous work on AADH³¹). This difference in reaction energy, and different barrier shapes, will result in quite different RTEs for the 2 proton transfers and consequently different KIEs. Our previous (lower-level) calculations³¹ gave similar potential barriers for MADH with methylamine (15.3 kcal/mol) and AADH with tryptamine (15.5 kcal/mol), but very different KIES (11 vs 30), largely due to the differences in reaction energy for the two systems. While the models of the solution reaction here should not be overinterpreted, the energy profiles

suggest that there would be a significant contribution from tunneling for an equivalent uncatalysed reaction, suggesting that the contribution of tunneling to catalysis (rate acceleration) is small.¹⁰¹

There is very little change in the structure of the enzyme during the reaction.³¹ Also, the barrier and reaction energetics differ very little between the 5 MD snapshots, showing that there is not much affect of protein conformational variation. The structures obtained with the two the two DFT functionals (B3LYP and BH&HLYP) are similar, despite the energetic differences, so, only the BH&HLYP/6-31G(d)MM results are compared here with previous semiempirical calculations. Hydrogen bonds are generally shorter at the higher level of theory e.g. $d(\text{D128}\beta \text{ O2} - \text{T172}\beta \text{ HG1}) = 1.77 \text{ \AA}$ in the reactant at the BH&HLYP/6-31G(d)/MM level of theory and $d(\text{D128}\beta \text{ O2} - \text{T172}\beta \text{ HG1}) = 1.91 \text{ \AA}$ at the PM3/MM level.³¹ At both the PM3/MM and BH&HLYP/6-31G(d)/MM levels, the hydrogen bond between D128 β O2 and T172 β HG1 is shorter than that between D128 β O1 and W160 β HN. These hydrogen bonds involving the catalytic base change the most during the reaction: protonation of O2 increases hydrogen-to-acceptor distance by $\sim 0.1 \text{ \AA}$ / $0.2 \text{ \AA}$ for the hydrogen bonds with T172 β HG1 and W160 β HN, respectively. Protonation of D128 β O1 leads to a $\sim 0.4 \text{ \AA}$ increase in hydrogen-to-acceptor distance for the W160 β HN hydrogen bond, but only a $\sim 0.1 \text{ \AA}$ in the hydrogen bond between O2 and T172 β . The hydrogen bond between D128 β O1 and W160 β HN is of a similar length in the product of proton transfer to O2 at the PM3/MM and BH&HLYP/6-31G(d)/MM levels [$d(\text{D128}\beta \text{ O1} - \text{W160}\beta \text{ HN}) = 2.15(\pm 0.09) \text{ \AA}$ BH&HLYP/6-31G(d)/MM and $d(\text{D128}\beta \text{ O1} - \text{W160}\beta \text{ HN}) = 2.17 (\pm 0.23) \text{ \AA}$ PM3/MM³¹]. With PM3/MM, the

change in the D128 β O2 – T172 β HG1 hydrogen bond is larger (average $d(\text{D128}\beta \text{ O2} - \text{T172}\beta \text{ HG1}) = 2.68 (\pm 0.68) \text{ \AA}$ in the product ($d(\text{D128}\beta \text{ O2} - \text{T172}\beta \text{ HG1}) = 1.91 \text{ \AA}$ BH&HLYP/6-31G(d)/MM)).

The contributions of W160 β and T172 β to the electrostatic stabilization from DFT QM/MM are similar to those at the PM3/MM level.³¹ The interaction energies are similar at all levels of theory: e.g. the average residue contribution of W160 β to the electrostatic stabilization of the reactant is $-17.2/-16.7 \text{ kcal/mol}$ with B3LYP/MM; $-15.4/-17.3 \text{ kcal/mol}$ with BH&HLYP/MM (for O2/O1 pathways, respectively) and -13.1 kcal/mol with PM3/MM.³¹ A similar decrease in interaction energy along the reaction coordinate is observed at all levels of theory. In the product of proton transfer to O2, W160 β contributes -5.9 kcal/mol to the electrostatic energy and T172 β -3.4 kcal/mol , at the PM3/MM level³¹. At the BH&HLYP/6-31G(d)/MM level, T172 β makes a larger contribution (-8.5 kcal/mol) to the electrostatic stabilization energy of the product than W160 β (-4.1 kcal/mol). NB the PM3/MM interaction energies are not exactly comparable, being averaged over a much larger number of structures from an umbrella sampling simulation than the 5 structures used in the adiabatic mapping/NEB pathways here.

Comparison of results for enzyme with those for the same (QM) structures using continuum solvent models provides a simple analysis of the effects of the environment on the reaction energetics.¹⁰² The barriers in solution calculated with BH&HLYP are higher than those from B3LYP and the reactions are more exothermic. The SM8⁸⁹ continuum solvent model gives barriers 1-2 kcal/mol lower than in the enzyme and also significantly more

exothermic (> 6 kcal/mol) (Table 9), while the PCM model gives even lower barriers (4-8 kcal/mol lower than in the enzyme). The reactions in solution are exothermic (with either the PCM or SM8 solvent models). The otherwise large differences in the results with the two continuum solvent models suggests that results obtained with continuum models should be treated with caution.

The standard deviation of the barrier heights and reaction energies is less than 1 kcal/mol in both the enzyme and solution environments. The biggest difference between the two environments is in the reaction energy (not surprisingly because of the charge transfer in the reaction). The reaction is endothermic in the enzyme, but exothermic in solution. The standard deviation of the average reaction energy is smaller in the solution model e.g. ± 1.10 kcal/mol in the enzyme BH&HLYP/6-311+G(d)/MM and ± 0.26 kcal/mol with SM8 model at the BH&HLYP/6-311+G(d) level of theory (for proton transfer to O2); this is because of variations in the enzyme structure. The difference in reaction energy predicted for the two proton transfers is smaller in the solution models than in the enzyme, which is of course because the carboxylate oxygens (and other QM atoms) have specific, different hydrogen bond interactions in the enzyme. The BH&HLYP/6-311+G(d)/MM average reaction energies for O2 and O1 of D128 β are 6.52 ± 1.10 and 5.52 ± 0.61 kcal/mol, in the enzyme and -4.71 ± 0.46 kcal/mol and -4.21 ± 0.38 kcal/mol in solution (SM8). Thus, without the specific hydrogen bonding network provided by the enzyme, the two oxygens of the catalytic base are effectively indistinguishable, as expected. D128 β is the base in this step of the reaction but it is also important in several other steps in the reaction mechanism⁶, and potentially either oxygen atom of the carboxylate sidechain (O2 or O1) may be

involved in other steps. The enzyme environment makes these two oxygens distinguishable (with different basicities) by hydrogen bonding with T172 β and W160 β .

Conclusions

Here, we have presented multiple fully optimized potential energy profiles for two distinct proton transfer pathways in AADH, using correlated ab initio QM/MM methods up to the coupled cluster level of theory to obtain accurate energetics. The profiles show very little sensitivity to fluctuations in the enzyme conformation. We have optimized geometries with two different DFT functionals, initially using a reaction coordinate involving the difference of two distances: $Z = d(\text{D-H}) - (\text{H-A})/\text{\AA}$, where A is either O2 or O1. Refinement with NEB⁷³ and (particularly) CINEB⁷⁴ techniques to obtain true TSs shows that this reaction coordinate is a good choice for the proton transfers described here. The structure of the TS from adiabatic mapping is very close to the true TS in all cases, differing by a maximum of 0.07 $\text{\AA}$ in the value of Z.

The results show that two distinct reaction pathways are kinetically and thermodynamically accessible, and therefore both may contribute to reaction (and KIEs) in AADH. The potential energy barriers are 16.7 kcal/mol for proton transfer to O2 of D128 β and 14.0 kcal/mol for transfer to D128 β O1 at the LCCSD(T)/(aug)cc-pVTZ/MM//B3LYP/6-31G(d)/MM level of theory. DFT/6-31G(d)/MM (particularly B3LYP), MP2 and LMP2/(aug)cc-pVTZ/MM methods significantly underestimate the energy barriers, as is often (but not always) observed for these methods. The use of local approximations does not affect the quality of ab initio results. The BH&HLYP/6-311+G(d)/MM//BH&HLYP/6-31G(d)/MM results are reasonably close to the

coupled cluster energies, showing this to be the best choice of DFT functional for this system but a reasonably large basis set should be used. When the effects of ZPE and tunneling from lower level modeling (-3.1 kcal/mol or -2.4 kcal/mol for the tunneling contribution O2/O1) are included the barriers are reduced to 11.5 kcal/mol and 8.84 kcal/mol for transfer to D128 β O2/O1, respectively. These results agree well with the apparent experimental free energy barrier of ~12.7 kcal/mol (at 300K) for AADH with tryptamine.⁶ It is important to note that exact agreement should not be expected between potential energy barriers and activation energies derived from experimental kinetics, because the latter include effects such as entropy and quantum tunneling. Our aim here is not to calculate free energy barriers but rather to provide a firm basis for future calculations. Tunneling contributions can be calculated e.g. by VTST/MT calculations, but high levels of theory such as CCSD(T) are prohibitively expensive.

The LCCSD(T)/MM results presented here are the most accurate potential energy surfaces calculated to date for reaction in the important model enzyme, AADH. The B3LYP/MM method does not give very good agreement with the higher level methods for the barrier shape even with a larger basis set. BH&HLYP/6-311+G(d)/MM gives a better description, but the ab initio SCS-MP2/MM method gives results much more similar to the LCCSD(T)/MM barrier shape. (The MP2/MM method consistently predicts lower barrier heights and more exothermic reaction energies, leading to narrower barriers; MP2 is not recommended for this and similar enzyme systems, instead SCS-MP2 should be used). BH&HLYP gives better results for these proton transfers than B3LYP, but shows significant differences

(particularly for the reaction energy) from the most accurate (LCCSD(T)/MM) results. Caution should therefore be applied in drawing quantitative conclusions from lower-level calculations on this and similar enzyme-catalysed reactions. These findings demonstrate that it is necessary to go beyond DFT for accurate calculations of potential energy surfaces (e.g. for calculations of tunneling contributions or reaction dynamics) in AADH. To obtain accurate energetics, a correlated ab initio method is required (ideally at the coupled cluster level, or if that is not feasible, SCS-MP2), for AADH and for other enzymes.

The results demonstrate that two distinct pathways are energetically feasible for proton transfer in AADH. These pathways show significantly different features (e.g. different barrier heights and shapes) and thus will individually give rise to quite different tunneling behaviour. The contributions of both pathways should be considered in any investigation of the temperature dependence of the KIEs in AADH with any of its several alternative substrates, in MADH and also in the very many other enzymes in which a carboxylate group acts as a base: this effect is potentially of wide importance in experimental and computational investigations of tunneling in enzyme-catalysed reactions.¹⁶

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Supporting Information

Additional Table S1-2, Figures S1-11 and details of MM MD simulations and of the estimation of the activation entropy.

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