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Vibrational analysis of the chorismate rearrangement: relaxed force constants, isotope effects and activation entropies calculated for reaction in vacuum, water and the active site of chorismate mutase†

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ABSTRACT: We present results derived from vibrational Hessians calculated for the reactant complex, transition structure and product complex of the rearrangement of chorismate to prephenate under different conditions. The AM1 semiempirical MO and B3LYP/6–31G* density functional methods were employed for calculations in vacuum, whereas a hybrid QM/MM method AM1/CHARMM/TIP3P was used for calculations in water and within the active site of *B. subtilis* chorismate mutase. Kinetic and equilibrium isotope effects and entropies of activation and reaction were investigated as a function of the increasing size of the Hessian, as the system is expanded to include not only the atoms of chorismate/prephenate itself but also an increasing number of surrounding water molecules (up to 99) or active-site residues (up to 225 atoms). Primary ^{13}C and ^{18}O isotope effects are not sensitive to the size of the Hessian, but secondary $^{3}H–C^{5}$ and $^{3}H_2–C^{9}$ effects require the inclusion of at least those atoms directly involved in hydrogen bonds to the substrate or, better, a complete first solvation shell or cage of active-site amino acid residues. Pauling bond orders for the breaking CO and making CC bonds are remarkably similar for the transition structures in all three media. Relaxed force constants for stretching of these bonds (which allow meaningful comparisons to be made along a reaction path) give a significantly different picture of the bonding changes in the transition structures. The ratio of logarithms of kinetic and equilibrium isotope effects does not agree with measures of transition-state structure derived from Pauling bond orders or from relaxed force constants. There is no simple relationship between kinetic isotope effects and transition-state structure for this Claisen rearrangement. The calculated vibrational entropy of activation for the enzymic reaction agrees well with an experimental value for *E. coli* chorismate mutase. Vibrational entropy reduces the free energy barrier for the catalysed reaction by about 1 kJ mol^{-1} at 333 K.
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KEYWORDS: enzyme catalysis; transition state; solvation; isotope effects; relaxed force constants; vibrational entropy; cut-off rule; QM/MM Hessian

INTRODUCTION

In 1971, Page and Jencks pointed out[1] that the increased rates for intramolecular reactions as compared with intermolecular reactions in solution are due to the losses of translational and rotational entropy involved in the latter; large 'effective molarities' in enzyme-catalysed reactions could be rationalized in terms of these entropy changes. The entropically unfavourable association of an enzyme with its substrate is offset by a reduction in the free energy of binding of the complex from its intrinsic enthalpy of binding to the observed value. The intramolecular enzymatic reaction then proceeds with a free energy of activation that is reduced with respect to the intermolecular non-enzymatic reaction, since the adverse entropic component has already been 'paid for'.[2] A recent review of enzyme action described[3] this work as 'a milestone in enzymology of comparable importance to the role of quantum mechanics in chemistry'.

While not denying the truth of the insights provided by the Page and Jencks analysis, one may query their assumption that vibrational entropy changes do not contribute significantly. The example originally considered was cyclopentadiene dimerization, a reaction for which the entropy of activation is almost identical with the entropy for formation of the covalently bonded adduct (i.e. the transition state is very tight). Similarly, the molecules employed by physical organic chemists to account for the large rate enhancements characteristic of enzyme-catalysed reactions tend to be small, light and rigid.

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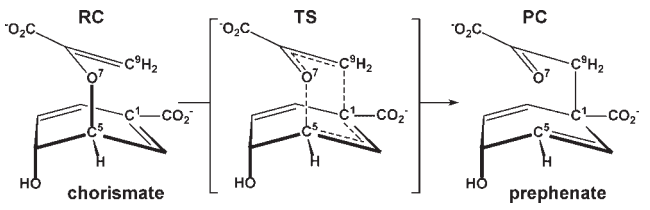
A typical model designed to illustrate intramolecular reactivity juxtaposes two reacting groups attached by strong covalent bonds to a small, rather rigid ring system.[4] Such a molecule would possess little vibrational entropy in the first place, and would therefore neither gain nor lose much on going to its transition state. In dramatic contrast, enzymes are large, heavy and floppy molecules and possess many very low-frequency entropy-rich modes of vibration.[5]

In 1974, Cook and McKenna suggested the potential importance of vibrational activation entropy in enzyme catalysis.[6] They hypothesized that alteration in bonding between an enzyme–substrate complex and its transition state could affect many vibrational modes, in such a way as to decrease the magnitude of low-frequency modes in the latter, which could lead to a significant positive change in vibrational entropy. However, at that time it was simply not possible to perform meaningful calculations of vibrational frequencies for the Michaelis complex and transition state of an enzymatic reaction. Hence the possible role of vibrational activation entropy in enzymatic catalysis has never been properly assessed.

Advances in computer hardware and software now make it feasible to perform the necessary calculations to assess the hypothesis outlined above. As the first stage of a larger program of research aimed at evaluating contributions from vibrational activation entropy to catalysis, this paper presents results of calculations based upon consideration of harmonic vibrational frequencies for subsets of atoms within a larger system. We consider vibrational Hessians calculated for the reactant complex (RC), transition structure (TS) and product complex (PC) of the rearrangement of chorismate to prephenate (Scheme 1) under different conditions, namely the gaseous phase, aqueous solution, and within the active site of *B. subtilis* chorismate mutase (BsCM). Reaction pathways and free energy profiles,[7] and kinetic isotope effects (KIEs),[8] for these rearrangements have been computed by means of a hybrid quantum-mechanical/molecular-mechanical (QM/MM) method. This is one of the few examples of an enzyme that catalyses a pericyclic reaction: the concerted Claisen rearrangement proceeds through a chair-like TS with asynchronous cleavage of a carbon–oxygen bond (C5O7) and formation of a carbon–carbon bond (C1C9). Although chorismate mutase is probably not an enzyme that might be expected to manifest any particularly noteworthy effects in respect of McKenna's hypothesis, nonetheless this study serves several purposes, not least that it provides a first example of this type of vibrational analysis.



Scheme 1

A second purpose for the present study was to provide a re-assessment of the 'cut-off rule' for calculations of KIEs and equilibrium isotope effects (EIEs). Wolfsberg and Stern[9] noted that it was possible to leave out parts of a large molecule without significantly affecting the value of a calculated KIE or EIE, provided that (1) the temperature was around room temperature, (2) the omitted atoms were more than two bonds distant from the position(s) of isotopic substitution where force constants changed on going from RC to TS and (3) the force constants for that portion of the molecule retained were correct (i.e. the same as they would be in the whole molecule without the cut-off).[10] Melander and Saunders discussed the practical usefulness of this simplification, as a means by which to reduce the computational cost of isotope-effect calculations, but added a wise precautionary note that the cut-off procedure would be valid only if it were justifiable to ignore the influence of medium effects on the isotope effect.[11] We now present results from calculations designed to investigate exactly this point.

A third purpose for this work was to examine the relationship between KIEs and TS structure. It has been commonly assumed that such a relationship exists;[12] for example, the ratio of (the logarithms of) a secondary KIE and EIE is often considered as a measure of progress of the TS along the reaction coordinate between RC and PC.[13,14] However, some doubt has recently been cast upon the generality of this relationship on the basis of *ab initio* molecular orbital (MO) calculations for deuterium[15] and heavy-atom[16] KIEs for *E*2 elimination. Most correlations between isotope effects and structure focus solely upon aspects of geometric structure, for example changes in bond length or bond angle, and most discussions of the non-synchronous or asymmetric character of pericyclic reactions, such as Claisen rearrangements, involve comparisons of bond lengths or derived Pauling bond orders.[17] However, bearing in mind that the three parameters necessary to define a Morse curve (as a model for a chemical bond) are the well depth, the equilibrium distance and the force constant at the energy minimum, an equally valid measure of TS structure is provided by changes in bond stiffness.

Relaxed force constants provide a physically meaningful way to compare the stiffness of bonds within a system undergoing chemical change, avoiding the ambiguities associated with conventional force constants in the presence of redundancies among the valence coordinates used to describe the bonding.[18,19] By consideration of the changes in the relaxed force constants for stretching of the making and breaking CC and CO bonds along the reaction coordinate for the rearrangement of chorismate to prephenate, we therefore have an independent measure of the degree of asymmetry and the tight/loose character of the TS. Relaxed force constants derived from Hessians computed for the rearrangement

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of chorismate to prephenate in vacuum, water and the active site of BsCM are compared with computed KIEs and EIEs (secondary ^{3}H and primary ^{13}C and ^{18}O) to provide complementary insight to that afforded by consideration of bond lengths or bond orders.

COMPUTATIONAL METHODS

Gas-phase calculations were carried out with the AM1 semiempirical MO method[20] and the B3LYP/6–31G* level[21] of DFT using the Gaussian 98 package of programs.[22] Hessian matrices of second derivatives of energy with respect to Cartesian displacements were evaluated analytically. QM/MM calculations in solution were carried out using the CHARMM24b2[23] and GRACE[24] programs. The diaxial conformer of the chorismate/prephenate QM system (24 atoms) was treated by AM1 and surrounded by a box of 711 water molecules described by non-rigid TIP3P empirical potentials[25] (note that CHARMM contains a modified TIP3P potential that includes intramolecular terms). Hessians were computed numerically for specified subsets of atoms of RC, TS and PC optimized as previously described:[7,8] those considered included 0, 11, 20, 31, 46, 60, 71, 80 and 99 water molecules selected by a distance criterion from the solute [Fig. 1(a)]. The largest Hessian was therefore of dimension 963×963. It is important to recognize that in every case the Hessian was computed using full QM/MM gradient vectors for the entire system.

QM/MM calculations for the BsCM enzyme (Protein Data Bank ID code 1COM)[26] complex with diaxial chorismate substrate were performed with CHARMM24b2 and GRACE. The QM region, treated by AM1, comprised the substrate only, while the MM region comprised the enzyme trimer plus crystallization and solvation water molecules (5654 enzyme atoms plus 3835 non-rigid TIP3P water molecules). Again the Hessians were computed numerically for specified subsets of atoms of RC, TS and PC optimized as previously described.[7,8] The smallest subset comprised the QM atoms of the substrate alone; in the larger subsets, amino acid residues were selected by a distance criterion from the substrate, such that the whole residue was included if it contained a single atom within a certain distance of any atom of the substrate. The largest Hessian contained a total of 13 residues (four arginines, two alanines, two cysteines, threonine, valine, glutamic acid, leucine and phenylalanine) and an active-site water molecule, together with the substrate [Fig. 1(b)], and was of dimension 747×747. Again, in every case the Hessian was computed using full QM/MM gradient vectors for the entire system.

KIEs and EIEs were evaluated from the optimized geometries and computed subset Hessians using our CAMISO program.[18] Unwanted contamination by spurious translational and rotational contributions, which give rise to small non-zero frequencies for translational and rotational motion, were eliminated by a projection method. The resultant pure vibrational frequencies for isotopomeric species satisfied the Teller–Redlich product rule, being entirely consistent with the masses and moments of inertia obtained from the molecular geometries.[27] This provides a stern and unambiguous test for the correctness of the procedures employed in the isotope-effect calculations. Partition functions were evaluated with the harmonic-oscillator, rigid-rotor, ideal-gas approximations and were utilised within the standard semi-classical transition-state theory of isotope effects.[11]

Hessians were transformed from Cartesian coordinates to non-redundant internal coordinates. The latter comprised generally of a sub-set of bond stretches, angle bends and torsions. Inversion of the non-singular matrix of internal coordinates yielded the compliance constant

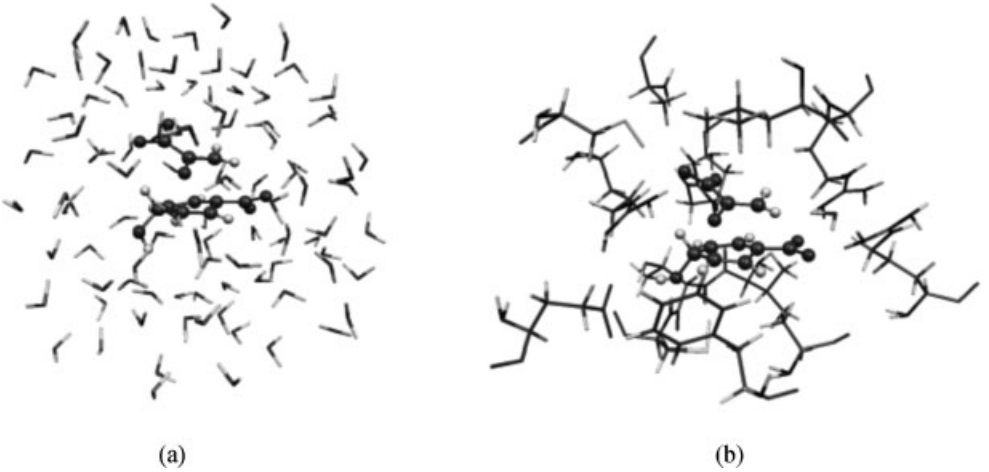

Figure 1. (a) Chorismate with 99 solvent water molecules included in largest Hessian for rearrangement in aqueous solution. (b) Chorismate with active-site residues included in largest Hessian for rearrangement catalysed by *B. subtitlis chorismate* mutase

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Table 1. Optimized bond lengths, Pauling bond orders and relaxed stretching force constants for breaking C5O7 and making C1C9 bonds in reactant complex, transition structure and product complex for rearrangement of chorismate to prephenate in vacuum, water and the active site of *B. subtilis* chorismate mutase

		Bond length (Å)			Pauling bond order[a]			Relaxed force constant (md Å^{-1})		
Method	Coordinate	RC	TS	PC	RC	TS	PC	RC	TS	PC
B3LYP/6–31G* in vacuum	C5O7	1.445	2.275	3.800	**1.00**	0.25	0.02	3.74	0.19	0.03
	C1C9	4.404	2.719	1.578	0.01	0.15	**1.00**	0.12	1.15	2.89
AM1 in vacuum	C5O7	1.429	1.854	3.637	**1.00**	0.49	0.03	6.49	1.16	0.08
	C1C9	4.124	2.164	1.536	0.01	0.35	**1.00**	0.15	0.51	5.11
AM1/TIP3P in water	C5O7	1.459	1.874	5.090	0.95	0.48	0.00	5.70	2.40	0.15
(Hessian = QM atoms only)	C1C9	3.401	2.141	1.530	0.04	0.36	1.01	0.20	1.28	6.70
AM1/CHARMM/TIP3P in	C5O7	1.445	1.879	3.303	0.97	0.47	0.04	5.80	2.10	0.20
enzyme (Hessian = QM atoms only)	C1C9	3.389	2.161	1.536	0.05	0.35	1.00	0.03	0.30	4.90

[a] Bold face indicates bond order = 1 by definition; $c = 0.6$.

matrix, the reciprocals of the diagonal elements of which are relaxed force constants. Relaxed valence force constants were obtained by back-transformation of the compliance matrix from non-redundant internal coordinates to redundant valence coordinates.[11] No scaling of force constants was performed.

RESULTS AND DISCUSSION

Table 1 contains optimized bond lengths, Pauling bond orders, and relaxed stretching force constants for the breaking C5O7 and making C1C9 bonds in RC, TS and PC in the three media. The Pauling bond order $n(\mathrm{C}\cdots\mathrm{X})$ for a $\mathrm{C}\cdots\mathrm{X}$ bond is given by $n(\mathrm{C}\cdots\mathrm{X}) = \exp[(r_1 - r_n)/c]$, where r_1 and r_n are bond lengths for C—X bonds of order 1.0 and n, respectively. Although a value of 0.3 for the coefficient c has been widely employed, we have noted elsewhere that this value correlates to changes in bond orders >1, due largely to changes in the degree of π-bonding. A different value is more appropriate for changes in bond order <1 which are largely due to changes in the degree of σ-bonding;[28] there is support for value of $c = 0.6$ as being more appropriate for bonds with $0 < n < 1$.[29] The C5O7 bond for RC and C1C9 bond for PC in vacuum are taken to have $n = 1.0$ by definition for the purposes of this study. The relaxed force constants in water and enzyme are derived from the Hessians for the largest systems considered.

Table 2 contains KIEs and EIEs calculated for the rearrangements in the three media, together with the mass-moment-of-inertia (MMI), excitational (EXC) and zero-point energy (ZPE) factors. Four isotopic substitutions are considered, corresponding to the effects previously calculated[8] and which have been measured experimentally for the enzyme-catalysed rearrangement: k_{12}/k_{13} at C5, k_{16}/k_{18} at O7, $k_{\mathrm{H}}/k_{\mathrm{T}}$ at C5 and $k_{\mathrm{H}}/k_{\mathrm{T}_2}$ at C9. For the sake of consistency, all isotope effect calculations were carried out for a temperature of 333 K, corresponding to the conditions of the experimental measurements in aqueous solution. This temperature is significantly higher than that used for experimental measurements of the enzyme-catalysed rearrangement, but the purpose of this paper is not to make comparisons of calculated results directly with experiment but rather with calculated results for different media.

Table 3 contains the calculated vibrational entropies of activation and reaction at 333 K for the rearrangements in the three media as evaluated for the differently sized Hessians.

It has been noted previously that the B3LYP/6–31G* method gives calculated KIEs (in vacuum) in very good agreement with experimental values determined for the Claisen rearrangement (in neat liquid) of allyl phenyl ether and of allyl vinyl ether.[17] In contrast, AM1 gives poor KIE predictions for the latter rearrangement and a TS structure with significantly shorter distances for both the breaking CO and making CC bonds. The same trend in the bond lengths is found in the present study (Table 1) with AM1 giving shorter distances for both C5O7 and C1C9 in RC, TS and PC. However, the purpose of this work was not to demonstrate agreement between calculation and experiment, but rather to investigate the sensitivity of the results to the size of the system included in the Hessian. Therefore, despite its clear limitations, it is adequate to employ the AM1 method for this purpose, especially since it was not computationally feasible to employ a DFT/MM method for the systematic evaluation of Hessians of ever increasing size. However, it is worth noting that the B3LYP/6–31G* method predicts the TS to be markedly looser than does AM1 for the reaction in vacuum.

Cut-off rules for isotope effects in condensed media

Inspection of Table 2 clearly shows that neither the (primary) KIEs nor the EIEs for the heavy-atom isotopic substitutions at C5 and O7 change significantly in value as the number of solvent water molecules included in the Hessian increases from 0 to 99 in aqueous solution, or as an essentially complete cage of active-site amino acid

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Table 2. Calculated kinetic and equilibrium isotope effects at 333 K for rearrangement of chorismate to prephenate in vacuum, water and the active site of *B. subtilis* chorismate mutase

Method	Isotopic substitution	No. of H_2O in Hessian (water) or cumulative residues in Hessian (enzyme)	KIE	MMI	EXC	ZPE	EIE	MMI	EXC	ZPE
B3LYP/6–31G* in vacuum	^{13}C5		1.021	0.997	0.999	1.027	1.004	1.000	1.002	1.001
	^{18}O7		1.036	0.996	0.994	1.046	1.009	0.990	0.977	1.043
	^{3}H–C5		1.189	0.993	0.947	1.264	1.261	0.990	0.909	1.401
	$^{3}H_2$–C9		0.900	1.008	1.113	0.802	0.693	1.022	1.178	0.576
AM1 in vacuum	^{13}C5		1.040	0.997	0.999	1.043	1.012	1.000	0.999	1.013
	^{18}O7		1.045	0.997	0.998	1.050	1.024	0.991	0.974	1.061
	^{3}H–C5		1.054	0.993	0.988	1.075	1.197	0.991	0.907	1.330
	$^{3}H_2$–C9		0.870	1.014	1.145	0.749	0.729	1.017	1.197	0.600
AM1/TIP3P in water	^{13}C5	0	1.018	1.001	1.001	1.016	0.996	1.003	0.997	0.997
		11	1.017	1.000	1.000	1.017	0.996	1.001	0.997	0.997
		20	1.017	1.000	1.000	1.017	0.996	1.001	0.997	0.997
		31	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
		46	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
		60	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
		71	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
		80	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
		99	1.017	1.000	1.000	1.017	0.996	1.000	0.998	0.998
	^{18}O7	0	1.042	0.998	0.996	1.048	1.021	0.990	0.979	1.053
		11	1.041	0.999	0.997	1.045	1.020	0.998	0.973	1.050
		20	1.042	0.999	0.998	1.045	1.020	0.999	0.972	1.051
		31	1.042	0.999	0.998	1.045	1.020	0.999	0.972	1.051
		46	1.042	0.999	0.998	1.045	1.020	0.999	0.972	1.051
		60	1.042	0.999	0.998	1.045	1.020	0.999	0.971	1.051
		71	1.042	0.999	0.998	1.045	1.020	0.999	0.971	1.051
		80	1.042	0.999	0.998	1.045	1.020	0.999	0.971	1.051
		99	1.042	0.999	0.998	1.045	1.020	0.999	0.971	1.051
	^{3}H–C5	0	1.120	0.997	0.971	1.157	1.188	0.999	0.979	1.215
		11	1.138	0.999	0.989	1.152	1.170	1.000	0.979	1.195
		20	1.144	1.000	0.995	1.150	1.171	1.000	0.978	1.197
		31	1.146	1.000	0.995	1.151	1.173	1.000	0.978	1.199
		46	1.145	1.000	0.995	1.151	1.174	1.000	0.978	1.200
		60	1.145	1.000	0.995	1.151	1.174	1.000	0.978	1.200
		71	1.145	1.000	0.995	1.151	1.172	1.000	0.977	1.200
		80	1.145	1.000	0.995	1.151	1.172	1.000	0.977	1.200
		99	1.145	1.000	0.995	1.151	1.172	1.000	0.977	1.200
	$^{3}H_2$–C9	0	0.899	1.004	1.108	0.808	0.771	1.015	1.178	0.645
		11	0.920	1.002	1.110	0.827	0.757	1.000	1.207	0.627
		20	0.923	1.001	1.109	0.831	0.756	1.000	1.213	0.623
		31	0.925	1.000	1.108	0.835	0.757	1.000	1.213	0.624
		46	0.926	1.000	1.108	0.836	0.756	1.000	1.212	0.624
		60	0.926	1.000	1.108	0.836	0.757	1.000	1.213	0.624
		71	0.926	1.000	1.108	0.836	0.757	1.000	1.213	0.624
		80	0.926	1.000	1.108	0.836	0.757	1.000	1.213	0.624
		99	0.926	1.000	1.108	0.836	0.757	1.000	1.213	0.624
AM1/CHARMM/ TIP3P in enzyme	^{13}C5	Substrate	1.038	0.999	0.998	1.041	1.007	0.997	0.992	1.017
		+Arg7	1.039	0.999	0.998	1.042	1.006	0.999	0.993	1.015
		+Arg63, Arg90	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
		+Arg116	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
		+Thr74	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
		+Val73, Glu78, Cys88, Leu115, H_2O	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
		+Ala59	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
		+Ala9, Cys75, Phe57	1.038	1.000	0.997	1.042	1.006	0.999	0.993	1.015
	^{18}O7	Substrate	1.043	0.999	0.996	1.049	1.018	0.998	0.976	1.045
		+Arg7	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
		+Arg63, Arg90	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
		+Arg116	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
		+Thr74	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045

(Continues)

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Table 2. Continued

Method	Isotopic substitution	No. of H_2O in Hessian (water) or cumulative residues in Hessian (enzyme)	KIE	MMI	EXC	ZPE	EIE	MMI	EXC	ZPE
		+Val73, Glu78, Cys88, Leu115, H_2O	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
		+Ala59	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
		+Ala9, Cys75, Phe57	1.043	1.000	0.994	1.049	1.018	0.998	0.976	1.045
	^{3}H–C5	Substrate	1.124	0.997	0.970	1.163	1.140	0.994	0.934	1.228
		+Arg7	1.112	0.999	0.988	1.127	1.122	0.996	0.939	1.200
		+Arg63, Arg90	1.115	1.000	0.996	1.119	1.122	0.996	0.939	1.200
		+Arg116	1.114	1.000	0.996	1.118	1.122	0.997	0.940	1.197
		+Thr74	1.114	1.000	0.996	1.118	1.123	0.998	0.941	1.196
		+Val73, Glu78, Cys88, Leu115, H_2O	1.115	1.000	0.996	1.119	1.123	0.998	0.941	1.196
		+Ala59	1.114	1.000	0.996	1.118	1.123	0.998	0.941	1.196
		+Ala9, Cys75, Phe57	1.114	1.000	0.996	1.118	1.123	0.998	0.941	1.196
	$^3H_2$–C9	Substrate	0.876	1.013	1.142	0.758	0.718	1.020	1.203	0.586
		+Arg7	0.899	0.999	1.145	0.786	0.714	1.008	1.221	0.580
		+Arg63, Arg90	0.880	1.001	1.162	0.756	0.713	1.002	1.236	0.576
		+Arg116	0.880	1.001	1.162	0.756	0.707	0.999	1.238	0.572
		+Thr74	0.879	1.001	1.161	0.757	0.709	0.997	1.249	0.569
		+Val73, Glu78, Cys88, Leu115, H_2O	0.879	1.000	1.164	0.755	0.709	0.997	1.249	0.569
		+Ala59	0.879	1.000	1.164	0.755	0.709	0.997	1.248	0.568
		+Ala9, Cys75, Phe57	0.879	1.000	1.164	0.755	0.709	0.997	1.248	0.568

residues is built up around the substrate in the BsCM enzyme. This is consistent with the observation that atoms more than one bond removed from the site of a heavy-atom isotopic substitution may be adequately truncated in model calculations of isotope effects.[11]

In water, there is a small but significant effect on both the (secondary) KIE and EIE for the isotopic substitution of tritium in place of protium attached to C5 as the number of solvent molecules included in the Hessian increases from zero, with the KIE becoming more normal (1.120 → 1.145) and the EIE less normal (1.188 → 1.172). Once a complete first solvation shell, in the range of 20–31 waters, is included both the overall isotope effects and their MMI × EXC × ZPE factors reach converged values, unchanged by inclusion of further molecules up to 99. Similarly, there are small but non-trivial changes to both the (secondary) KIE and EIE, for the isotopic substitution of two tritiums in place of the protiums attached to C9, as the number of solvent molecules included in the Hessian increases from zero,

Table 3. Calculated entropies of activation and reaction ($J\,K^{-1}\,mol^{-1}$) for rearrangement of chorismate to prephenate in vacuum, water and the active site of *B. subtilis* chorismate mutase

Method	Water: No. of H_2O in Hessian Enzyme: residues in Hessian (cumulative)		$\Delta S^{\ddagger}$	ΔS°
B3LYP/6–31G* in vacuum			−18.62	+26.37
AM1 in vacuum			−17.79	+9.35
AM1/TIP3P in water		0	−11.81	+46.89
		11	−20.04	+28.19
		20	−17.88	+26.44
		31	−18.54	+27.33
		46	−18.96	+27.27
		60	−17.88	+27.44
		71	−17.54	+27.35
		80	−17.29	+27.44
		99	−17.54	+27.35
AM1/CHARMM/TIP3P in enzyme	Substrate		−14.04	−11.06
	+Arg7		−15.13	−6.90
	+Arg63, Arg90		−12.97	−5.99
	+Arg116		−12.39	−5.07
	+Thr74		−13.80	−4.66
	+Val73, Glu78, Cys88, Leu115, H_2O		−13.97	−4.99
	+Ala59		−13.39	−4.82
	+Ala9, Cys75, Phe57		−13.88	−4.82

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with the KIE becoming less inverse (0.899 → 0.926) and the EIE more inverse (0.771 → 0.757).

In the enzyme, the ^{3}H–C5 KIE and EIE become slightly less normal (1.124 → 1.114 and 1.140 → 1.123, respectively) as the closest residues (arginines 7, 63, 90 and 116) are introduced into the Hessian but remain essentially unchanged as further residues are introduced. Inclusion of a single arginine residue changes the $^{3}H_2$–C9 KIE in the enzyme significantly, but again, once all four arginines are included both the KIE and EIE reach stable values.

A subset of N_s atoms within the environment of a larger N-atomic system possesses $3N_s$ vibrational modes, of which $3N_s - 6$ correspond to internal motions within the subset and six correspond to motions (librations) of the subset relative to its environment. Even though the entire system lies at a stationary point on the $3N - 6$ dimension potential energy hypersurface, in general the N_s-atomic subsystem is not at a stationary point within the $3N_s - 6$ dimensional subspace, and the 6 librations are not separable from the $3N_s - 6$ vibrations. Our procedure ignores all coupling between the subset atoms and their environment, but yields six translational and rotational modes with zero eigenvalues and $3N_s - 6$ genuine vibrations, and satisfies the Teller–Redlich product rule.[11] This procedure is consistent but necessarily approximate, since in reality the subset is coupled to its environment. Li and Jensen have recently described[30] a method for 'partial Hessian' vibrational analysis of large systems that is similar to our procedure but significantly different in detail. Their method yields three small but non-zero eigenvalues (typically $<10\,\mathrm{cm}^{-1}$) for motion of the subsystem relative to its environment and $3N_s - 3$ modes with significant eigenvalues for internal motions and for motion of the subsystem relative to its environment. This method is also approximate but is not consistent, since it treats the coupling of the subset with the environment neither as six librations nor as six translations and rotations, and it would not satisfy the Teller–Redlich product rule.

Medium effect on isotope effects

Substitution of ^{13}C and ^{13}O at C5 and O7 gives heavy-atom KIEs for the rearrangement in vacuum in the normal sense and with magnitudes greater than the corresponding EIEs, in accord with expectation; these effects are dominated by the ZPE factor. In water the ^{13}C5 KIE is normal but much smaller, and the EIE is slightly inverse. In the BsCM active site the normal ^{13}C5 KIE and EIE are each similar in magnitude to those in vacuum. In water and in the enzyme both the ^{18}O7 KIE and EIE are normal and similar in magnitude to those in vacuum. These heavy-atom primary isotope effects therefore show no medium effect with the exception of ^{13}C5 in water.

Substitution of tritium at the hydrogen attached to C5 gives rise to normal KIEs and EIEs in all three media, consistent with the change in hybridisation at this centre from tetrahedral to trigonal. The magnitude of the ^{3}H–C5 EIE decreases from 1.197 in vacuum to 1.172 in water and 1.123 in the enzyme, whereas the KIE increases from 1.054 in vacuum to 1.145 in water and 1.114 in the enzyme. Replacement of the two hydrogens attached to C9 by two tritiums leads to inverse KIEs and EIEs in all three media, consistent with the hybridization change from trigonal to tetrahedral at this centre. The magnitudes are similar in vacuum and in the enzyme (KIEs of 0.870 and 0.879, and EIEs of 0.729 and 0.709, respectively) but the values in water are less inverse (0.926 and 0. 757 for the KIE and EIE, respectively). Again, aqueous solution seems to cause the most significant medium effect.

Medium effect on TS structure: Pauling bond orders

As expected, the Pauling bond orders (Table 1) show values close to zero for the C1C9 bond in RC and the C5O7 bond in PC in all three media, corresponding to essentially no bonding. The fully formed covalent bonds C5O7 in RC and C1C9 in PC have bond orders of unity by definition in vacuum, and values close to unity in the condensed media. The TS bond orders in vacuum, water and enzyme are remarkably similar: the C5O7 bond is about half broken and the C1C9 bond is about one-third made. Remarkably, there is essentially no medium effect upon the TS structure as determined by the geometrically derived Pauling bonds orders.

Medium effect on TS structure: relaxed force constants

Just as a Pauling bond order tends to zero as a bond is stretched, but retains a finite value for all finite bond distances, so also a relaxed force constant may possess a non-zero value for pairs of atoms that are not formally connected by a covalent bond. Indeed, it is possible to determine physically meaningful and unambiguous values of relaxed force constants for stretching of each of the six bonds involved in the pericyclic rearrangement in each of RC, TS and PC. It is important to appreciate that this could not be done using conventional bond stretching force constants defined as the force engendered by unit extension of a particular bond while all other bonds and angles remain unaltered. The cyclic arrangement of six bonds necessarily involves a cyclic redundancy among the valence coordinates, since only five may be varied independently. In order to describe either RC or PC in a kinetically complete manner but avoiding redundancy, the obvious procedure would be to include stretching coordinates of the five covalent bonds in each case and to exclude the stretching coordinate for C1C9 in RC and C5O7 in PC. However, the value of the force constant for any other bond, for example O7C8, depends upon the

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choice of the other four coordinates; thus, in general, the force constant for stretching O7C8 will have a different value depending upon whether C1C9 or C5O7 is excluded. This is true for all three species RC, TS and PC and demonstrates that conventional valence force constants have no physical significance in the presence of redundancies among the valence coordinates. In contrast, the relaxed force constants, defined as the force engendered by unit extension of a particular bond while all other bonds and angles are allowed to relax to zero force, may be determined unambiguously for each bond in each species. Comparison of the values for any particular bond in RC, TS and PC provides meaningful physical insight into the bonding changes occurring along the reaction coordinate.

The fully formed C5O7 bond in the RC and C1C9 bond in the PC have relaxed force constants (Table 1) characteristic of covalent single bonds; the CO bond is slightly stiffer than the CC bond in vacuum and in enzyme, but the other way around in water. As expected, the fully broken C1C9 bond in the RC and the C5O7 bond in the PC have very small relaxed force constants. In the TS both coordinates have relaxed force constants with values clearly indicative of partially made or broken bonds, and the absolute values both in vacuum and in the enzyme seem to indicate rather loose bonding in a TS with dissociative character. The picture in water is, however, somewhat different: although the C5O7 bond in the TS is only slightly stiffer than in the enzyme, the C1C9 bond is considerably stiffer in water than in either the enzyme or vacuum.

Is there a relationship between KIEs and TS structure?

If the change in each relaxed force from RC to TS is expressed as a fraction of the total change from RC to PC, the resultant normalized value ϕ may be considered to be a measure of the degree of progress of TS along the reaction coordinate. By this measure, the C5O7 has almost completely loosened up in the TS for rearrangement in vacuum ($\phi_b = 0.83$) whereas C1C9 is still almost as completely loose as it is in RC ($\phi_m = 0.07$). In the enzyme TS, the degree of loosening of C5O7 is somewhat less ($\phi_b = 0.66$) whereas the looseness of C1C9 is unchanged ($\phi_m = 0.06$). In water, the degree of bond breaking of C5O7 is slightly smaller still ($\phi_b = 0.59$), but the degree of bond making of C1C9 is larger ($\phi_m = 0.17$). However, in comparison with the description provided by the Pauling bond orders, the relaxed force constants suggest a larger degree of bond breaking for C5O7 and a smaller degree of bond making for C1C9. Either of these bonding descriptors could be used to locate the TS on a More O'Ferrall-Jencks diagram[31] for the Claisen rearrangement, in the manner employed by Gajewski.[14] However, it is clear that each would locate the TS in a different place on such a diagram (Fig. 2). Remarkably, the bond-order measure places the TS at essentially the same spot on the map in vacuum, water, and enzyme: there is a very clear cluster of points at ($\phi_m \approx 1/3$, $\phi_b \approx 1/2$). In contrast the relaxed force constant measure of TS structure provides a very loose cluster of points for the three media: all indicate a more dissociative TS, with less bond making and more bond breaking. Obviously, these two measures of transition-state structure do not agree with each other. It is true that the tightness/looseness character of the Pauling bond orders could be altered by changing the coefficient c in the defining equation from the value of 0.6 adopted throughout this work. For example, a value of $c = 0.2$ would bring the bond-order (ϕ_m, ϕ_b) points into rough agreement with the relaxed force constant point in vacuum. However, a different value for c would be needed for each medium. Although there is scope for further investigation of this issue, the immediate and clear conclusion is that Pauling bond orders and relaxed force constants give different descriptions of transition-state structure. The generality of the commonly assumed[32] linear relationship between stretching force constants and bond orders must be called into question; while it may be reliable for bonds in stable molecules, it may be inappropriate for partial bonds in transition structures.

Figure 2 also shows (ϕ_m, ϕ_b) points determined by the ratios ln KIE/lnEIE for, respectively, $^3H_2$–C9 and ^{3}H–C5 in the three media. It is obvious that this isotope-effect measure of TS structure does not agree with either the bond order or the relaxed force constant descriptor. In part the problem may be with the intrinsically two-dimensional nature of the More O'Ferrall-Jencks

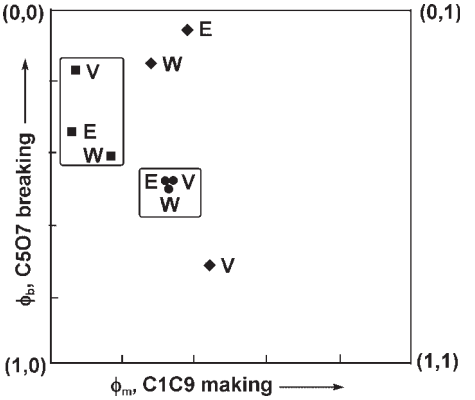

Figure 2. More O'Ferrall-Jencks diagram for the rearrangement of chorismate to prephenate in vacuum (V), water (W) and enzyme (E). RC is located in the bottom-left and PC in the top-right corner. The top-left corner represents the intermediates in a completely dissociative mechanism and the bottom-right corner the intermediate in a completely associative mechanism. ●, Location of TS as determined by the fractional change in Pauling bond order; ■, location of TS as determined by the fractional change in relaxed force constant; ◆, location of TS as determined by the normalized value (ln KIE/lnEIE) of the isotope effects, k_H/k_T at C5 for C5O7 and k_H/k_{T_2} at C9 for C1C9

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diagram, the construction of which excludes consideration of the cyclically delocalized (aromatic) character of transition states.[33] More fundamentally, the problem is the multidimensionality of the structural changes occurring during the rearrangement. Individual descriptors of bonding changes, e.g. bond orders, force constants and isotope effects, capture different and very partial views of the whole process. We are bound to concur with Jensen and co-workers[15] and with Saunders[16] that, for the Claisen rearrangement studied here as for the *E*2 eliminations they each considered, 'there is no obvious answer to the question of whether TS geometries can be inferred from KIEs' and 'the dependence of KIEs on TS structure appears too complex to be expressed well by simple relationships with bond orders' or other descriptors.

Vibrational entropies

The calculated entropy changes shown in Table 3 are for the rearrangement of the diaxial conformer of chorismate. Thus the B3LYP/6–31G* value of 26.4 $J\,K^{-1}\,mol^{-1}$ for the entropy of reaction in vacuum at 333 K differs from that of 3.3 $J\,K^{-1}\,mol^{-1}$ previously reported (at 298 K),[34] since the latter refers to rearrangement of the diequatorial conformer. Clearly there is an adverse-entropy change of $-23.1\,J\,K^{-1}\,mol^{-1}$ for the equilibrium between diequatorial and diaxial conformers. The activation entropy is predicted to be about $-18\,J\,K^{-1}\,mol^{-1}$ (at 333 K) for both B3LYP/6–31G* and AM1 methods.

The AM1/TIP3P calculated values for both $\Delta S^{\ddagger}$ and ΔS° in water (-17.5 and 27.4 $J\,K^{-1}\,mol^{-1}$, respectively, at 333 K) with 99 solvent molecules included in the Hessian are very similar to the B3LYP/6–31G* values in vacuum. However, it is clear that it is necessary to include at least the first solvation shell of water molecules, since the values calculated for the substrate alone are considerably different. Although it is the total entropy that is calculated for each species, the entropy changes are completely dominated by the vibrational entropy contribution. The uncatalysed rearrangement of chorismate to prephenate in water has an experimental activation entropy of $-54\,J\,K^{-1}\,mol^{-1}$ at 298 K,[35] but again this refers to reaction of the diequatorial not the diaxial, conformer. Comparison with the calculated entropy of activation would require the entropy change for the diequatorial–diaxial conformational equilibrium in water to be computed, which, in turn, would presuppose that the chorismate conformation of lowest free energy had been determined. Although the necessary molecular dynamics simulations are in progress, the results of this investigation will be reported elsewhere.

The entropies of activation and reaction calculated for the enzyme-catalysed rearrangement depends upon the number of amino acid residues included in the Hessian. The effect upon the reaction entropy is particularly marked, changing from -11 to $-5\,J\,K^{-1}\,mol^{-1}$ at 333 K as the number of included residues increases. The values seem to have converged for the largest Hessian employed in this study, for which the AM1/CHARMM/TIP3P calculated $\Delta S^{\ddagger} = -13.9\,J\,K^{-1}\,mol^{-1}$ at 333 K. This agrees well with the experimental value of $-12.6 \pm 6.7\,J\,K^{-1}\,mol^{-1}$ determined from the temperature dependence of k_{cat} between 283 and 310 K for the *E. coli* chorismate mutase,[36] but not so well with the value of $-38 \pm 5\,J\,K^{-1}\,mol^{-1}$ determined similarly for the *B. subtilis* enzyme between 278 and 318 K.[37] However, it has been shown that the latter enzyme is partially diffusion controlled,[38] so the measured activation parameters do not directly reflect the transition state for the chemical rearrangement; therefore the comparison between the present calculated result and the *E. coli* enzyme may be reasonably valid.

Finally, the difference in calculated activation entropies for the rearrangements of diaxial chorismate in water and in enzyme (at 333 K) is just 3.7 $J\,K^{-1}\,mol^{-1}$, corresponding to a free energy difference of $-1.2\,kJ\,mol^{-1}$ in favour of chorismate mutase. This rather small effect is not unsurprising, and is consistent with Warshel and coworkers' assertion (based on computational simulations for subtilisin) that 'entropic effects do not contribute in a major way' to the reduction of the free energy of activation.[39] However, in our view it would be dangerous to generalize this statement from only two examples; whether other enzymes may display the type of effect proposed by Cook and McKenna[6] remains an open question.

CONCLUSIONS

Calculated heavy-atom ^{13}C and ^{18}O KIEs and EIEs for the chorismate–prephenate rearrangement are insensitive to inclusion within the Hessian of solvent molecules or of chorismate mutase active-site residues. On the other hand, secondary ^{3}H KIEs and EIEs for this reaction do depend upon the size of the Hessian. In general, it is necessary to include the equivalent of a first solvation shell in order to obtain converged values for the isotope effects and their MMI, EXC and ZPE factors. More particularly, it may be sufficient to include only those atoms involved in specific interactions (e.g. hydrogen bonds) to the substrate.

The sensitivity of substrate isotope effects to inclusion of atoms of the 'environment' within the Hessian is not exerted through the values of the force constants, since all Hessians are computed using the full QM/MM gradient vector of the entire system. It may arise as the result of truncating a large system by including only the internal motions of the selected subset of atoms while neglecting the librational motions of the subset with respect to its environment. In this sense it may be considered as the price to pay for isotope effects that satisfy the Teller–Redlich product rule.

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The importance of these librational modes for accurate computation of isotope effects may indicate a source of error present in all calculations based upon continuum solvation models which inherently neglect specific interactions between a substrate and its environment.

There are significant medium effects upon secondary ^{3}H KIEs and EIEs for the chorismate–prephenate rearrangement. To obtain agreement between calculated and observed isotope effects it may be essential to include solvation effects explicitly.

Relaxed force constants provide an independent measure of the bonding changes occurring during the rearrangement, and suggest TSs much looser (less stiff) than indicated by Pauling bond orders for the making CC and breaking CO bonds. There is no obvious relationship between relaxed stretching force constants and Pauling bond orders for these bonds in the TS.

Just as the relaxed force constants and the Pauling bond orders do not agree with each other as measures of transition-state structure, so also neither agrees with that deduced from the ratios ln KIE/lnEIE of secondary ^{3}H isotope effects for the making CC and breaking CO bonds. There is no obvious relationship between KIEs and transition-state structure.

The geometry of a transition structure is not the only property that may be validly and usefully employed to describe the nature of a transition state. Changes in relaxed force constants provide complementary insight into the reorganization of chemical bonds along a reaction coordinate, and may offer a significantly different view upon the transition state.

Vibrational entropies of activation are sensitive to the size of the Hessian in a manner similar to that the isotope effects. The AM1/CHARMM/TIP3P calculated $\Delta S^{\ddagger}$ for *B. subtilis* chorismate mutase is in good agreement with the value determined experimentally for the *E. coli* enzyme for which the chemical rearrangement is rate limiting.

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