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Semiempirical calculations of the oxygen equilibrium isotope effect on binding of oxamate to lactate dehydrogenase

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Abstract. Semiempirical methods have been used in an attempt to predict theoretically the experimentally observed value of 0.9840 for the oxygen isotope effect on binding of oxamate to lactate dehydrogenase. The overall strategy involved vibrational analysis of oxamate in two different environments; that of the active site residues and in aqueous solution. The comparison of calculated values with the experimentally determined isotope effect proved the AM1 Hamiltonian to be superior to the PM3 Hamiltonian in this modelling. While most tested methods of accounting for solvent effects on the vibrational frequencies of the solute yielded similar results it turned out that what was crucial for the purpose of determination of the isotope effect was the model of oxamate in the active site of the enzyme. In particular, the major factor responsible for the inverse value of this isotope effect can be ascribed to the formation of an ordered, bifurcated hydrogen bond between the oxamate carboxylate and the guanidinium group of the active site histidine.

Key words: Isotope effects – Oxygen-18 – Oxamate – Lactate dehydrogenase

Introduction

Computer-based methods of predicting molecular properties have gained wide acceptance in many areas of chemistry; the results obtained using some methods have a status equivalent to those determined experimentally. The ultimate goal of computational simulations is the accurate prediction of the properties of systems inaccessible to direct experimental measurements. Non-covalent, short-lived enzyme-substrate complexes are an ideal example of such systems. Unfortunately, the most dependable quantum mechanical ab initio calculations, which rely directly on the (approximate) solution to the Schrödinger's equation, are not capable of handling such macromolecular complexes owing to the limitations in computer time.

In order to provide quantum-mechanics based methods suitable for the calculations of properties of medium-size molecules at a reasonable amount of computer time, a series of so called semiempirical methods has been developed. Semiempirical methods, while also rooted in the molecular orbital framework, require, unlike the ab initio approach, a set of experimentally determined parameters in order to compensate for the simplifications used in the course of calculations. A series of semiempirical self-consistent field molecular orbital methods originated by Dewar and further developed by his successors consists now of a set of Hamiltonians, the latest of which are MNDO (Dewar and Thiel 1977a, b), AM1 (Dewar et al. 1985), PM3 (Stewart 1989a). The results of calculations carried out using these methods, despite their quite rigorous teoretical basis, are still in the phase of being confronted with ab initio and experimental data in order to evaluate their accuracy. Their applicability to the modelling of the course of enzymatic reactions in a predictive manner is therefore still limited.

In this paper we attempt to test the ability of available semiempirical methods, especially those developed recently which permit simulations in solutions, to reproduce isotope effects on enzyme-ligand association. Theoretical evaluation of isotope effects is one of the most sensitive criteria of the reliability of a computational method. Since such evaluation depends on moments of inertia and normal modes of vibrations, both geometry optimization and force field calculations can be tested. Furthermore, equilibrium isotope effects are easier to examine and more straightforward to interpret than kinetic ones, thus providing the best grounds for testing purposes.

We have recently reported [Gawlita, Paneth and Anderson, submitted for publication] the experimental determination of the equilibrium oxygen isotope effect on binding of oxamate to lactate dehydrogenase, LDH. This equilibrium process is represented by the following equation:

$$(\text{oxamate})_{aq} \overset{K_A}{\Leftrightarrow} (\text{oxamate})_{LDH} \qquad (1)$$

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where "aq" and "LDH" subscripts indicate aqueous and bound states of the ligand, respectively, and K_A is the association constant. The experimentally determined value of the oxygen equilibrium isotope effect on this constant is 0.9840±0.0027.

We have undertaken theoretical computation of this effect in an attempt to evaluate some of the physical phenomena underlying the process of enzyme-ligand association. The calculations were carried out to yield optimized geometries and corresponding vibrational analysis of oxamate in the two different environments necessary for evaluation of the equilibrium isotope effect; in aqueous solution and within the active site of the enzyme. The semiempirical level of calculations was dictated by the number of atoms included our models. The availability of a high resolution crystal structure of the ternary LDH-NADH-oxamate complex provided a good starting point for the active site-ligand interactions modelling. Several methods of accounting for solvent effects on a semiempirical level have been recently described in the literature (Karelson et al. 1989; Cramer and Truhlar 1991b, 1992; Luzkhov and Warshel 1992) but their accuracy has been primarily tested by evaluating their ability to reproduce free energies of solvation. Only the latest few studies attempted to predict such phenomena as tautomeric or conformational equilibria in solution (Katritzky and Karelson 1991; Urban et al. 1992). The applicability of these methods to the vibrational analysis of molecules in solution is still debatable. We have therefore decided to employ currently available solvation models, namely SCRF, SM2 or SM3, and COSMO as well as the supermolecule approximation, to carry out simulations of the oxamate ion in water. This was done with the hope that the studies presented below will provide guidance concerning the choice of the method which is able to account for the solvent-induced perturbations in the solute vibrational frequencies.

Methods

Calculations of the equilibrium isotope effect

The minimal information neccessary to calculate an equilibrium isotope effect is two sets of normal modes of vibrations related to the isotope effect by the so called complete equation (Melander 1960):

$$\frac{K_{16}}{K_{18}} = \prod_{i}^{3n^{P}-6} \frac{u^{P}_{i(16)} \cdot \sin(u^{P}_{i(18)}/2)}{u^{P}_{i(18)} \cdot \sin(u^{P}_{i(16)}/2)} \times \prod_{i}^{3n^{S}-6} \frac{u^{P}_{i(18)} \cdot \sin(u^{S}_{i(16)}/2)}{u^{P}_{i(16)} \cdot \sin(u^{S}_{i(18)}/2)} \qquad (2)$$

where K_{16}/K_{18} is the equilibrium oxygen isotope effect; n are numbers of atoms in substrate or product; and $u=h\nu/kT$, where h and k are Planck and Boltzman constants, respectively, T is the absolute temperature and ν_i are the frequencies of normal vibrations. Subscripts "16" and "18" correpond to ^{16}O or ^{18}O substituted species, respectively, while the "S" and "P" superscripts indicate the properties of substrate or product molecules.

All data necessary to calculate the isotope effect is available from semiempirical results which include geometry optimization and force field calculations and were extracted from the appropriate output files using our ISOEFF program[1]. All calculated frequencies were used, the actual number depending on the size of model used (see below). However, only those frequencies which exhibit isotopic shift contribute to an isotope effect, as can be seen from Eq. (2). The isotope effect was calculated at the temperature of 277 K. Since there are two indistinguishable carboxylic oxygen atoms per molecule, the isotope effects were calculated for doubly substituted oxamate. Values of the isotope effect reported here are square roots of these, as implied by the rule of geometric mean (Bigeleisen 1955).

Calculations of the properties of reactants

Two quantum chemical methods, ab initio and semiempirical, are able to predict frequencies of normal vibrations and moments of inertia of isotopic molecules necessary for isotope effect evaluation. Both these techniques, based on the molecular orbital (MO) framework, have been successfully applied to theoretical calculations of the effect of isotopic substitution on the reaction rate constants or equilibrium constants (Jones and Urbauer 1991). Only the semiempirical treatment, however, is capable of handling systems consisting of hundreds of atoms at a reasonable cost in computer time. This is therefore a methods of choice for simulations of properties of a ligand bound to an enzyme active site.

The state-of-the-art semiempirical techniques offer possibilities to carry out the calculations for isolated molecules in the gas phase or, at the expense of longer simulation times, for molecules in solvents of given macroscopic characteristics. A gas phase approximation is believed to be suitable for the description of properties of a ligand molecule buried inside the active site of an enzyme. This is because the environment of enzyme binding pockets is highly hydrophobic and characterized by a low dielectric constant in which it resembles a vacuous rather than an aqueous medium. Recent developments in the application of MO methods to condensed phases, on the other hand, has made possible a realistic treatment of the properties of a ligand surrounded by solvent molecules.

In the initial state, oxamate is surrounded by a random network of water molecules in solution, while in the bound state this surrounding network is replaced by a more ordered environment of amino-acid residues in the enzyme active site. Thus, two separate evaluations of the vibrational characteristics of oxamate were needed. The comparison of results from these two determinations on a semiempirical level yields the theoretical value of the isotope effect on the association constant K_A (K_{16}/K_{18}).

Several programs were used throughout calculations; MOPAC ver. 5, 6 and 93 (Stewart 1990) and AMSOL ver.

[1] ISOEFF ver. 6.0 program can be obtained upon request from P. Paneth (ppaneth1@plearn.edu.pl). This program, written in FORTRAN, extracts all data necessary for calculations of isotope effects from outputs of quantum chemistry packages. MOPAC/AMPAC, AMSOL, SIBIQ, GAMESS and GAUSSIAN formats are currently supported

3.1 (Cramer and Truhlar 1991a) packages from QCPE were used for geometry optimizations and force field calculations, our own program ISOEFF (ver. 4.0) for calculations of isotope effects, and INSIGHT II from BIOSYM, San Diego, CA, for visualization purposes. The following platforms were used: Silicon Graphics IRIS 4D under Irix 4.05 System V, PC486/50 MHz under DOS 5.0 or SCO Open Desktop 2.0, and IBM 3090 J under VM/XA 2.1.

To ensure convergence we have found it necessary to turn the acceleration procedures off by invoking the NODIIS keyword. Oxamate contains the – NHCO – arrangement typical for the peptide linkage. Semiempirical methods underestimate the barrier to rotation of such a linkage. This can be corrected by introducing a Molecular Mechanics correction. For simplicity, this correction was not applied in our studies.

Construction of the LDH active site model with bound oxamate. The crystal structure of the ternary complex of dogfish LDH with NADH and oxamate deposited as 1ldm file in the Brookhaven Protein Data Bank (pdb) was used as a basis for the construction of the starting geometry of oxamate-LDH active site complex. The complete 1ldm.pdb file contains the coordinates of 2787 heavy atoms and it was highly impractical to optimize geometry and compute the force field for such a large system. However, the influence of amino acids located outside the active site on the bound ligand is insignificant and can be neglected. The INSIGHT II molecular graphics package was used to select the relevant amino acid residues and extract them from the original pdb file forming the oxamate-NADH-LDH active site model. A 5Å radius centered on the oxamate molecule contained, besides oxamate itself, the following amino acids: His-193, Arg-106, Arg-169, Asn-138 as well as the NADH molecule. This selection overlaps, to a large extent, with related models of the LDH active site, especially that describing the residues important for catalysis and binding, on the basis of site-directed mutagenesis studies (Clarke et al. 1989a, b). The differences in numbering between that model and ours are result of distinct primary structures of the bacterial and dogfish muscle enzymes. However, the residues in the active sites of LDH from dif-

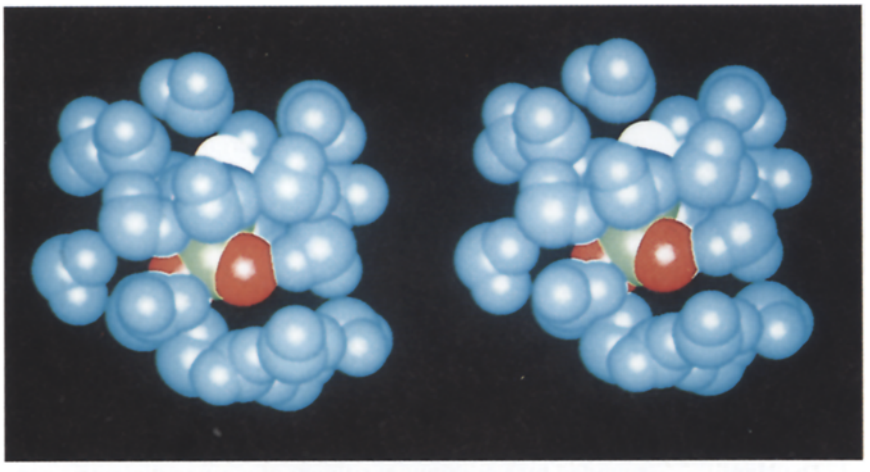

Fig. 1. Model of oxamate in water used in semiempirical calculations. The TIP4P water molecules are cyan, while the oxamate is colored by element: oxygen is red, nitrogen is blue, carbon is green, and hydrogen is white. The spheres are drawn at their full van der Waals radius

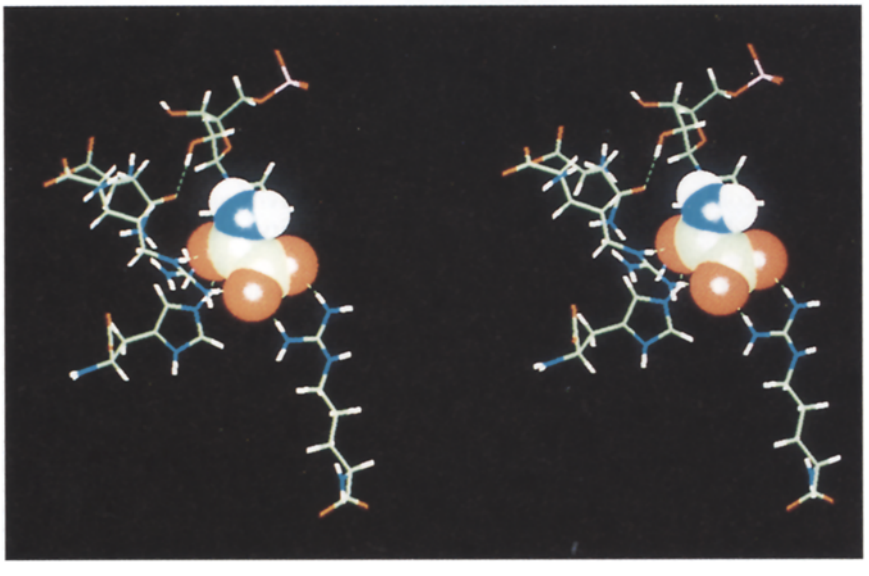

Fig. 2. Hydrogen bonding between oxamate and the model of LDH active site. The H-bond between Arg-169 and the oxamate carboxylate is shown at lower right. The H-bonds from the oxamate amide carbonyl to Arg-106 and His-193 are present at left. The NAD+ ribose-5′ phosphate (phosphorous is magenta) is the residue at the top with the nicotinamide being behind the oxamate

ferent sources are conserved; thus, for example, His-193 in the LDH from dogfish muscle corresponds to His-195 in the most popular bacterial LDH. These similarities indicate that the subspace created by the radius of 5Å centered on the oxamate molecule produces a meaningful representation of the ligand environment. The amino acid residues were cut from the original file along the peptide bonds and the appropriate N- or C-terminals atoms were then added. In order to further decrease the number of atoms in the active site model only the nicotinamide portion of the coenzyme molecule was taken into account. The file constructed in this way lacked the hydrogen atoms as the X-ray crystallographic coordinates explicitely define only positions of atoms heavier than hydrogen. Missing hydrogen atoms were therefore added a required positions at the standard C-H, N-H or O-H bond lengths of 1.10, 1.01 and 0.97 Å respectively. Their positions were subsequently optimized. The final model of the LDH active site with bound oxamate contained a total of 132 atoms and is illustrated in Fig. 2.

Geometry optimization and force field calculations. MOPAC ver. 6 was used for all calculations involving oxamate in the LDH active site. During the geometry optimization procedure, only the positions of oxamate atoms were allowed to be changed, while the remaining atoms of the amino acid residues and the NADH molecule were "frozen" in their initial coordinates (with the exception of added hydrogen atoms, as mentioned above). This simplification is justified because the crystallographic coordinates represent the enzyme that had already undergone conformational changes accompanying the ternary complex formation and the positions of enzyme atoms are not expected to change significantly. In contrast, the ligand (oxamate) may still retain some degree of conformational freedom even after the molecule is constrained in the active site. Analysis of the most likely protonation states of the oxamate carboxyl group as well as ionizable portions of the four amino-acid residues and NADH molecule at pH 7.0 resulted in applying a net charge of –3. The Hamiltonians AM1, PM3 and MNDO were used for the geometry optimization. The force field calculations were performed for all the obtained optimized geometries using the analytical derivatives.

Force fields were calculated in two different ways. One method was to calculate the force field for the oxamate atoms only, using the geometry optimized in the presence of the active site amino-acids and the truncated NADH molecule. In an alternative approach, the force field was computed for all atoms. The same force field was then used to calculate the vibrational frequencies of oxamate substituted with ^{18}O in both carboxyl oxygens (using the ISOTOPE and RESTART keywords). The files resulting from these calculations were then used directly for calculations of the isotope effect.

"Supermolecule" approach. The most straightforward method of taking into account the effect of a solvent is the so-called "supermolecule" approach (Cramer and Truhlar 1992; Luzkhov and Warshel 1992; Kitaura and Morokuma 1976) where a large but limited number of solvent molecules is explicitly included in the energy minimization procedure. The input file for MOPAC calculations was again prepared using Insight II. The oxamate molecule was extracted from the LDH ternary complex to provide the initial coordinates. 22 explicit water molecules were defined by using the "soak" option in the Insight software with the 5 Å radius around the oxamate molecule. This option allows one to reproduce realistically, in a stochastic manner, the positions of water molecules inside a cavity of a defined radius according to the TIP4P model (Jorgensen et al. 1983) of water. This model of the "supermolecule" is shown in Fig. 1. The geometry optimization was then carried out in essentially the same manner and using the same computer resources as described for the active site model, i. e. only positions of oxamate atoms, but not those of water molecules were allowed to be optimized. Since in an aqueous environment only oxamate could be ionized, the –1 net charge was applied to the system in this and following simulations. The force field calculations were performed in two versions analogous to those described for the active site models. In one version the force field was calculated for all the atoms of which the "supermolecule" consists, while in the other method only oxamate atoms were taken into account.

Alternative methods of including a solvent in semiempirical calculations limit the quantum mechanical treatment to a solute, while the effect of a solvent is represented by effective potentials which are included in the solute Hamiltonian. The three approaches emerging from these considerations differ in details of the physical properties of a solvent which are taken into account to describe the solute-solvent interactions.

Self-consistent reaction field (SCRF). The combination of Self-Consistent Reaction Field theory with AM1 and PM3 methods developed by Katritzky and co-workers (Katritzky and Karelson 1991) makes use of the macroscopic dielectric properties of a solvent. A solute molecule is treated as being in a spherical or ellipsoidal cavity surrounded by a continuum solvent of a given dielectric constant. A possible drawback of this method might thus be an arbitrary choice of cavity radius.

The same coordinates of the oxamate molecule prepared from the 1ldm.pdb file that were used for the "supermolecule" simulations were the basis for the SCRF calculations. The SCRF and EPS=87 keywords were used to invoke the method for simulations in aqueous solution. The additional specific options ELLIPS and AUTOCAVITY attained the use of an elliptical cavity and the choice of the automatic calculation of the cavity size.

Solvation models (SM2, SM3). The new semiempirical model of solvation developed by Truhlar and co-workers (Cramer and Truhlar 1991b, 1992), called AM1-SM2 and PM3-SM3 (Solvation Model 2 and 3), includes two terms in the solute Hamiltonian: a surface tension term based on solvent – accessible surface area for dispersion and a cavity formation term for polarization effects. This method thus takes into account additional physical effects, not accounted for by the macroscopic dielectric constant, by assuming a proportionality between hydrophobicity and the

solvent accessible surface area. These calculations were done using AMSOL. AM1 with SM2 and PM3 with SM3 keywords were used to invoke the Solvation Models implemented for AM1 or PM3 Hamiltonians, respectively. Use of the DERINU option was necessary in order to numerically rather than analytically calculate derivatives.

Conductor-like screening model (COSMO). The most recent model (Klamt and Schüürmann 1993) is a non-iterative approach in which solvation energy is treated as a quadratic expression with respect to charge distribution within the cavity formed by the solute. Thus it can be easily included in the Hamiltonian of the solute and allows for the calculation of analytical gradients without constraints regarding cavity shape. Calculations based on this model were performed with the aid of MOPAC 93. The EPS=78 keyword was used together with EF and ANALYT to accomplish optimization of oxamate geometry.

Gas phase calculations. These calculations were performed in an analogous manner, starting with the same trial geometry of the oxamate ion, but without any keywords corresponding to either solvation model.

Results

Vibrational analysis of oxamate in the LDH

An attempt was made to carry out the simulations of the conformation of oxamate in the rigid active site of LDH using all Hamiltonians available in the MOPAC package. However, the use of the MNDO option did not produce any stable structure. This is not surprising since this Hamiltonian is known to behave very poorly for hydrogen-bonded structures (Stewart 1989b). The MNDO Hamiltonian was therefore eliminated from the subsequent studies.

The remaining two Hamiltonians, AM1 and PM3, led to slightly different geometries of oxamate in the environment of the enzyme. In both cases the energy gradient, despite achieving self-consistency, remained relatively high, a phenomenon frequently encountered during geometry optimization of a large systems. It should also be noted that despite a limited number of atoms being allowed to change positions, calculations of such a large system were extremely time-consuming and sometimes took up to 25 h CPU time. Following geometry optimization, force field calculations were carried out for both AM1 and PM3 optimized structures as described in the Methods. However, only the AM1 Hamiltonian yielded two alternative force fields, which will be later called AM1 (full) and AM1 (cut). The indexes "full" and "cut" indicate respectively whether the force field was calculated for the entire active site model or only for the oxamate atoms, which were cut from the output file after the geometry optimization but prior to the force field evaluation. We were unable to calculate the force field for the entire active site model using the PM3 Hamiltonian. This is due to the existence of linear arrangements of atoms. Calculations which use internal coordinates, as defined in Z-matrix, are incapable of handling such structures. The usual alternative is to perform calculations using cartesian coordinates of the system, but these would be prohibitively lengthy. Thus only the PM3 (cut) results were taken into further consideration. The common feature of the force fields obtained for oxamate in the active site was the presence of a number of relatively low (in the range of 0–90 cm^{-1}) and in some cases even negative frequencies. Such frequencies are not likely to represent the normal modes of vibrations. A structure with one and only one negative (imaginary) frequency is normally considered a transition state and structures with more than one imaginary frequency do not represent extreme points on the potential surface. In this case, however, their presence is the result of leaving a major part of the model unoptimized. However, all normal modes of vibrations associated with the molecule of interest, oxamate, were above this range. Full optimization of the active site could probably partly correct this deficiency but, at the same time, it would result in changing its geometry, which was believed to be correct.

Most of the remarks concerning difficulties in handling large systems at a semiempirical level apply, albeit to a lesser extent, to the simulations carried out for the "supermolecule" consisting of oxamate and 22 water molecules. Both AM1 and PM3 yielded force fields in the "full" and "cut" versions for oxamate and water molecules or oxamate alone, respectively. The force fields are denoted EXP in conjunction with the (full) or (cut) indexes.

It was possible to obtain force fields for oxamate in aqueous solution using all available continuum models of the solvent. The SCRF approach proved relatively undemanding in CPU time. The simulations which used Solvation Models parameterized separately for AM1 (SM2) and PM3 (SM3) were much more time consuming. This was a result of obligatory use of options that attained the numerical rather than analytical calculations of derivatives. The striking feature of the force field calculated using the Solvation Models was that essentially all determined frequencies of vibrations, even those of bonds far removed from the carboxyl group, have changed upon substitution of carboxyl oxygens with ^{18}O. This was also true with the COSMO model.

The comparison of calculated normal vibrations of oxamate in the LDH active site with the available IR spectrum of oxamate in the crystalline state (Chouteau 1953) is given in Table 1. Relevant frequencies of normal vibrations were identified in the MOPAC output files by noting their isotope sensitivity. Only frequencies which differed upon substitution with ^{18}O by more than 10 cm^{-1} are listed. In the "cut" models of the active site usually only one frequency in a given range was isotope sensitive. In the AM1 (full) model, however, each range of frequencies corresponding to a particular vibrational mode contained several distinct isotope sensitive frequencies. Furthermore, a number of vibrations of low frequencies, in the range of 500–800 cm^{-1}, generated by the AM1 (full) model, turned out to be isotope sensitive. These frequencies are not listed in Table 1 as they are not easily attributable to specific vibrational modes.

The results of calculations of the equilibrium ^{18}O isotope effect on the association constant of oxamate with LDH have been summarized in Table 2.

Table 1. Selected frequencies of normal vibrations in oxamate

vibr. mode	exp.[a]	AM1 (cut)	AM1-(full)	PM3 (cut)
C–O	1200	1520	1545 1551	1370
C···O	1500–1600	1961	2094 2103	1636
C=O (carbonyl)	1666	1798	1602 1624 1634 1644	1600 1645
N–H	2850–3000	3196 3259		3202 3303

[a] after Chouteau (1953)

Table 2. Summary of the calculated values of the isotope effect

→ Active site models → ↓ Solution models ↓	AM1 (full)	AM1 (cut)	PM3 (cut)
EXP (full)	0.9860	0.9999	–
EXP (cut)	0.9849	0.9988	1.0015
SCRF	0.9837	0.9975	1.0007
SM2/SM3	0.9936	1.0075	1.0216
COSMO	0.9810	–	–
Gas phase	0.9840	–	–

Discussion

Theoretical simulations of kinetic isotope effects have been previously used to test the usefulness of newly developed theoretical methods (Paneth 1991; Jones and Urbauer 1991; Liu et al. 1993). A rationale for this is provided by the way in which theoretical isotope effects are calculated. Since the theoretical values can be obtained from frequencies of vibrations of isotopic molecules, even crude results of calculations should yield reasonable values of isotope effects as both isotopomers have the same geometry and force field. A simulation of a equilibrium isotope effect offers additional advantages over corresponding simulations of kinetic effects because it avoids problems of locating the transition state on the reaction potential surface. Semiempirical methods, especially those making use of the most recently developed Hamiltonians, namely AM1 and PM3, have proven effective in predicting vibrational properties of molecules in the gas-phase (Coolidge et al. 1991), including those of hydrogen-bonded systems (Rzepa and Yi 1990). However, the usefulness of novel semiempirical techniques of taking into account solvent effects in predicting molecular properties other than solvation energies is still questionable. Thus our results provide some guideline as to the applicability of such methods in calculating frequencies of vibrations in molecules in aqueous solutions.

As can be seen from data compiled in Table 1, vibrational frequencies obtained using the PM3 Hamiltonian more closely resemble those experimentally determined. The AM1 method overestimates the absolute values of calculated frequencies. Nonetheless, only isotope effects calculated using this Hamiltonian reproduce the experimentally determined values (see Table 2). Virtually all results obtained using the PM3 method have proven either not significantly different from unity or normal, i. e. larger than unity, contrary to the experiment. This may be surprising as PM3 was thought to better reproduce the geometry of the hydrogen-bonded structures (Zheng and Merz 1992). At the same time, however, it has been noted a number of times that in general the PM3 method performs very poorly for nitrogen containing structures, particularly if nitrogen is directly involved in hydrogen bonding (Schröder 1991). Several points of comparison between the AM1 and PM3 Hamiltonians (as well as their relation to the results obtained by means of several ab initio basis sets) have been supplied by a complex computational study of hydrogen-bonding interactions (Zheng and Merz 1992). For example, it has been noted that AM1 favors bifurcated arrangement for the O–H···O and N–H···O types of hydrogen bonds, leading in some cases to disagreement with experimentally observed geometries. In the presently studied case, however, such a bifurcated structure between the arginine guanidinium group and the oxamate carboxylate can be observed in the X-ray crystal structure of the LDH ternary complex. This may be a reason why the AM1 method has proven superior over its PM3 counterpart. It should also be recognized that the failure of the PM3 Hamiltonian to reconstruct the modelled isotope effect may result from our inability to calculate the force field for the entire active site model. Perhaps, if this was feasible, the results yielded by this method would be more favorable.

We noted above that the results coming from the less accurate AM1 method, in terms of absolute values of frequencies, give better results than those obtained with the more accurate PM3 method. Such a situation is not unreasonable since the magnitute of an isotope effect depends less on the accuracy of frequencies but relies primarily on the precision in determination of isotopic shifts. Generally, it is hard to estimate the precision of calculations. We, therefore, illustrate this point with the following example. The last line in the Table 2 lists a calculated isotope effect of 0.9840, the result obtained using the molecule of oxamate in the gas phase (no solvent treatment) as the model of the reactant. The dominant contribution to the isotope effect for this model is stretching vibration of C–O bonds in the carboxylic group, which were found to be equal to 2104.7 cm^{-1} for ^{16}O and 2068.6 cm^{-1} for ^{18}O isotopomers, respectively. If we increase the first of these frequencies by 10 cm^{-1} (to equal 2114.7 cm^{-1}) without changing any other frequency of either reactant or product (LDH-oxamate complex) the calculated isotope effect changes dramatically to 1.0056. Alternatively, if we do the same perturbation to the frequency corresponding to the heavy isotope, the calculated isotope effect drops to 0.9638. If both these changes are introduced simultaneously, however, the isotope effect changes insignificantly to 0.9845. Thus, obviously the exact position of a frequency in the spectra has only a negligible influence on the calculated isotope effect. In contrast, small errors in calculations of isotope shifts can lead to unrealistic values of isotope effects. Fortunately, calculations of isotopic shifts are

less prone to errors, since they arise from different mass weighing of second derivatives around the same potential energy surface, than calculations of absolute positions of frequencies, which mathematically come as small differences between large numbers.

The manifestation of the importance of taking into account the entire active site model in the force field determination was demonstrated in the case of the AM1 method. While the difference between the isotope effect calculated using the "full" and "cut" versions of the force field for the "supermolecule" model appears negligible, the analogous treatment of the active site models leads to significant change in value, and sometimes in direction, of the calculated isotope effect. This indicates that the relatively expensive procedure of force field calculations for the entire active site model cannot be avoided without significant alteration of results. Close inspection of isotopically sensitive frequencies of vibrations calculated through the use of the AM1 (full) model revealed a number of low vibrational frequencies. Most likely, these vibrations appeared as a result of distortions of vibrations of bonds linked to atoms involved in hydrogen bonding. Since this is the most apparent distinction between the vibrational frequencies obtained for the "full" and "cut" models, they can be pointed to as a direct source of the calculated isotope effect. The "splitting" of vibrational frequencies corresponding to a particular vibrational mode into several isotope sensitive vibrations can be indicated as the alternative vibrational origin of the simulated value.

Except for SM2/SM3 all methods of including solvent effects yielded acceptable values of the isotope effect. The failure of the Solvation Models to predict the observed isotope effect indicates that the SM2 and SM3 methods may not yet be suitable for predicting vibrational frequencies of molecules in the presence of a solvent. This is an unexpected conclusion as the Solvation Models utilize a more sophisticated model of solvent-solute interactions than the SCRF and COSMO methods and were tested primarily on simple organic and inorganic molecules similar to oxamate. Thur our results indicate that continuum models SCRF and COSMO can be successfully used for modelling of isotope effects while allowing one to avoid prohibitevely long computations due to a large number of explicitely defined solvent molecules.

So far our discussion has concentrated on the evaluation of modern computational techniques based on comparison of the computational results with the experimental value. Since we were able to draw some general conclusions regarding their applicability it is now possible to use these theoretical values in order to shed some light on the origin of equilibrium isotope effects on binding of a reactant to an enzyme. A lactate dehydrogenase inhibitor, oxamate, offered a suitable case for studying this problem. The analysis of a high resolution X-ray crystal structure of lactate dehydrogenase complexed with oxamate indicates that the oxamate carboxylate is involved in a bifurcated hydrogen bond with the catalytic site. This observation, as well as site-directed mutagenesis studies, suggest that the formation of this hydrogen bond is a primary event in the process of binding. Thus, the effect of isotopic substitution of one of the carboxylate oxygens was expected to be significant and directly correlated with the formation of a well defined hydrogen bond.

The key role in the interpretation of the origin of the isotope effect is the observation that the value obtained without any solvent model (result labeled gas phase in Table 2) does not differ substantially from those which were obtained with SCRF, COSMO, or explicit models. This implies that there is not much isotopic fractionation in going from gas phase to aqueous phase, a conclusion which is in agreement with the known negligible values of phase transfer isotope effects. Yet another confirmation of this conclusion comes from the comparison of 'full' versus 'cut' models. While there is not much difference between results obtained with these models for aqueous solution, analogous results for the active site differ significantly, which indicates again the importance of interactions between oxamate and the residues of the enzyme. Thus the main source of the inverse isotope effect is the increased bonding of the carboxylic oxygens of oxamate in the active site of the enzyme compared to "free" oxamate. This increased bonding can be assigned to the extremally strong, bifurcated hydrogen bond with the guanidinium group of arginine. The conclusions coming from theoretical modelling of the isotope effect support, therefore, those made on the basis of experimental observations.

In conclusion, our results indicate that the theoretical derivation of isotope effects on binding processes can be used to learn details of enzyme-reactant interactions and also for the evaluation of the usefulness of novel computational methods. Further work is necessary to augment the conclusions drawn here.

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