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THEORETICAL CHARACTERIZATION OF TRANSITION STRUCTURE FOR THE ENZYME-CATALYZED REACTION AT THE ACTIVE CENTER OF LACTATE DEHYDROGENASE. GEOMETRY AND TRANSITION VECTOR DEPENDENCE UPON COMPUTING METHOD AND MODEL SYSTEM

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A theoretical study of the catalytic mechanism of lactate dehydrogenase enzyme on different model systems was carried out with the help of the PM3 semi-empirical procedure and an *ab initio* method at the 4–31G and 6–31G basis sets at a Hartree–Fock (HF) level of theory.**

The geometry, transition vector (TV) and electronic structure of the transition structure (TS) for the acid-catalysed hydride reduction were obtained. The dependence of these properties on the computing method and model system is analysed and discussed. Proton transfer is much more advanced than hydride transfer occurring in roughly perpendicular planes. All the TSs render very similar structural features, the control of the chemical reaction being associated with the hydride transfer process. A comparison among simple and sophisticated molecular models shows that the TS seems to be structurally a rather robust entity. There is a minimal molecular model with a TS which describes the essentials of the chemical interconversion step in a given enzyme mechanism and the corresponding TV is an invariant feature.

INTRODUCTION

Theoretical chemistry provides the basis for the design of sound computer-assisted simulation tools, such as modern statistical mechanics techniques and advanced quantum chemistry, contributing to a better understanding of enzyme catalysis by introducing basis concepts and increasingly accurate calculations.[1] Different overviews of the current state of the art in theoretical approaches to understanding enzyme mechanisms have been offered by Warshel,[2] Kollman[3] and Merz and Kollman.[4]

The understanding, at the molecular level, of reaction mechanisms for enzyme catalytic processes requires a detailed knowledge of transition structure (TS).[5] Whereas in the past TS were difficult to obtain, contemporary computational quantum chemistry offers a unique way to determine them with high accuracy, allowing for the calculation of analytical gradients (forces) and Hessian matrices that, once diagonalized, provide essential information to characterize the nature of stationary points, reactants, TS, products and possible intermediates on the potential energy surface (PES). This provides an independent source of information concerning the geometry, stereochemistry, charge distribution and the reactive fluctuation pattern (dynamics); the last aspect is embodied in the transition vector (TV). TS are stationary points on the reactive PES with well defined geometries. Once the Hessian, i.e. the matrix of the second order derivatives of the electronic energy with respect to geometric parameters, is diagonalized, the TS is a saddle point of index one with only one negative eigenvalue and the eigenvector associated with that eigenvalue is dubbed the TV. The amplitudes of the eigenvectors assign weights to the atomic fluctuations around the stationary point. All except the negative one are associated with stationary fluctuations. Distinct geometric information is obtained together with the fluctuation pattern around the quadratic zone for such systems. Fluctuations along the TV may conduct the system either to reactants or product basins.

Recently, a quantum description based on the role of TS characterization of chemical reactions for enzyme catalysis was presented.[6,7] The key hypothesis of our work is that the *in vacuo*-calculated TS informs us on the geometry that the activated complex will have at the

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active site of the given enzyme and the reactive fluctuation pattern there. The description of TS on the basis of experimental evidence is the central, if sometimes elusive, goal of most studies of reaction mechanisms. The value of TS for understanding enzyme catalysis is widely used.[5] Following Pauling's hypothesis,[8-10] in 1969 Jencks[11] proposed and later Lerner *et al.*[12] demonstrated experimentally a practical entry into designed catalysis, provided by antibodies raised against stable TS analogues of chemical reactions. In recent years, based on this approach, a new field has emerged with the expressed aim of designing reaction types and substrates.[13-15] In this sense, this is a highly interdisciplinary field, ranging from the study of biological systems via synthetic chemistry to computational chemistry.

In recent years, quantum mechanical calculations have been gradually recognized as a useful tool in research related to elucidation of molecular reaction mechanisms.[16-22] A complete application of *ab initio* methods is generally prohibitive in terms of computational effort for any except the smallest of systems. Furthermore, semi-empirical procedures are intended for studying large molecular systems of chemical or biological interest. The different features of the various semi-empirical methods arise from the different approximations introduced, the different parameterization included and the different sets of molecules used to obtain the parameters. Semi-empirical methods have progressed over the past few years to a surprising level of accuracy and reliability, considering the limitations of the underlying approximations.[23,24]

The transfer of a hydride equivalent from the reduced pyridine dinucleotide coenzymes NADH and NADPH to a variety of substrates is catalysed by enzymes of the dehydrogenase family.[25] The lactate dehydrogenase (LDH) enzyme, belonging to this family, is an ideal vehicle to explore the effects of computing methods and model systems on the calculated geometry, TV and electronic structure of TS for the molecular mechanisms that take place in its active site. The purpose of the present work was to compare the important features and behaviour of the TS for the molecular mechanism catalysed by this enzyme. Analytical gradient SCF MO with PM3 semi-empirical and *ab initio* methods were applied.

ENZYME MECHANISM, METHODS AND MODELS

LDH enzyme mechanism

Reactions catalysed by enzymes are not single processes but normally follow a catalytic mechanism containing a number of molecular and electronic steps. The structural and electronic theory of enzyme catalysis is directed at obtaining a rational explanation for the chemical interconversion process where the fundamental chemistry (bond making/forming) is carried out. Before arriving at the chemical interconversion step, and after passing it, there may be a number of molecular rearrangements, acid–base reactions and/or precursor chemical interconversions; these molecular and/or chemical events usually serve to prepare the enzyme to perform the fundamental chemical act and determine the efficiency of the enzyme.

LDH is a nicotinamide adenine dinucleotide (NAD^+)-dependent enzyme that reversibly catalyses the oxidation–reduction reaction of the pyruvate to L-lactate[26-30] (Figure 1):

$$CH_3COCOO^- + NADH + H^+ \rightleftharpoons CH_3CHOHCOO^- + NAD^+$$

Although the overall kinetics of the reaction in LDH (EC 1.1.1.27) have been the subject of exhaustive experimental investigation,[31-33] and the roles of active-site enzyme residues and the molecular details of the mechanism have been obtained from site-generated mutagenesis and protein engineering studies,[34-36] the catalytic steps in the active site of the enzyme present many questions about the mode of action of LDH.[37,38] These experimental facts invite complementary investigation by computational methods.

The chemical conversion has two essential components: hydride and proton transfer processes. The substrate interacts with the active site residues and is positioned such that it can accept a hydride ion from the nicotinamide ring of NADH while the histidine residue is the proton donor or acceptor in the reaction.[39]

Our previous theoretical studies[40-42] indicate that the TS that controls the overall process is dominated by the hydride transfer from nicotinamide adenine dinucleotide to pyruvate carbonyl carbon. The proton transfer process takes place along a pre-existing hydrogen bond between hydrogen attached at the nitrogen atom of the imidazole ring and the in-plane sp^2 lone pair on the carbonyl oxygen of the pyruvate substrate. In the TS, the hydride transfer step has progressed to only a small degree whereas the proton transfer has been completed, both processes being kinetically coupled but dynamically uncoupled. These results are in agreement with theoretical studies of Wilkie and Williams,[43,44] Gready and co-workers[45,46] and Almarsson and Bruice[47] and are in accordance with experimental data reported by Coleman *et al*;[48] but are in conflict with results obtained by Yadav *et al.*,[49] who evaluated the very large effect of the enzyme environment.

Methods and models

PM3 semi-empirical and *ab initio* MO calculations were performed using the Gaussian92 program[50] on a cluster of Hewlett-Packard 730 workstations. All TSs were determined by gradient search techniques, without constraints, and using the Berny algorithm.[51,52] These

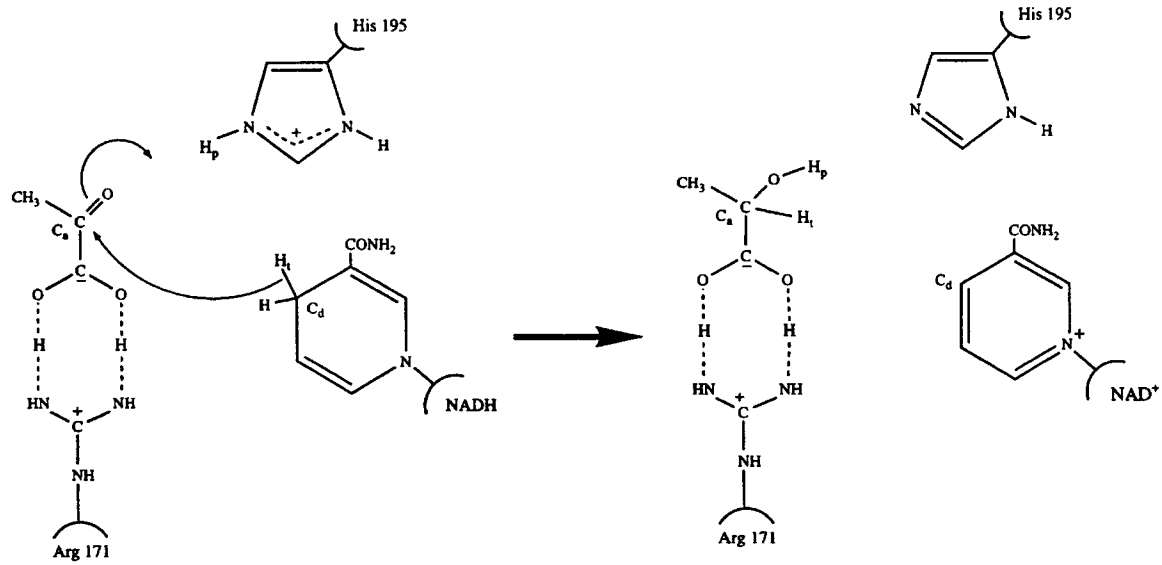
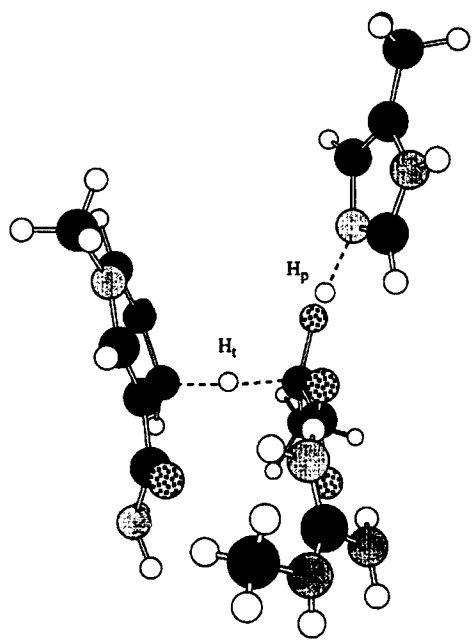


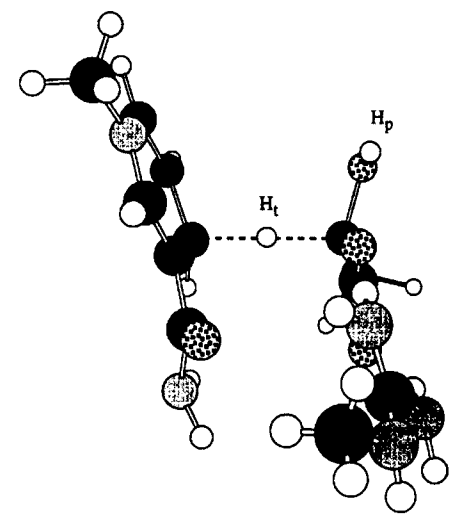
Figure 1. Model reaction for the reduction of pyruvate by lactate dehydrogenase

stationary structures on PES were shown to be saddle points of index one by virtue of possessing a single imaginary vibration frequency for the reaction coordinate mode.

The physical region where the primary interconversion process takes place is the active site. The regions occupied by other moieties of the substrates after binding to the enzyme are usually larger than that occupied by the active site. This complementary region is then referred to as the substrate binding site. To elaborate this point further, a fundamentally different type of information about the primary interconversion step is required. This can be generated by studying



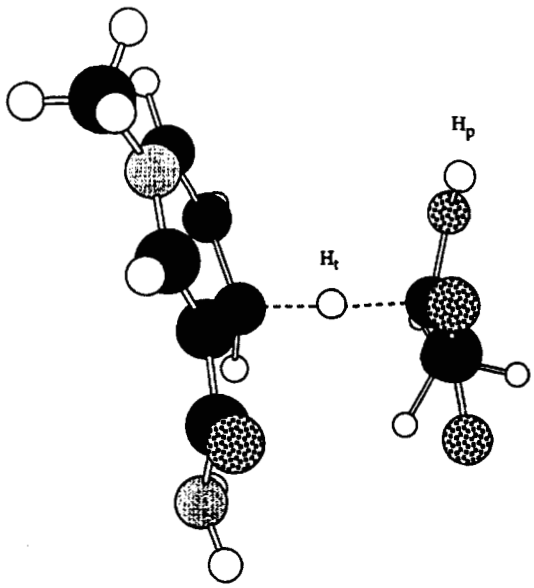
Model I



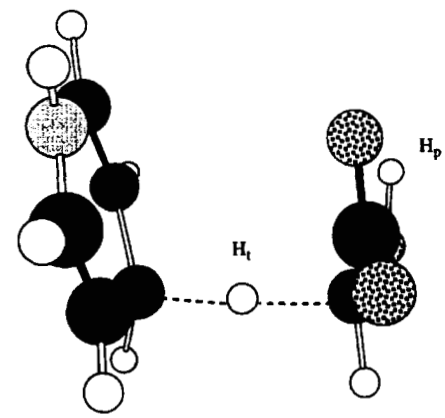
Model II

appropriate minimal molecular models sustaining the actual chemical reaction. These systems may contain the intrinsic potentialities of the real system. In the terminology used by us in calculating activated complexes, the atoms participating in the actual interconversion are named reactive space atoms (or degrees of freedom belonging to the control space); those related to the specificity of the substrates and bound outside the active site are named non-reactive atoms (or degrees of freedom in the complementary space).

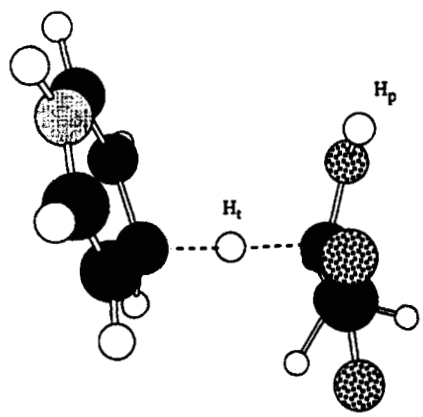
Seven model systems were selected. They can be ordered, from the most complex to the simplest structure as follows. In model **I**, the guanidine part of Arg 171 was conserved and the moiety of this amino acid was replaced by a methyl group. The imidazole ring substituted by the methyl group was positioned in place of His195 and *N*-methyl-1,4-dihydronicotinamide played the role of NADH. The starting geometry of the complete supermolecule comprising 55 atoms was established according to our previous results,[53] which were based on X-ray findings.[27,54,55] In model **II**, the imidazole ring has been discarded, maintaining the protonation in pyruvate fragment. In model **III**, the Arg171 has been discarded. In model **IV**, the methyl and amide groups of NADH residue have been removed. In Model **V**, the methyl group of pyruvate fragment has been discarded. In model **VI**, the carboxylate group of pyruvate residue has been removed. In the model **VII**, the cyclopropenyl ring plays the role of the pyridinium ring.



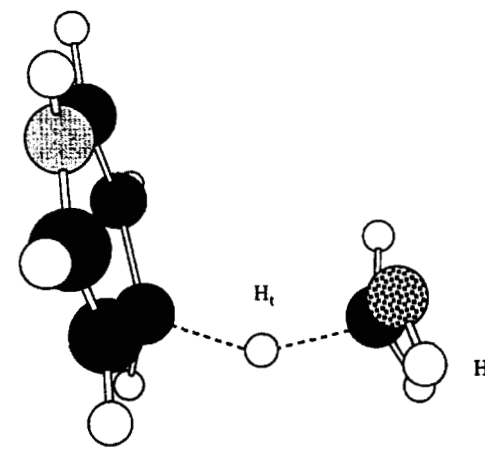
Model **III**



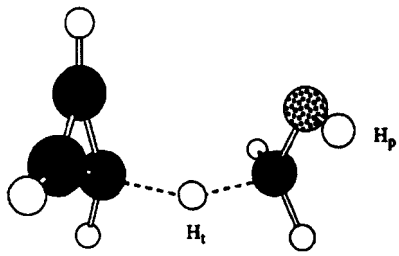
Model **V**



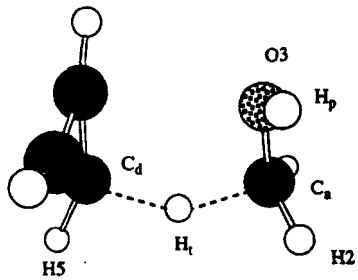
Model **IV**



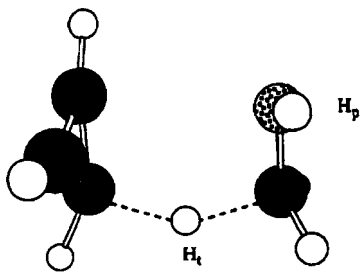
Model **VI**



PM3



HF / 4-31G



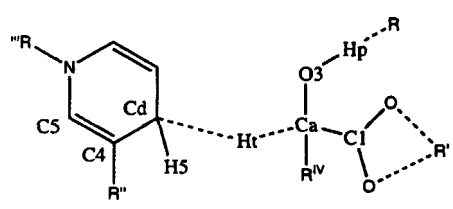
HF / 6-31G**

Model VII

RESULTS AND DISCUSSION

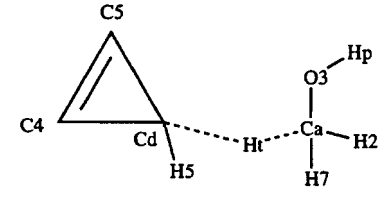
The TSs for the different model systems are depicted in Figure 2. The TV renders very concisely the essentials of the chemical process under study: the corresponding TV and force constants, geometric values for the variables defining the control space, imaginary frequency and the unique negative eigenvalue are reported in Tables 1–3. The completely optimized geometries are available from the authors on request. The results provided good hints for a qualitative and semi quantitative analysis. The selected geometrical parameters for the different model systems, **I–VII**, reveal slight differences. The donor and acceptor fragments have an *endo* conformation and their intermolecular distance, C_a—C_d, is in the range 2·721–2·753 Å. The forming bond, C_a—H_t, is longer than the breaking bond, C_d—H_t for models **I–V**, whereas an opposite trend is obtained for models **VI** and **VII**. There are two set of values for the C_a—H_t—C_d bond angle and Ht—Ca—O—Hp dihedral angles for models **I–V** and models **VI** and **VII**: 173·2–176·8° and 88·5–88·8° and 144·7° and 91·6–108·4°, respectively.

The normal-mode analysis of these structures yields a relatively low imaginary frequency (1017i–1400i cm^{-1}). For all model systems, the negative eigenvalue arises from the cross terms in the force constant matrix, diagonal force constants are all positive. An analysis of the components of TV shows that there is a strong coupling between the Ht position in the bridge, the internuclear distance, Ca—Cd, and the Ht—Ca—C1 bond angle, these variables being the major components of the TV. The minimal set of coordinates capable of producing the TS are the Ht advance and the rehybridization coordinates at both the acceptor and donor centres. This result is obtained after reducing the size of the control space followed by the diagonalization of the corresponding Hessian matrix.



Models I-VI

In Model **VI**, C1 is a hydrogen atom.
Atoms D1, D2 and D5 are dummy atoms used in our Z-matrix.



Model VII

Atoms D1, D2 and D5 are dummy atoms used in our Z-matrix.

Figure 2. Schematic representation of the transition structures for the enzyme-catalysed reaction at active site of lactate dehydrogenase obtained with the different computing methods and model systems

Table 1. Selected geometric parameters, and net atomic charges (a.u.) for the TS structures of the different models

Parameter	I	II	III	IV	V	VI	VII-A	VII-B	VII-C
Distances (Å):									
Ca—Cd	2·7207	2·7530	2·7207	2·7207	2·7366	2·7368	2·7368	2·7368	2·7368
Ca—Ht	1·4173	1·4174	1·4173	1·4174	1·4174	1·4174	1·4173	1·4174	1·4174
Ht—Cd	1·3082	1·3967	1·3082	1·3082	1·3234	1·4544	1·4544	1·4544	1·4544
Bond angles (°):									
Ca—Ht—Cd	173·15	176·82	173·15	173·72	173·72	144·72	144·73	144·73	144·72
Dihedral angles (°):									
Ht—Ca—O3—Hp	88·80	88·47	88·80	88·81	88·56	92·74	91·55	108·40	98·57
Net atomic charges (a.u.):									
Ca	0·21	0·13	0·17	0·17	0·02	0·04	0·09	0·04	0·10
O3	−0·27	−0·26	−0·25	−0·25	−0·25	−0·28	−0·24	−0·71	−0·54
Hp	0·15	0·27	0·29	0·29	0·28	0·25	0·27	0·46	0·39
Ht	0·02	0·01	0·04	0·04	0·02	0·02	−0·07	0·02	−0·06
Cd	0·05	0·06	0·04	0·03	0·05	0·02	−0·02	−0·20	−0·13

Table 2. Imaginary frequencies (cm^{-1}), force constants (K, in a.u.) and corresponding eigenvector (e) associated with the unique negative eigenvalue for the TS of molecular models I–VI

Parameter	I		II		III		IV		V		VI	
Imaginary frequency (cm^{-1})	1134·43i		1400·24i		1016·51i		1021·42i		1196·58i		1035·72i	
	e	*K*	*e*	*K*	*e*	*K*	*e*	*K*	*e*	*K*	*e*	*K*
r(Ca—C1)	−0·085	0·409	−0·029	0·311	−0·115	0·249	−0·123	0·251	−0·107	0·178	−0·152	0·019
r(Hp—O3)	−0·098	0·522	−0·063	0·520	−0·113	0·410	−0·117	0·450	−0·044	0·506	−0·054	0·540
r(Ht—Ca)	−0·263	0·318	−0·366	−0·015	−0·675	0·018	−0·731	0·018	−0·858	0·004	−0·747	0·031
r(H5—Cd)	−0·080	0·325	−0·153	0·303	−0·323	0·305	−0·351	0·308	−0·306	0·305	−0·441	0·308
∠Ca—C1—D1	0·109	5·274	0·087	0·879	0·096	0·376	0·044	0·365	0·038	0·394	0·041	0·825
∠Ht—Ca—C1	0·701	3·085	0·704	0·299	0·408	0·394	0·122	0·384	0·041	0·328	0·001	0·204
∠Cd—D5—D1	−0·079	0·147	−0·096	0·277	−0·030	0·299	−0·011	0·311	−0·011	0·273	−0·007	0·119
∠H5—Cd—C4	−0·321	0·287	−0·220	0·210	−0·131	0·208	−0·002	0·195	−0·029	0·192	−0·020	0·189
∠Ca—C1—D1—D2	−0·086	3·343	−0·080	0·610	−0·058	0·983	−0·090	0·949	−0·038	0·810	−0·195	0·130
∠Hp—O3—Ca—C1	−0·088	0·173	−0·013	0·027	−0·036	0·041	−0·020	0·040	−0·005	0·041	−0·009	0·014
∠Ht—Ca—C1–O3	−0·470	3·309	−0·462	0·297	−0·380	0·399	−0·464	0·392	−0·185	0·340	−0·297	0·132
∠Cd—D5—D1—D2	−0·074	0·558	−0·037	0·148	−0·027	0·164	−0·028	0·150	−0·005	0·111	−0·009	0·050
∠H5—Cd—C4—C5	0·178	0·220	0·187	0·121	0·200	0·142	0·193	0·138	0·041	0·122	0·199	0·154

It is important to note that while the atoms participating in the TSs are always the same, those entering in the complementary space are not. If the latter do not modulate in first order the force constants in the control space, the TV is invariant. The invariance is to be understood in terms of preservation of the reactive fluctuation patterns. Fluctuations in one direction lead the system downwards in energy towards the reactants and in the opposite direction to the products.

A close look at the crystallographic structure of a number of dehydrogenases suggests that the TS for hydride transfer have a geometric arrangement similar to that theoretically determined for LDH. The *endo* configuration for the TS seemed to be a general feature of dehydrogenases. This idea has been successfully used by Sustmann *et al.*[56] in gluthatione reductase and can be also observed in liver alcohol dehydrogenase (LADH),[57,58] formate dehydrogenase (FDH)[59] and dihydrofolate reductase (DHFR).[60]

In the case of LDH enzyme, where a particle is exchanged between donor and acceptor centers, the model system has transportable geometric parameters within a small tolerance. In this respect, the TS presents similar geometric properties for the different model systems. One of the interesting results obtained is a sort of geometric invariance of the fragments participating in a first-order saddle-point geometry. This effect has been found by us and other workers.[61] Structural invariance

Table 3. Imaginary frequencies (cm^{-1}), force constants (K, in a.u.) and corresponding eigenvectors (e) associated with the unique negative eigenvalue for the TS of molecular models **VII** obtained with the PM3, HF/4–31G and HF/6–31G** methods

	PM3		HF/4–31G		HF/6–31G**	
Imaginary frequency (cm^{-1})	1165·28i		1355·45i		1397·39i	
	e	K	e	K	e	K
r(Ca—H2)	−0·252	0·321	−0·223	0·396	−0·211	0·392
r(Hp—O3)	−0·055	0·531	−0·145	0·569	−0·090	0·618
r(Ht—Ca)	−0·845	0·009	−0·858	0·004	−0·862	0·004
r(H5—Cd)	−0·343	0·298	−0·290	0·316	−0·301	0·323
∠Ca—D5—D2	0·011	0·914	0·059	0·640	0·051	0·553
∠Ht—Ca—D1	0·039	0·347	0·094	0·303	0·091	0·275
∠Cd—D5—D1	−0·034	0·099	−0·058	0·158	−0·058	0·129
∠H5—Cd—C4	−0·022	0·123	−0·061	0·196	−0·054	0·172
∠Ca—D1—D5—D2)	−0·110	0·200	−0·038	0·330	−0·046	0·291
∠Hp—O3—Ca—H7)	−0·020	0·023	−0·023	0·021	−0·006	0·027
∠Ht—Ca—D1—D2	−0·020	0·253	−0·073	0·355	−0·071	0·332
∠Cd—D5—D1—D2	−0·029	0·067	−0·005	0·167	−0·006	0·141
∠H5—Cd—C4—C5	0·143	0·065	0·096	0·095	0·099	0·099

of the TS structure fragments is an important result from the technical viewpoint. It helps in setting up the search of TSs.

From an electronic point of view, in hydride transfer reactions, the geometrical arrangement found at the active site results in an optimal frontier orbital interaction. A maximum overlap between the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals is achieved in this conformation. This result adds to the other examples reported in the literature which tend to show that the principle of maximum overlap of HOMO–LUMO[57,58,62–65] may be used as a guide to build up putative TS in hydride transfer reactions. On the other hand, for electron transfer reactions *endo* configurations facilitating HOMO–LUMO overlap are common. Thus, the enzymes belonging to the family of flavin-containing disulphide oxidoreductases having as coenzyme either NADP or nicotinamide adenine dinucleotide (NAD)[66] present an *endo* configuration between the nicotinamide plane and the isoalloxazine ring of the flavin. Glutathione reductase, trypanothione reductase and lipoamide dehydrogenase are three important examples of this family.[67,68] Another fairly general example is to be found in the hydrolysis taking place on a carbonyl carbon. This process goes systematically via a tetrahedral arrangement for the TS.

Factors influencing TSs for hydride transfer in model systems have been studied by us[41,42,59,60,64,65,69] and Williams an co-workers.[43,44,70,71] Normal-mode analysis showed that experimental data concerning kinetic isotope effects are well rationalized by the theoretical TS.[72] Pauling's lemma, stating that the shape of the active site is complementary to the form of the activated complex for the reaction catalysed by the enzyme, can be extended in the following sense: the geometry of the activated complex corresponds to the TS describing the chemical step *in vacuo*. It follows then, that, in almost all situations, the geometry of reactants and/or products are not necessarily those pertaining to their equilibrium conformations, but they will be moulded by the enzyme into conformations resembling the TS. Support for this idea is emerging from the study of enzyme model systems, especially with the finding made for LDH. From all the results hitherto obtained, it can be concluded that the relative orientation imposed by the active site constraints to the reactants in LDH (*endo* configuration) is optimal in polarizing the scissile C—H bond and situate the system in the neighbourhood of a saddle point of index one.

From this new perspective, a unified viewpoint emerges, thereby giving an explanation to enzyme catalysis phenomena that can be described as a specific binding process. This positioning eliminates the decrease in entropy required for catalysed reactions and is in agreement with proposals concerning Raman spectroscopic studies by Callender *et al.*[73] The binding energy is partly used to activate the substrate to a geometry that looks like the TS of the chemical interconversion step catalysed by the enzyme. Strictly, the binding energy of an enzyme for a TS is the force that drives enzymatic catalysis,[74,75] so the calculated activation energy is not real. The barrier height cannot be directly related to the activation energy parameter for a real enzyme reaction.

It is important to note that our arguments are based on the gas phase. The TS discussed here give an explanation for the complex behaviour of LDH enzyme in the

framework of a catalysis theory emphasizing the role of a TS-related active complex via its selective binding by the protein. However, the inclusion of the environment effects produced by the enzyme can be important and medium effects on chemical reactivity are still a debate area. This topic has been extensively studied by Warshel and co-workers[2,76–78] In addition, a recent overview on classical electrostatics in biology and chemistry was published by Honig and Nicholls.[79] Note that the solute can be taken as a classical external electrostatic source to the surrounding medium. For this approximation to be accurate, the solute wavefunction must be fairly well localized in the volume assigned to the solute system; overlap with the surrounding medium must be minimal.

CONCLUSIONS

Quantum chemically, the characterization of TS may be rationalized to discuss enzyme-catalysed reactions. While the gas-phase study of isolated substrate and cofactor species permits several relative conformations of the reactants, the active site residues in the enzyme would impose directional constraints on the approach of the reactants. Following an initial computational investigation of isolated substrate and cofactor analogues, the active site residues have been introduced in stages. At each stage, the possibility of TSs involving hydride transfer step were characterized. The gas phase model cannot mimic the true enzyme environment and the relevance of these results can be summarized as follows: there exists a minimal molecular model with a TS which describes the essentials of the chemical interconversion step in a given enzyme mechanism and the corresponding TV, that encodes the fundamental information relating reactive fluctuations patterns, is an invariant feature. Theoretical calculations of these types of stationary points on PES permit the actual determination of geometric structures and corresponding force constants. One constructs, in this manner, not an artificial situation, but a minimal model to produce testable information. It is also clear that more studies should be conducted to verify our results and to examine the validity of different methods and models. It seems reasonable to assume that this work should give reliable results in studies of hydride transfer reactions in enzymes. Computations at a higher level (post-HF) of theory or alternative procedures (DFT) are under way to test the stability of the results obtained. Also, a more complete investigation incorporating the entire enzyme environment and solvent is being undertaken, using a combined quantum and molecular mechanical approach.

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