Transition state structures for the molecular mechanism of lactate dehydrogenase enzyme

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The possible reaction pathways of the molecular mechanisms for the transformation from pyruvate to lactate in the active site of the lactate dehydrogenase (LDH) enzyme have been characterized by means of the PM3 and AM1 semiempirical methods. The energies and optimized geometries of the stationary points have been calculated on the potential energy surface. Medium effects have been estimated by means of AMSOL calculations.

Both PM3 and AM1 methods indicate that the transition state structure that controls the overall process is dominated by the hydride transfer from nicotinamide adenine dinucleotide to the pyruvate carbonyl carbon. The transition vector and the reaction pathways show that the hydride and proton transfers are kinetically coupled but dynamically uncoupled.

The AM1 and PM3 results can be summarized as follows: (*i*) there are differences in the representation of the interaction associated with proton transfer from the imidazole ring to the pyruvate carbonyl oxygen and the substrate fixation controlled by weak H-bonds between pyruvate and a guanidine residue, and (*ii*) *ab initio* and PM3 results fulfil the principle of maximum overlap of HOMO–LUMO for hydride-transfer reactions for this and related reactions.

Introduction

In recent years, computational chemistry has made a significant contribution to the understanding of enzyme catalysis because it is able to model catalytic reactions in enzymes in a quantitative way.[1–4] The most successful approaches have involved theoretical results complemented with data extracted from experimental studies. Thus, chemical processes in enzymes can be described, in principle, by SCF-MO approaches.[1–4] From a theoretical point of view, detailed analysis of a reaction path within the enzyme active site needs an appropriate potential energy surface (PES) which involves an electronic rearrangement that has to be determined quantum mechanically. The computation of PESs in chemical systems is of general interest, within the Born–Oppenheimer approximation; this requires the calculation of the change in potential energy of a molecular system as a function of changes in its nuclear coordinates. This allows the modelling of macromolecular interactions *via* the use of models of the active site, as well as the qualitative treatment of the bond-breaking and/or bond-forming processes which characterize catalysis.[1–4]

In the PES, the structure and the relative energy of the transition-state structure (TS) are of prime importance in predicting and controlling the course of the chemical reaction as it commands both the direction and the rate of chemical change. A major advantage of theoretical calculations is precisely their ability to give detailed descriptions of these structures. The development of efficient algorithms[5] during recent years, particularly of analytical methods for determining the gradients and curvatures, have made the location of TSs on PESs relatively routine. On the other hand, one of the challenges in computational chemistry is to develop accurate solutions to the Schrodinger equation for large molecular systems. If possible, the TS should be refined to any desired accuracy, although practical considerations usually put rather strict limits on both size and the level of sophistication.

Determining reaction paths together with studying TS structures and associated energetics using methods of quantum chemistry has until recently been prohibitively expensive. In the last few years, taking advantage of the availability of the computational resources in theoretical chemistry, semiempirical molecular orbital methods[6] have been applied with considerable success in enzyme model studies, as was shown by Merz *et al.*,[7] Alex and Clark,[8] and Kollman *et al.*[9] Nonetheless, one of the problems with semiempirical approaches is that, by adjusting parameters to fit some experimental properties, others may be poorly represented. For example, MINDO/3[10] is not adequate for reproducing hydrogen bonding systems because these systems were not included in the parametrization set.

Proton and hydride transfers are known to play a leading role in biological systems, and more generally these processes occur in the course of many enzymic reactions and are variously involved in the generation of enzyme catalytic power.[11] The enzyme lactate dehydrogenase (LDH) (EC 1.1.1.27) catalyses the interconversion of lactate and pyruvate employing nicotinamide adenine dinucleotide (NAD) as cofactor according to equilibrium (1). The X-ray structure of LDH has been resolved

$$CH_3CHOHCOO^- + NAD^+ \rightleftharpoons CH_3COCOO^- + NADH + H^+ \quad (1)$$

with great accuracy in its apo, binary and ternary forms.[12–15] The molecular mechanisms that take place in the active site of lactate dehydrogenase (LDH) have these 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.[16–18]

Our interest in LDH is of long standing,[19,20] and we have also made a detailed study of different models of interaction by means of *ab initio* calculations[21,22] and X-ray diffraction data.[23] The reaction process, including slightly truncated models of the residues making up the catalytic mechanism, was

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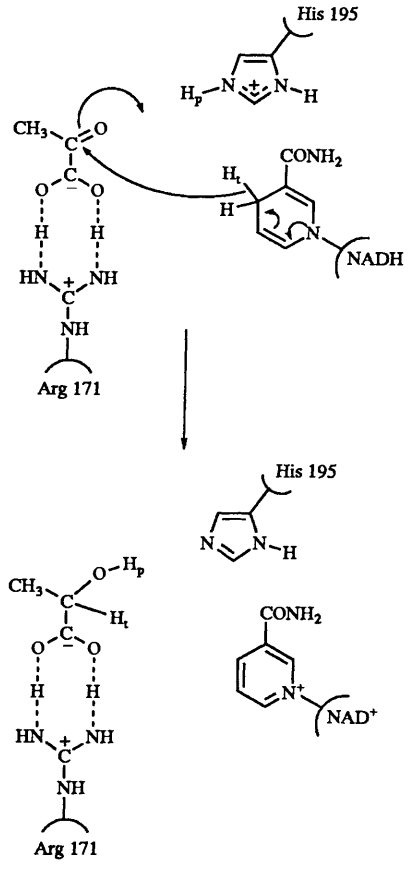

Fig. 1 Model reaction for the reduction of pyruvate by lactate dehydrogenase

established in detail by the use of X-ray diffraction analysis[24] and, as a consequence, practical applications are expanding considerably.[25] The assumed catalytic mechanism[13] is given in Fig. 1.

On the other hand, our related theoretical studies[3] have concentrated solely upon the hydride-transfer aspects. For instance, this type of process has been fully characterized in the enzyme alcohol dehydrogenase (LADH)[3b,c] and formate dehydrogenase (FDH),[3d] the main purpose being the determination of the transition-state structure in the alcohol and formate oxidation, respectively. The difference between LADH and LDH enzymes is that the active site of LADH has a zinc atom, serving as a Lewis acid, which coordinates with the oxygen atom of the carbonyl group, while in the LDH, the protonated imidazole of the histidine residue acts as a general acid during the reduction processes.[26] Recently, Onciul and Clark[27] have carried out AM1 calculations to investigate the oxidation of alcohols at the active site of LADH and Wilkie and Williams have published an AM1 study solving the situation of the transition state of a highly simplified model for LDH in which formaldehyde plays the role of the substrate.[28a] They have also performed an extensive study of the geometries of TSs for acid-catalysed hydride reduction of formaldehyde by means of semiempirical and *ab initio* computer methods. This comparative analysis is reported in this issue.[28b] We have recently published a PM3 characterization on a similar subject using a realistic model[29a] and a theoretical study in which the kinetic isotope effects for the hydride-transfer step in LDH are characterized and discussed.[29b]

It is well established that MINDO/3[10] and MNDO,[30] fail to model hydrogen bonds accurately. The AM1 method, although in principle designed to overcome MNDO's defects in this and other respects, appears not to have achieved its objectives, at least as far as hydrogen bonds are concerned.[31] PM3 seems to obtain better results,[32] but owing to its recent appearance this has not yet been rigorously tested. Therefore, this work should be a good test of the reliability of both methods describing the molecular mechanisms of LDH, in particular the nature of alternative routes for hydride, H_t, and proton, H_p transfers, that take place in the active centre of this enzyme (*cf.*, Fig. 1).

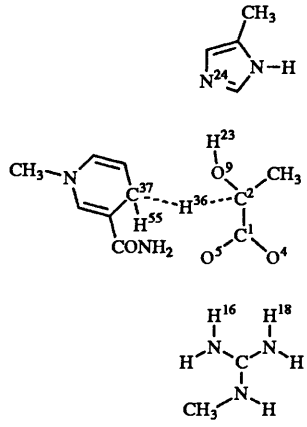

Fig. 2 Numbering of the model molecular system

Methods and model

The calculations were carried out using the standard PM3[33] and AM1[34] procedures as contained in the GAUSSIAN 92 package.[35] AM1 and PM3 derive from the same theoretical framework, *i.e.*, they use the same basic approximations (MO-LCAO HF combined with the NDDO approximation)[36] and differ mainly in the way they are parametrized. In AM1, additional Gaussian terms occur in the core repulsion function defined in MNDO with the aim of correcting the excessively long-range core–core repulsions, whereas in PM3 a new technique for obtaining optimized parameters is applied.[37]

The geometry optimizations were carried out by means of Berny analytical gradient optimization routes.[38] The requested convergence on the density matrix was 10^{-9} au, and the threshold value of maximum displacement was 0.0018 Å and that of maximum force was 0.000 45 hartree $bohr^{-1}$. The nature of the TS was established by calculating analytically and diagonalizing the Hessian matrix.

A model of the LDH active centre for the course of pyruvate reduction was assembled as follows: the guanidine part of Arg171 was conserved and the moiety of this amino acid was replaced by a methyl group. The imidazole (Im) nucleus substituted by the methyl group was positioned in the place of His195 and *N*-methyl-1,4-dihydronicotinamide (Ni) played the role of NADH. The starting geometry of the complete supermolecule comprising 55 atoms was established according to our previous results,[20] which were based on X-ray findings.[24] Five dummy atoms were used to define the geometry. Numbering of the system is depicted in Fig. 2.

It is well known that the solvent is a leading factor in chemical reactivity both in reaction rate and in the reaction mechanism. Although almost all the reactions take place in solution, theoretical calculations normally refer to isolated systems. For this reason many theoretical chemists have felt it necessary to propose different methods for evaluating the solvation of the reacting substrate by the enzyme microenvironment. Solvation effects may be estimated either by simulation studies (Monte Carlo or molecular dynamics), in which the solvent molecules are considered explicitly, or alternatively by models which consider the solvent as a dielectric continuum. In this case, the

resulting solvent–solute electrostatic interactions may be readily incorporated into self-consistent field molecular orbital (SCF-MO) methods and allow solute properties, such as structure and energetics to be predicted.[39–44] Such continuum methods have been developed within the context of AM1 and PM3 semiempirical methods. In this work, the effects of the solvent environment have been considered in a qualitative way by means of the AMSOL program[45] within continuum solvent model representation. This method[46–48] includes the solvation effects directly into the Fock matrix of AM1 and PM3 Hamiltonians and is parametrized to produce solvation-free energies.

Results and discussion

Since there has been some recent controversy over the differences between and advantages of the different semi-empirical parameter sets,[49] we have undertaken this comparative study of the AM1 and PM3 semiempirical molecular orbital methods for the determination of stationary points, in particular TS structures in the catalytic mechanism of LDH.

The course of the reaction can be described in terms of the hydride and proton transfers: the H36 (Ht) originating in the nicotinamide ring and the H23 (Hp) transferred from imidazole to form the –OH group of lactate, respectively. The stationary structures localized and characterized on PES can be schematically represented as follows.

Im-Hp Pyruvate Ht-Ni ⟶ Im Hp-Pyruvate ··· Ht ··· Ni ⟶ Im Hp-Pyruvate-Ht Ni

P **TS** **L**

A more detailed diagram of the stationary structures is shown in Fig. 3. Alternative routes for hydride- and proton-transfer reactions to form lactate from pyruvate were considered. However, no energy minima were located corresponding to the 'Im Hp-Pyruvate Ht-Ni' structure or the transition-state structure for the proton-transfer process, 'Im ··· Hp ··· Pyruvate Ht-Ni', which would form part of the intermediate structures occurring in stepwise mechanisms where Hp precedes Ht or *vice versa*. The reaction mechanism is determined by the hydride transfer, Ht, from NADH towards the pyruvate carbonyl carbon. These results complement a theoretical study of the possible mechanism and corresponding intermediates for this reaction carried out by Norris and Gready.[50]

The TS structures obtained with PM3 and AM1 are depicted in Fig. 4. By means of intrinsic reaction coordinate (IRC) calculations,[51] the unique mode with imaginary frequency of the full second derivative matrix at the saddle point determines the initial direction away from the TS. In the present study, displacements from this point, either in the direction of the reactants or of the products, led only to respective **P** and **L** minima. Both AM1 and PM3 calculations are in agreement with our previous studies,[29] and no evidence was found for any other minimum on the PES.

In Table 1 selected geometrical parameters for stationary structures, obtained *in vacuo*, are listed. The completed optimized PM3 and AM1 geometries are available from the authors on request. The proton and hydride transfers occur in roughly perpendicular planes. The TS structure takes a boat shape with a *syn* arrangement of carbonyl and the π-system of the reductant[52] as has been proposed by Lehn *et al.*[53] for the nucleophilic attack to a carbonyl π-system and as previous theoretical studies show.[3,54,55] The forming H36–C2 bond is longer than the breaking H36–C37 bond. There is a strong coupling between the H36 position in the bridge, the intermolecular distance, C2–C37, and the C37H36C2 bond angle.

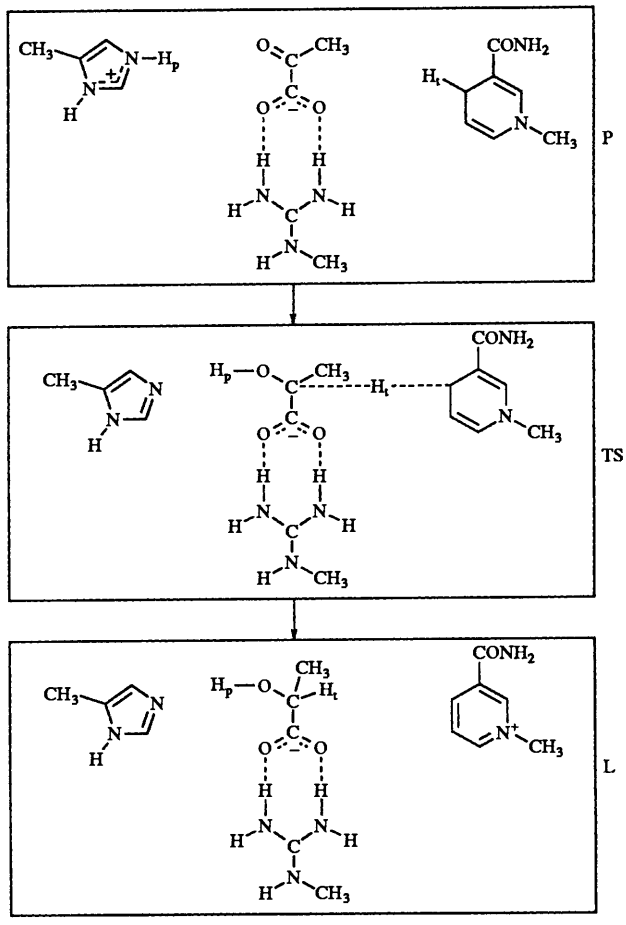

Fig. 3 Schematic diagram of the stationary points

Table 1 Selected parameters of the optimized structures, reactants (pyruvate, P), transition state (TS) and products (lactate, L) obtained with both methods, PM3 *vs.* AM1. Distances in Å, and bond angles and dihedral angle in degrees.

	PM3	AM1	PM3	AM1	PM3	AM1
	H36–C2		H36–C37		C37–C2	
P	2.8492	4.1592	1.1140	1.1374	3.7894	5.0336
TS	1.4173	1.3903	1.3082	1.3479	2.7207	2.7034
L	1.1208	1.1278	4.7231	5.2307	5.4482	5.7102
	H23–O9		H23–N24		O9–N24	
P	1.8191	2.1909	1.0078	1.0012	2.6766	2.8190
TS	0.9927	0.9820	1.7047	2.5013	2.6471	3.4064
L	0.9717	0.9662	1.7937	2.5401	2.7612	3.3676
	H16–O5		H18–O4			
P	1.7653	2.1219	1.7650	2.0494		
TS	1.7188	2.1231	1.7104	2.0159		
L	1.7260	1.9920	1.7180	2.0055		
	O9–H23–N24		C2–H36–C37		H36–C2–09–H23	
P	140.75	119.26	141.98	135.47	83.71	69.12
TS	157.03	153.16	173.15	161.71	88.80	85.91
L	173.44	143.67	125.31	109.69	−168.91	−161.66

The proton transfer process takes place along a pre-existing hydrogen bond between proton donor (imidazole ring) and an in-plane sp^2 lone pair on the carbonyl oxygen (O9 of the pyruvate substrate). The greatest and fundamental difference within the AM1 and PM3 results appears in the distance H23–N24 that controls the proton transfer (Hp) in the TS and the H16–O5 and H18–O4 distances, which are responsible for the

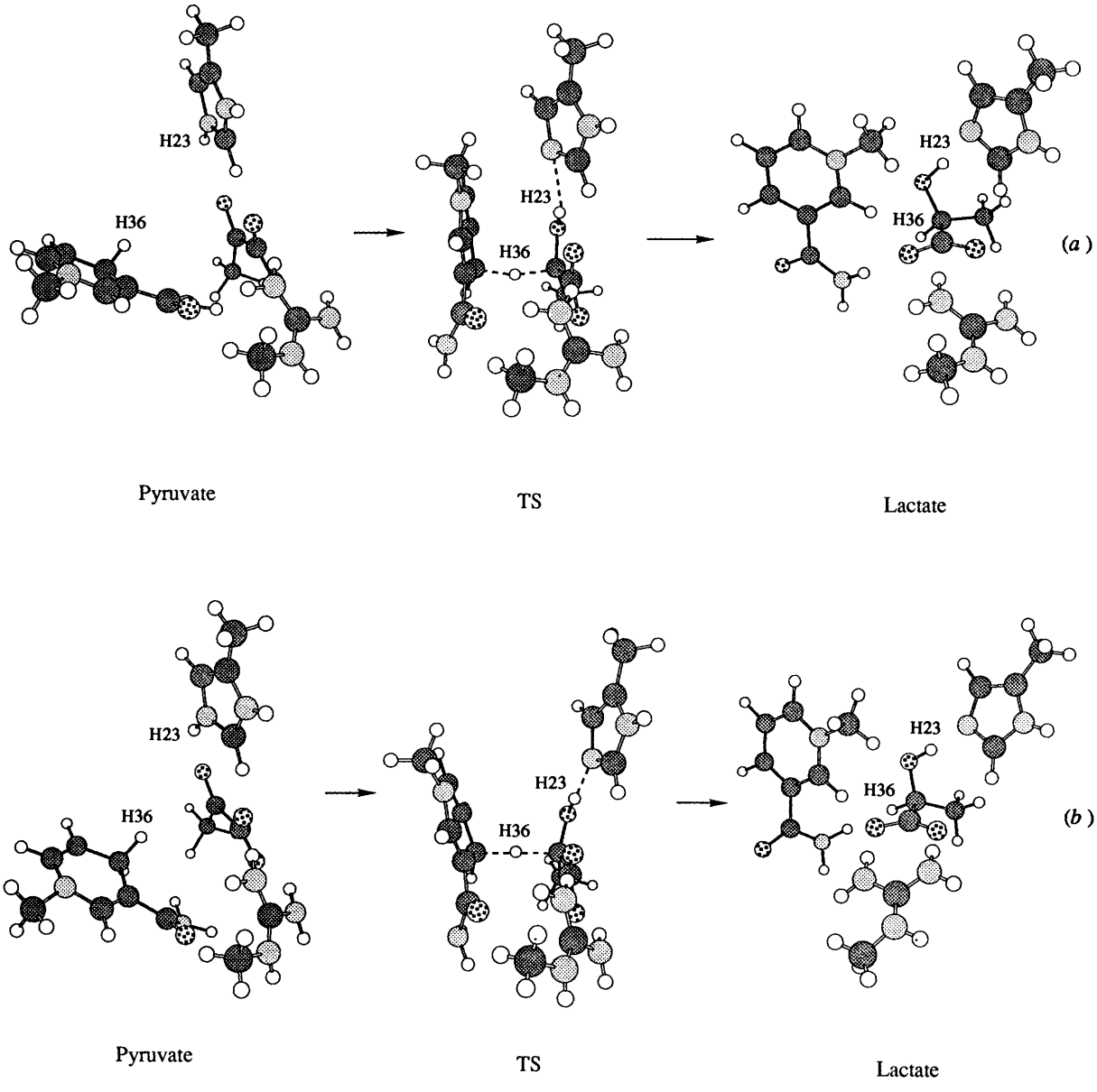

Fig. 4 Representation of supermolecule arrangement in stationary points obtained with both AM1 and PM3 semiempirical methods

substrate fixation to a guanidine residue. The AM1 data produce a larger distance between the imidazole ring and the substrate, H23–N24, than the PM3 values, 2.5 and 1.7, respectively. At the same time, the H16–O5 and H18–O4 distances are larger for the AM1 method than for PM3. These distances indicate the possibility of a weak intermolecular H-bond interaction with PM3. In the corresponding AM1 structures, a much weaker H-bond interaction appears. This behaviour of the AM1 method has been noted previously in the literature.[31,56-59]

There is striking agreement between the PM3 TS structure and previous results reported by us for LADH[3b] and FDH[3d] and Shulz *et al.*[60] for glutathione reductase. The separation of the atoms, C37–C2, between which Ht is exchanged, is around 2.7 Å in *ab initio* calculations.[3b,3d] The PM3 and AM1 results produce similar values. However this distance for **P** obtained by the AM1 method (5.0 Å) is very different from the PM3 (3.8 Å and 3.2 Å) and *ab initio* (3.3 Å) results.[61]

The activation and reaction energy for the structures listed in Table 1 are presented in Table 2. The activation energy ranges from 42.0 to 38.0 kcal mol^{-1} while the reaction energy corresponds to an endothermic reaction, oscillating between 6.4 and 4.9 kcal mol^{-1} for PM3 and AM1 data, respectively.

Table 2 Relative energies of system (in kcal mol^{-1}) for the reactants (pyruvate, **P**), transition state (TS), and products (lactate, **L**) obtained with the AMSOL program. The total energy of **P** in the gas phase is −1.165 and 30.389 kcal mol^{-1} for PM3 and AM1, respectively, while the results when the environment is considered decreases to −57.689 and −24.992 kcal mol^{-1}, respectively

	P		TS		L	
	PM3	AM1	PM3	AM1	PM3	AM1
Isolated	0	0	38.0	42.0	4.9	6.4
Environmental (AMSOL)	0	0	39.2	43.6	2.4	1.3

To include in some way the effect of the environment, calculations by means of the AMSOL program developed recently by Cramer and Truhlar[45-48] were performed for the optimized structures in the gas phase. In the case of the minima, the approximation of frozen geometry can often be considered as adequate.[62,63] On the other hand, this could be more questionable in the case of transition structures, but, since in our

Table 3 Total atomic charges (au) distribution in reactants (pyruvate, **P**), transition state (TS), and products (lactate, **L**) obtained with the help of the Mulliken population analysis

	P		**TS**		**L**	
	PM3	AM1	PM3	AM1	PM3	AM1
C2	0.30	0.23	0.21	0.10	0.00	−0.07
O9	−0.40	−0.41	−0.27	−0.30	−0.42	−0.42
H23	0.23	0.41	0.15	0.33	0.30	0.27
N24	0.32	−0.30	−0.18	−0.25	−0.19	−0.25
H36	0.11	0.12	0.02	0.02	0.09	0.17
C37	−0.10	−0.14	0.05	0.01	−0.07	−0.05
Pyr[a]	−0.80	−0.93	−0.65	−0.80	−1.20	−1.36
Im–H23[b]	0.67	0.55	0.08	0.00	0.03	0.00
Ni–H36[c]	−0.11	−0.11	0.32	0.49	0.95	0.97

[a] Pyr = ΣCl + C2 + C3 + O4 + O5 + H6 + H7 + H8 + O9. [b] Im–H23 = ΣN24 + C25 + C26 + N27 + C28 + H29 + C30 + H31 + H32 + H33 + H34 + H35. [c] Ni–H36 = ΣC37 + C38 + C39 + N40 + C41 + C42 + C43 + O44 + N45 + H46 + H47 + H48 + C49 + H50 + H51 + H52 + H53 + H54 + H55.

study we are only interested in the differential stabilization of the two methods, we will use the gas-phase geometries for all stationary points. The AMSOL program simulates the medium effect by means of the solvent continuum model of a solvent corresponding to an aqueous medium. The environment of active enzyme sites may be quite different from this representation but our results should be taken as just a guide to the qualitative changes from the molecular system *in vacuo* to a continuum medium. At present, quantitative results of the medium effects are still elusive for small systems[64] and even more for large systems,[65] like our model.

In our molecular model, the environmental effects have differential contributions for barrier height and reaction energy. For PM3 these are 1.2 and 2.5 kcal mol^{-1}, respectively, while for AM1 they are 1.6 and 5.1 kcal mol^{-1}, respectively (see Table 2). The main difference between both methods appears in the values of reaction energy; the variation due to the inclusion of the environmental effect is twice that for the AM1 procedure. AM1 predicts a value of reaction energy bigger than PM3 *in vacuo*, while in solution the opposite order is presented. This difference can be justified by the high values of the total atomic charges (see Table 3), and also by geometrical considerations. The major difference between the AM1 and PM3 methods appears in the value for the net atomic charge on the N24 atom of the imidazole ring for **P**; PM3 shows a positive value while AM1 presents a negative value. This can be explained by the fact that the AM1 method produces a **P** structure in which the imidazole ring is far away. From geometrical considerations, see Table 1, the distance between pyruvate and the imidazole ring are almost the same from **P** to **L** for PM3 results, while for the AM1 results this distance increases significantly, favouring the solute–solvent interaction. Similar conclusions may be obtained from the Pyr–Arg171 distances, being larger for the AM1 results.

Chemical events in the enzyme-catalysed reaction occur in small volumes, active sites, compared with the full extent of the biosystem. Main and side chain functional groups and other molecules provide the material basis for the chemical transformations that take place. In LDH, the imidazole ring of histidine, Im, provides the proton to the substrate in order that the hydride transfer between pyruvate and Ni can later take place.

It is important to note that the minima structures, **P** and **L**, have only computational interest. If one considers the seminal hypothesis of Pauling[66] for describing enzyme catalysis, the active site moulds the reactants into the structure of the transition state. Strictly speaking, the binding energy of an enzyme for a TS is the force that drives enzymatic catalysis,[67,68] so the calculated activation energy is not real. On the other hand, the theoretical model employed neglects the tunnelling effects but different authors[69,70] found experimental and theoretical evidence for tunnelling in enzymatic hydride transfers. Therefore, the barrier height cannot be directly related to the activation energy parameter for a real enzyme reaction.

Table 4 Harmonic frequencies (cm^{-1}) obtained for the transition state, and the most important eigenvectors associated with this frequency

	PM3	AM1
Frequency	−1134.43	−1057.35
Distances		
C2–C1	0.085	−0.076
H23–O9	0.098	−0.038
H36–C2	−0.263	0.077
H55–C37	0.080	−0.055
Angles		
C2–C1–D1	0.109	−0.083
H36–C2–C1	−0.701	0.527
C37–D5–D1	0.079	−0.067
H55–C37–C38	−0.321	0.295
Dihedral angles		
C2–C1–D1–D2	−0.086	0.125
H23–O9–C2–C1	0.088	−0.129
H36–C2–C1–O4	0.470	−0.645
C37–D5–D1–D2	−0.074	0.115
H55–C37–C38–C39	0.178	−0.324

We notice that among the three stationary structures characterized on the PES, only the TS fits into the active site of LDH. This fact can be proved by observing Figs. 4(*a*) and 4(*b*) of this work and Fig. 1 of ref. 50, where X-ray coordinates for the dogfish LDH·oxamate·NADH complex (Protein Data Bank 1LDM) are represented. The calculated TSs take an *endo* configuration where the imidazole and the *N*-methyl-1,4-dihydronicotinamide adopt a quasi-parallel position, while in **P** and **L** these moieties are far away from each other. Only the TS is complementary to the one obtained by Norris and Gready.[50]

The imaginary frequency for the TS is nearly the same in the PM3 and AM1 results, $\nu^{\#}$ = 1134i cm^{-1}, and $\nu^{\#}$ = 1057i cm^{-1}, respectively. These values, together with the components of the transition vector, are listed in Table 4. The coordinates capable of producing a saddle point are: (*i*) the distance C2–H36, *i.e.*, the hydride-transfer advance coordinate and (*ii*) the rehybridization coordinate at both the acceptor and donor centres, *i.e.*, the bond angle H55 and the dihedral angle C37. These results are very similar to those of the LADH[3b] and FDH[3d] molecular mechanisms studies. However, they disagree with the results reported by Wilkie and Williams,[28a] where

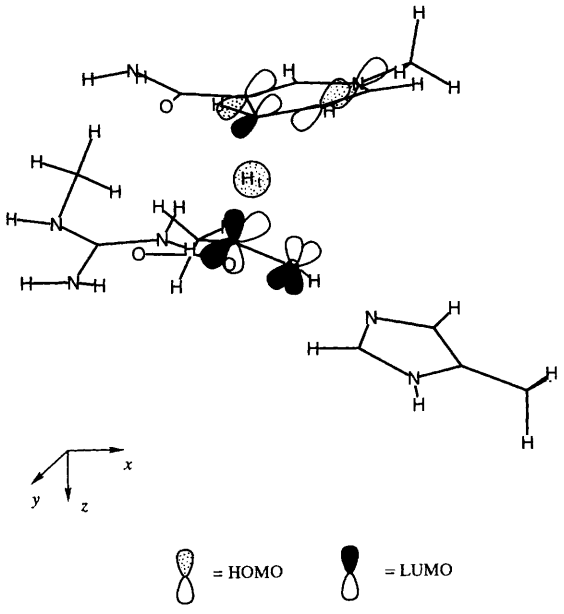

Fig. 5 Schematic representation of the frontier-orbital interactions obtained with the PM3 semiempirical method

a considerably smaller value of $\nu^{\#} = -287$ cm^{-1} is calculated.

From a geometrical point of view in the TS, the hydride-transfer process from Ni to pyruvate has progressed to only a small degree whereas the proton transfer from Im to pyruvate is completed. In this sense, we can conclude that these two processes are kinetically coupled but dynamically uncoupled.

The geometrical arrangement of the TS results in an optimal frontier orbital interaction.[55,60] A maximum overlap between the highest occupied (HOMO) and lowest unoccupied (LUMO) MOs is achieved in an *endo* conformation according to PM3 results. As shown in Fig. 5, the interaction of a hydride HOMO with the LUMO of the electrophilic centre, which would accept Ht during hydride transfer, occurs with a bent arrangement to maximize the overlap of these orbitals and to minimize interaction. However, AM1 results indicate different behaviour; the HOMO is associated to the imidazole ring and the LUMO corresponds to the electrophilic centre.

The present PM3 results, added to other examples reported in the literature using the PMC procedure[60] and the *ab initio* method,[3,55,61] tend to show that the principle of maximum HOMO–LUMO overlap may be used as a guide to build up the supposed TS in hydride-transfer reactions.

Conclusions

In this paper we have analysed the reliability of the AM1 and PM3 methods to study the molecular mechanisms for the transformation from pyruvate to lactate in the active site of the LDH enzyme. Optimized geometries of stationary points on the PES were characterized and compared with the ones obtained with *ab initio* data on simplified models.

The results can be summarized as follows: (*i*) The possible reaction pathways obtained by both the PM3 and AM1 methods are comparable, and show that the transition-state structure that controls the overall process is dominated by the hydride transfer from dihydronicotinamide to pyruvate carbonyl carbon. The energetic barriers are also similar. The transition vector and the reaction pathways show that the hydride and proton transfers are kinetically coupled but dynamically uncoupled.

(*ii*) The active site of LDH is complementary in structure to the characterized TS. Pauling's hypothesis is fulfilled.

(*iii*) The proton-transfer process that takes place along a pre-existing hydrogen bond between the imidazole ring and the pyruvate carbonyl oxygen, obtained by the AM1 method, is not correctly represented. While PM3 bond distances indicate the possibility of a weak intermolecular hydrogen bond, AM1 gives much longer lengths. The same trend is observed for the substrate fixation controlled by hydrogen bonds between the pyruvate and guanidine residue. Hence, from the structural point of view PM3 does show some improvement over AM1.

(*iv*) The analyses of the atomic charges are similar, and reveal that the unique difference can be explained by the fact that the AM1 method produces a **P** structure in which the imidazole is far away.

(*v*) By comparison with *ab initio* results on similar models, the PM3 method seems to be more appropriate than the AM1 procedure in order to show that the principle of HOMO–LUMO maximum overlap may be used as a guide to build up the supposed TS in hydride-transfer reactions.

(*vi*) Solvent-effect calculations carried out with the AMSOL program reveal a small influence on the activation energy. Reaction energies are significantly modified by the presence of a polar medium. The biggest changes are observed in the AM1 values: 6.4 kcal mol^{-1} *in vacuo* and 1.3 kcal mol^{-1} in water.

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