Dependence of enzyme reaction mechanism on protonation state of titratable residues and QM level description: lactate dehydrogenase

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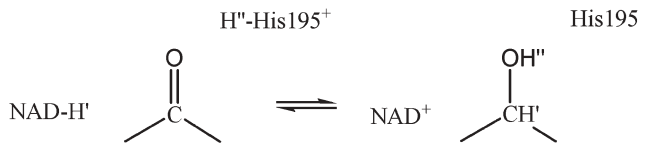
We have studied the dependence of the chemical reaction mechanism of L-lactate dehydrogenase (LDH) on the protonation state of titratable residues and on the level of the quantum mechanical (QM) description by means of hybrid quantum-mechanical/molecular-mechanical (QM/MM) methods; this methodology has allowed clarification of the timing of the hydride transfer and proton transfer components that hitherto had not been possible to state definitively.

It is observed macroscopically that proteins become unstable at extreme values of pH, but properties of enzymes are also pH dependent because their efficacy depends on the protonation state of their ionisable residues. The most expensive computational simulation may give meaningless results if an erroneous charge is selected for a single amino acid of the protein. Thus any theoretical analysis of protein structure or reactivity requires an adequate titration of all ionisable residues. Assignment of the protonation states of such residues is often based on the pK_a of the corresponding amino acids in aqueous solution. However, this practice may introduce significant artefacts into simulations because standard pK_a values of ionisable groups are shifted by local protein environments.[1]

LDH is a highly stereospecific metabolic enzyme that catalyses the interconversion of pyruvate and lactate using the NADH/NAD^+ pair as redox cofactor. This chemical step involves a hydride transfer (HT) from the dihydronicotinamide ring of NADH to the carbonyl C atom of pyruvate and a proton transfer (PT) to the carbonyl O atom from a N atom of the protonated His195 residue (Scheme 1).

Although the rate-limiting step of the full catalytic process in the wild-type enzyme is a conformational change involving closure of a surface loop (99–110) down over the active site,[2] nonetheless the chemical step catalyzed by LDH has been extensively studied both



Scheme 1

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experimentally[3] and theoretically.[4–8] Although the relative timing of the HT and PT components is a matter of interest in mechanistic enzymology, it is not yet possible to state definitively which mechanism is preferred.[8] While a stepwise mechanism is more likely than a concerted one, results from various computational groups differ in the order in which the HT and PT steps occur.[4–8]

Initially the oxamate inhibitor in the ternary complex X-ray crystal structure[9] of LDH tetramer from *B. stearothermophilus* with NADH cofactor was replaced by pyruvate. To assign protonation states for acidic and basic residues, two alternative strategies were considered. In the first, all ionisable groups were set at a state complementary to pH 7 using the pK_a values of the amino acids in aqueous solution, with the exception of His195, which was modelled in its doubly protonated form.

The second protocol involved recalculation of the pK_a values using the "cluster method",[1] as implemented by Field and coworkers,[10] according to which each titratable residue in the protein is perturbed by the electrostatic effect of the protein environment. After adding hydrogen atoms, the entire system was hydrated with a preformed 24 Å radius sphere of TIP3P water molecules centred on the pyruvate center of mass. Atoms 24 Å away from this centre of mass were kept frozen during the simulations. The resulting model had 22139 atoms of enzyme, co-factor, substrate and solvent, 14996 of them kept frozen. The entire chemical system was divided into a QM region, treated by the AM1 semiempirical MO method, and a MM region comprising the rest of the protein (CHARMM26 potentials)[11] and the water molecules. The generalized hybrid orbital (GHO)[12] method was used to treat those covalent bonds crossing the boundary between the QM and the MM regions. The QM region, formed by the dihydronicotinamide and the ribose ring of the NADH, Arg171, Arg109, the pyruvate and the His195 contained a total of 78 atoms. The full system was relaxed by means of a combination of minimizations and molecular dynamic simulations.

The potential energy surfaces (PESs) were obtained using two antisymmetric combinations of distances defining the HT and PT coordinates. Fig. 1 shows the PES obtained when the ionisable residues of the enzyme were titrated using the standard pK_a values in aqueous solution. The resulting competitive mechanistic pathways across this energy hypersurface, as well as the geometries of the stationary points and the barrier heights (see first two columns of Table 1) are similar to the results of our previous study.[8]

When the pK_a values of the ionisable residues were recalculated under the effect of the protein environment, as explained above, the protonation state of several ionisable groups at pH = 7 was found to have changed. In particular, Glu199 and His82 became

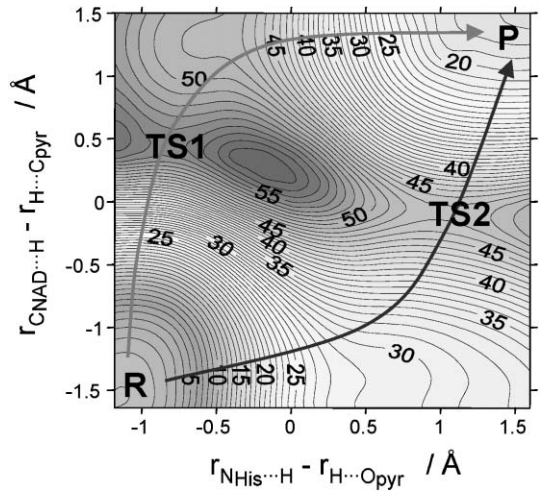

Fig. 1 PES 1: QM/MM contours (kcal mol^{-1}) for pyruvate (R) to lactate (P) LDH-catalyzed reaction. The protonation state of the ionizable groups in protein were calculated at pH = 7 using the standard pK_a values in aqueous solution.

Table 1 QM/MM selected interatomic distances (Å), and relative energies (kcal mol^{-1}) for the optimized geometries of the transition states of the reaction paths obtained at the two different protonation states of the ionizable groups of the protein

	PES 1		PES 2		PES 3
	TS1	TS2	TS1	TS2	TS1
$C_{NAD}\cdots H'$	1.78	1.36	1.69	1.27	1.43
$H'\cdots C_{pyr}$	1.22	1.39	1.21	1.47	1.31
$N_{His195}\cdots H''$	1.03	2.01	1.04	1.92	1.09
$H''\cdots O_{pyr}$	1.81	0.99	1.74	0.99	1.57
$\Delta H^{\ddagger}$	54	47	44	33	21

protonated, thereby changing their charges to neutral and positive, respectively.[13] The effect of this change in only two of the residues on the resulting PES is drastic, as shown in Fig. 2 and in the values listed in the third and fourth columns of Table 1. The main effect is to reduce the activation enthalpy by *ca.* 10 and 14 kcal mol^{-1} for TS1 and TS2 paths, respectively. This diminution in the reaction barriers is mainly a consequence of the added Glu199 proton close to Asp168, a residue that interacts directly to maintain His195 in a protonated state in preparation for substrate binding and turnover. Excess negative charge in this vicinity stabilizes the protonated imidazole of His195 in the reactant complex more than in the transition state or product, a situation exacerbated by negative charge on Glu199. The previously described ultra-simple electrostatic model, comprising only the substrate carbonyl group with the hydride and proton donors and suitably-located point-charges,[4] also predicted reduction in the AM1 calculated reaction barrier by *ca.* 10 kcal mol^{-1}, due to annulment of a negative charge in the vicinity of Asp168.[14]

The last phase of this study was motivated by the need to improve the description of the QM region beyond the present AM1 semiempirical level. In view of the size of our system a high theoretical level is still prohibitive, so we have performed single point calculations at the MP2/6-31G(d,p) level on the same grid of AM1/MM optimized structures used to obtain the PES displayed on Fig. 2.[15] The results (Fig. 3 and last column of Table 1) are impressive: the asynchronous concerted reaction pathway in which PT precedes HT disappears and the effective enthalpy barrier for the reaction mechanism now appears to be much more in accordance with the expected value for this biological reaction.[16]

We demonstrate in this work that reaction mechanisms and barrier heights of an enzymatic reaction can be wrong if protonation states for the titratable residues of the protein are selected on the basis of the pK_a of the corresponding amino acids in aqueous solution. From our results, obtained at high level MP2//AM1/MM, we have arrived at some conclusions about the timing of the hydride transfer and proton transfer components in the chemical step catalyzed by LDH that had not been possible to state definitively up to now.

Finally, accurate determination of transition-state properties would require statistical averaging over many configurations, each one individually being a transition structure[8] and, for this particular reaction, tunnel effects may also contribute to lowering the observed free energy barrier.

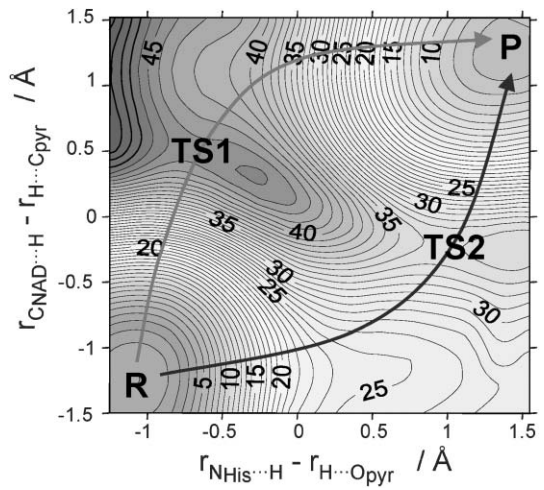

Fig. 2 PES 2: QM/MM contours (kcal mol^{-1}) for pyruvate (R) to lactate (P) LDH-catalyzed reaction. Protonation states of ionizable groups in protein were calculated following the method of Gilson *et al.*[1]

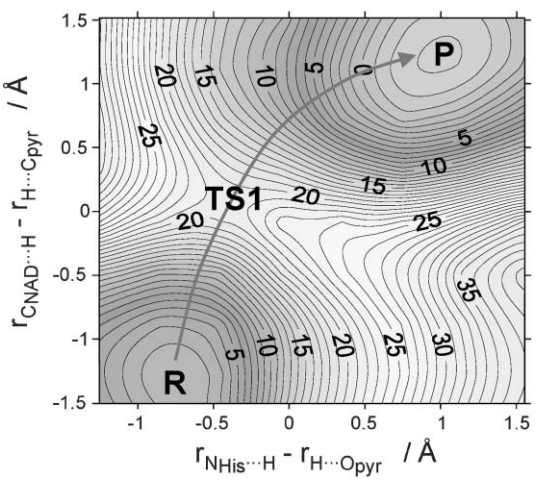

Fig. 3 PES 3: QM/MM contours (kcal mol^{-1}) for pyruvate (R) to lactate (P) LDH-catalyzed reaction, with MP2/6-31G(d,p) corrections. Protonation states of ionizable groups in protein are as for PES 2.

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