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Theoretical site-directed mutagenesis: Asp168Ala mutant of lactate dehydrogenase

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Molecular simulations based on the use of hybrid quantum mechanics/molecular mechanics methods are able to provide detailed information about the complex enzymatic reactions and the consequences of specific mutations on the activity of the enzyme. In this work, the reduction of pyruvate to lactate catalysed by wild-type and Asp168Ala mutant lactate dehydrogenase (LDH) has been studied by means of simulations using a very flexible molecular model consisting of the full tetramer of the enzyme, together with the cofactor NADH, the substrate and solvent water molecules. Our results indicate that the Asp168Ala mutation provokes a shift in the pK_a value of Glu199 that becomes unprotonated at neutral pH in the mutant enzyme. This change compensates the loss of the negative charge of Asp168, rendering a still active enzyme. Thus, our methodology gives a calculated barrier height for the Asp168Ala mutant 3 kcal mol⁻¹ higher than that for wild-type LDH, which is in very good agreement with the experiment. The computed potential energy surfaces reveal the reaction pathways and transition structures for the wild-type and mutant enzymes. Hydride transfer is less advanced and the proton transfer is more advanced in the Asp168Ala mutant than in the wild type. This approach provides a very powerful tool for the analysis of the roles of key active-site residues.

Keywords: quantum mechanics/molecular mechanics; enzymatic catalysis; lactate dehydrogenase

1. INTRODUCTION

Molecular simulations are a powerful tool to analyse biochemical processes in detail. In particular, modelling of enzymatic reactions can provide us with a deep knowledge of the chemical transformations taking place in the active site, allowing us to rationalize the basis of the amazing enzymatic ability to speed up chemical reactions with respect to their counterpart processes in solution. This knowledge can be used in turn to understand the consequences of mutations on the enzyme efficiency (and thus to explain the molecular basis of some genetic diseases), design new and potent inhibitors of a particular enzyme or guide the design of new biological catalysts for any desired reaction.

In this contribution, we illustrate the application of chemical modelling to the analysis of the effect of mutations on L-lactate dehydrogenase (LDH), a highly stereospecific enzyme that catalyses the interconversion of pyruvate and L-lactate using the NADH/NAD⁺ pair as redox cofactor. Enzymes such as LDH, involved in the metabolic pathways, have been the subject of extensive

research both experimentally and theoretically. While their properties are now relatively well known, details of their reaction mechanisms are still not completely known (Kedzierski *et al.* 2001).

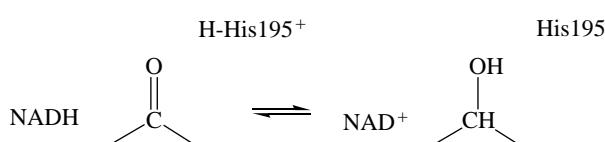
The chemical step of LDH (scheme 1) in the pyruvate to L-lactate direction involves a hydride transfer from the dihydronicotinamide ring of NADH to the carbonyl C atom of pyruvate and a proton transfer to the carbonyl O atom from a protonated histidine (His195) residue (Holbrook *et al.* 1975).

The whole catalytic process goes through several stages: substrate binding; unimolecular rearrangement, involving the closure of a surface loop (99–110) down over the active site; pyruvate reduction; reverse protein conformational change; and products release (Kedzierski *et al.* 2001). The loop closure enforces isolation of the active site from the bulk solvent, which is necessary for the reaction to proceed at a noticeable rate (Waldman *et al.* 1988; Dunn *et al.* 1991). In the wild-type enzyme, this conformational rearrangement is in fact the rate-limiting step (Dunn *et al.* 1991).

The advent of protein engineering has enabled the role of specific residues in the active site to be defined by substituting them with other amino acids presenting differences in charge and/or size (Holbrook & Ingram 1973; Clarke *et al.* 1986, 1988; Hart *et al.* 1987; Cortes

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One contribution of 9 to a Theme Supplement 'Biomolecular simulation'.



Scheme 1. Pyruvate to lactate transformation catalysed by lactate dehydrogenase.

et al. 1992; Sakowicz *et al.* 1995). Site-directed mutagenesis has proved to be a good tool not only to change the rate-limiting step of the mechanism, but also to demonstrate the feasibility of applying rational protein engineering to optimize and control the stereoselectivity of LDH for asymmetric synthesis (Sakowicz *et al.* 1995).

One of the most relevant early experiments was the chemical modification of histidine-195 in LDH (Holbrook & Ingram 1973); acylation of this residue inhibited the enzyme, an observation that was interpreted in terms of a model in which the substrates could bind only when His195 was protonated. Whereas the imidazole group of histidine is largely deprotonated at neutral pH in aqueous solution, the enzyme environment raises the pK_a of His195, thus favouring the protonated state. This is due to the close presence of the negatively charged carboxylate group of Asp168. The influence of this residue on the reaction was evaluated by replacing it with either Asn or Ala (Clarke *et al.* 1988). This study supported the hypothesis that Asp168 forms a strong interaction with His195 and thereby contributes to catalytic efficiency both by raising the pK_a of His195 and by anchoring it in the most favourable orientation to enhance its acid/base catalysis proton donating/accepting properties. Replacing the negatively charged Asp168 with a neutral residue destabilizes the ternary complex formed by the enzyme with the substrate and the cofactor and makes the chemical step rate limiting, in contrast to the wild-type enzyme; however, somewhat surprisingly, the pK_a of His195 does not change nor is the activity of the enzyme dramatically reduced.

Other mutations performed on LDH to investigate the role of various residues include: Arg171Lys that affects the catalytic properties of the enzyme by weakening the substrate binding (Hart *et al.* 1987); Arg109Gln that demonstrates the role of the arginine in polarizing the pyruvate carbonyl group (Clarke *et al.* 1986); Asn140Asp (Cortes *et al.* 1992); and the more recent Ala245Lys (Kedzierski *et al.* 2001).

Experimental studies can be complemented by means of computational simulations that provide details at the atomic level. The chemical reaction catalysed by LDH has also been extensively studied by several theoretical methods, either in gas phase or, more recently, considering the influence of the protein environment. Different computational groups' proposals differ in the order in which the proton and hydride ions are transferred. Some found a mechanism in which the hydride transfer preceded proton transfer in a stepwise manner (Yadav *et al.* 1991; Ranganathan & Gready 1997), while others predicted a concerted but asynchronous reaction in which the proton transfer was largely

completed in the transition state (TS) of the reaction (Wilkie & Williams 1992; Moliner *et al.* 1997; Turner *et al.* 1999; Ferrer *et al.* 2005a). A very flexible treatment of the environment (Moliner & Williams 2000) generated a potential energy surface (PES) showing the coexistence of both mechanistic pathways. The distinction in the timing of hydride transfer relative to proton transfer is a matter of interest in mechanistic enzymology but, at the time of this latter study, it was not possible to make a definitive statement as to which mechanism was preferred. Finally, we recently demonstrated that the chemical reaction mechanism of LDH depended on both the protonation state of the ionizable residues of the protein and the level of theory employed to describe the quantum region (Ferrer *et al.* 2005b). Using a higher level description of the reaction system rendered a PES that described an asynchronous concerted reaction pathway in which hydride transfer preceded proton transfer, with a remarkable reduction of the effective enthalpy barrier, much more in accordance with the expected value for the biological reaction.

In this paper, we carry out an exhaustive analysis of the LDH-catalysed reaction by obtaining the PES corresponding to the Asp168Ala mutant. Our results can be used to interpret the experimental studies already carried out in this system and to explain the predicted role of the ionized residues present in the active site of the enzyme. Finally, we suggest new mutations that could be carried out experimentally and which would definitely corroborate the proposed mechanism and the particular role of key active-site residues. We firmly believe that this example illustrates some of the possibilities offered by molecular simulation methods in order to rationalize and understand the chemical processes occurring in the complex environments, such as in enzymatic reactions. The use of these computational methods will be a fundamental tool for future research in biocatalysis.

2. COMPUTATIONAL METHODS

Nowadays, the most popular computational methodology to study enzymatic reactions is the combination of quantum mechanics and 'molecular' mechanics (MM). In these methods, the quantum mechanical (QM) description is reserved to a small portion of the system, the region where the most important chemical changes (i.e. bond breaking and forming processes) are taking place. The rest of the system can be described by means of classical MM potentials. In this combined quantum mechanics/molecular mechanics (QM/MM) approach, a very large number of atoms can be explicitly considered in the calculations. To describe a chemical reaction, we should be able to locate and characterize the set of stationary structures (reactants, products, TS and possible intermediates) that define a particular reaction mechanism. This is difficult owing to the large dimensionality of the system when the environment is explicitly included, but the exploration of the potential energy of the system as a function of some selected coordinates (to generate a PES) can provide a reasonable starting point to investigate the atomistic details of very complicated processes.

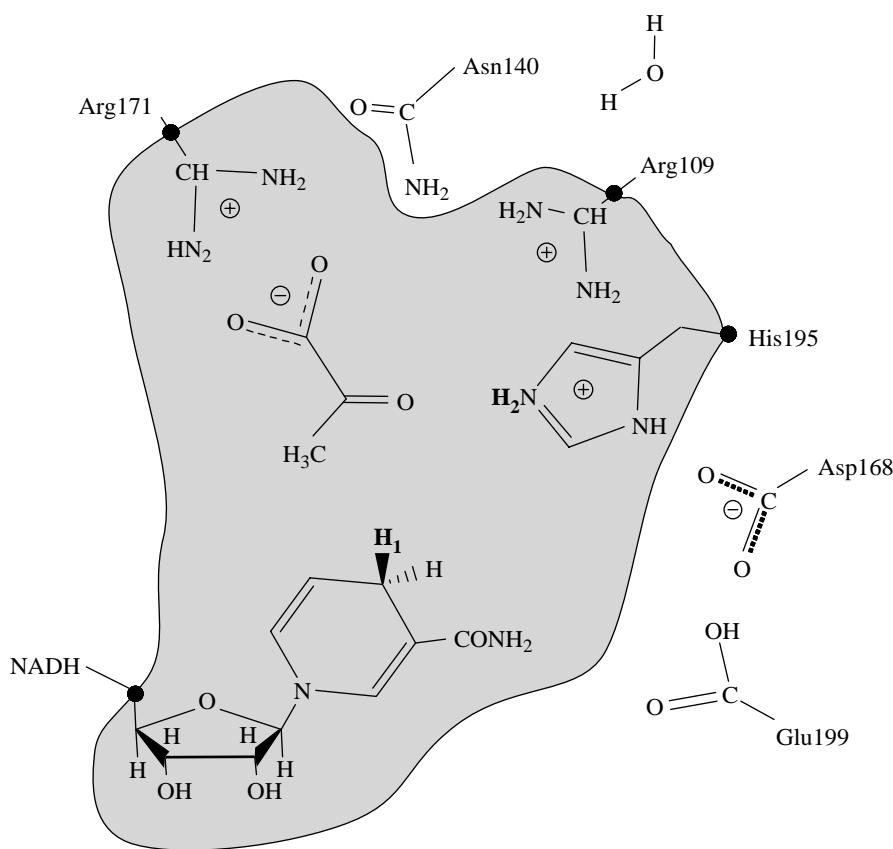


Figure 1. Schematic structure for cofactor (NADH), substrate (pyruvate) and some key residues of the active site. Generalized hybrid orbitals are indicated by filled circles, dividing the quantum region (shaded area) and the classical region. H₁ and H₂ are in bold.

QM/MM PESs for the LDH-catalysed reaction were obtained using the CHARMM29b1 program (Brooks *et al.* 1983). The starting geometry comes from the 2.5 Å resolution ternary complex X-ray crystal structure of the LDH tetramer from *Bacillus stearothermophilus* with NADH cofactor and oxamate inhibitor (Wigley *et al.* 1992). Oxamate was replaced by pyruvate and hydrogens were added to all titratable residues at a state complementary to pH 7. Owing to the fact that standard p*K*_a values of ionizable groups can be shifted by local protein environments (Ferrer *et al.* 2005*a,b*), an accurate assignment of the protonation states of all these residues was carried out by recalculating the standard p*K*_a values of the amino acids using the ‘cluster method’ (Gilson 1993; Antosiewicz *et al.* 1994) as implemented by M. J. Field *et al.* (2004, personal communication). According to this method, each titratable residue in the protein is perturbed by the electrostatic effect of the protein environment. As demonstrated in our previous study on LDH (Ferrer *et al.* 2005*b*), an inaccurate protonation state of only a few residues of the protein can render incorrect PESs and thus erroneous reaction mechanisms and/or activation barriers. The system was placed in a cavity deleted from a preformed 24 Å radius sphere of water molecules centred on the acceptor carbon and acceptor oxygen atoms. Water molecules within 2.5 Å of any non-hydrogen atoms of the protein, cofactor or substrate were removed. This procedure was repeated thrice by randomly rotating the water spheres to solvate potential cavities of a

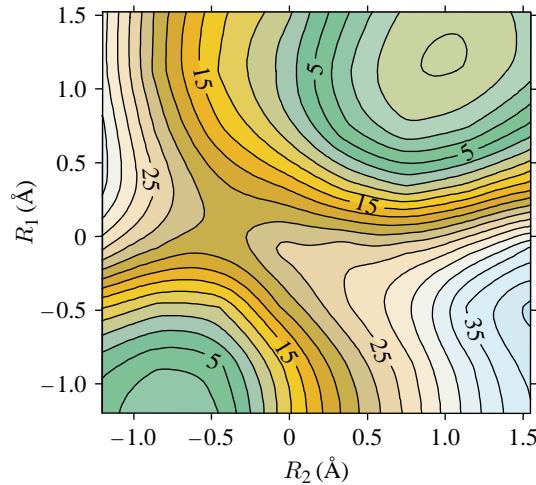


Figure 2. QM/MM energy contours (kcal mol⁻¹) for the interconversion of pyruvate and lactate in the wild-type LDH-catalysed reaction. Energies have been computed at the AM1/MM level adding MP2/6-31G(d,p) corrections as a two-dimensional spline function of R_1 and R_2 coordinates as explained in the text.

single water configuration. Then, after a short molecular dynamic simulation of 5 ps to relax unfavourable contacts, the solvation process was repeated to fill in additional cavities generated during the equilibration dynamics (Garcia-Viloca *et al.* 2004; Ferrer *et al.* 2005*a,b*). Once the entire system was built, those atoms that are 24 Å away from the centre of carbonyl

Table 1. Selected geometrical parameters (distances in Å) of the different stationary point structures on the QM/MM PES of the native-type LDH and the mutant. Potential energy barriers obtained at AM1/MM adding MP2/6-31G(d,p) corrections as a two-dimensional spline function of R_1 and R_2 coordinates as explained in the text (in kcal mol⁻¹).

	native LDH			Asp168Ala		
	pyruvate	TS	lactate	pyruvate	TS	lactate
C _{nic} ···H ₁	1.13	1.43	2.35	1.13	1.37	2.55
H ₁ ···C _{pyr}	2.33	1.31	1.13	2.44	1.38	1.13
N _{His195} ···H ₂	1.05	1.09	1.93	1.05	1.12	2.03
H ₂ ···O _{pyr}	1.78	1.57	0.99	1.79	1.49	0.98
O _{pyr} ···H _{Arg109}	2.13	2.08	2.15	2.14	2.06	2.10
O _{pyr} ···H _{Asn140}	2.11	2.01	3.00	2.89	2.56	3.06
O _{pyr} ···N _{E2His195}	2.72	2.61	2.77	2.75	2.58	2.88
ΔE ^a	21			24		

^aPotential energy barrier of the reaction.

C and O atoms of the pyruvate were kept frozen during the subsequent dynamics and optimizations. The resulting model had 22 141 atoms of enzyme, cofactor, substrate and solvent, of which 15 154 were kept frozen. The entire chemical system was divided into a QM region, treated by the AM1 semiempirical MO method (Dewar *et al.* 1985), and a MM region comprising the rest of the protein (described by CHARMM24 potentials) and the surrounding water (described by the TIP3P potential; Jorgensen *et al.* 1983). The generalized hybrid orbital (GHO) method (Gao *et al.* 1998; Amara *et al.* 2000) has been used to treat the covalent bonds crossing the boundary between the QM and the MM regions in order to satisfy the valence of the QM fragments. The QM region was formed by the dihydronicotinamide and the ribose ring of the NADH, pyruvate, His195 and arginines 109 and 171. A picture of the active site showing the partition in the QM and MM subsystems is presented in figure 1, where the QM region is shown as the shaded portion.

The mutation was carried out by substituting the original residue of the native structure by the new residue. Afterwards, the new tetramer was solvated by water molecules following the same simulation protocol as in the native LDH described above. A series of energy minimizations and molecular dynamics were then carried out in order to allow the system to be adapted to the mutation.

The PESs were obtained using two antisymmetric combinations of the distances between the transferring hydrogens with their donor and acceptor atoms. Thus, the reaction coordinate R_1 for the hydride transfer is defined (equation (2.1)) as the difference between the C_{nic}–H₁ and H₁–C_{pyr} bond distances (figure 1), and the reaction coordinate R_2 for the proton transfer is defined (equation (2.2)) as the difference between the N_{His}–H₂ and H₂–O_{pyr} bond distances,

$$R_1 = r_{\text{C}_{\text{nic}}} \text{H}_1 - r_{\text{C}_{\text{pyr}}} \text{H}_1, \quad (2.1)$$

$$R_2 = r_{\text{N}_{\text{His}}} \text{H}_2 - r_{\text{O}_{\text{pyr}}} \text{H}_2. \quad (2.2)$$

In order to correct for the known deficiencies of the AM1 Hamiltonian when describing hydride and proton transfer, we added MP2/6-31G(d,p) corrections as a two-dimensional spline function of the R_1 and R_2

Table 2. pK_a values of the different ionizable amino acids in aqueous solution and calculated for the native-type LDH and the Asp168Ala mutant.

	aqueous solution	native LDH	Asp168Ala
His195	6.0	7.6	7.3
Asp168	3.9	1.2	—
Glu199	4.1	7.4	4.2
Arg109	12.8	14.0	14.0

coordinates fitted to single-point MP2 energies at AM1 geometries (Ferrer *et al.* 2005a,b).

The CHARMM29b1 program was employed to carry out the grid scanning. Once the PESs were obtained, localized approximate TS structures were refined using the recently modified version of the GRACE software (Martí *et al.* 2005). A partial-rational-function-operator/adopted-basis-Newton-Raphson method was employed, using a Hessian matrix of the order 156 × 156, describing the curvature of the QM/MM energy hypersurface for a subset of the system (the QM atoms), together with a diagonal Hessian plus updates for the rest of the system. The r.m.s. residual gradient on the 78 atoms in the subset was less than 0.1 kcal mol⁻¹ Å⁻¹ in the optimized structure, while on the remaining atoms (approx. 3000) it was less than 0.005 kcal mol⁻¹ Å⁻¹. Finally, the intrinsic reaction coordinate path (Fukui 1970) was traced from the TSs in order to demonstrate conclusively that the reported structure was indeed the TS for the correct chemical step.

Finally, it should be mentioned that our analysis is based on the exploration of a single reaction path for the mutant and the wild-type enzyme and not on free-energy calculations that incorporate the effect of atomic fluctuations. We recently showed that different paths could be possible depending on the conformation of the enzyme (Ferrer *et al.* 2006) and thus the interpretation of experimental data would require a proper averaging over these conformations. In this work, we checked that the conformations of the flexible loop in the mutant and wild-type enzymes were equivalent, thus ensuring that they could be compared.

3. RESULTS AND DISCUSSION

3.1. Wild type

The PES obtained for the interconversion of pyruvate and lactate in the active site of the wild-type LDH enzyme is depicted in figure 2, while QM/MM energies and selected optimized interatomic distances for minima and saddle points located on the PESs are listed in table 1.

Figure 2 clearly corresponds to a PES with a concerted but asynchronous reaction pathway. In the TS, the hydride transfer is considerably more advanced than the proton transfer; this is to say that the hydride transfer precedes the proton transfer. Selected structures of this PES were used as starting points to optimize and characterize the stationary structures (reactants, transition structure and products). The potential energy barrier, provided in table 1, appears to be 21 kcal mol⁻¹ for the wild-type enzyme reaction.

From the analysis of the substrate–enzyme interactions listed in table 1, it is important to emphasize the significantly short distance established between the carbonyl oxygen atom of pyruvate with Arg109 and Asn140 at different stages of the reaction. As previously reported in the literature, these interactions may contribute to polarize the carbonyl bond thus facilitating the hydride transfer (Clarke *et al.* 1986). Furthermore, it is important to keep in mind the presence of a water molecule in the vacuole of the active site (detected by X-ray diffraction analysis; Wigley *et al.* 1992) together with some other important hydrogen-bond interactions established between the substrate and the Arg171, between His195 and the Asp168, or the interaction between Glu199 and Asp168. This series of interactions facilitate the favourable orientation of the ternary complex for the reaction to proceed. Furthermore, as demonstrated in our previous paper (Ferrer *et al.* 2005b), this local environment has important consequences in the acidity of the ionizable residues of the active site. It can be observed in table 2 how the electrostatic interactions in the enzyme shift the pK_a values of these residues from the standard pK_a values in aqueous solution. In particular, it is clear that the protein environment raises the pK_a of some residues with respect to aqueous solution (Glu199, His195 or Arg109), demonstrating that they must be protonated in the enzyme at pH 7, which favours the catalysis, and diminishes the value of Asp168, proving the deprotonated state of this residue which, as previously shown, favours the protonation state of His195.

As mentioned above, a deeper insight into the role of the different amino acids of the active site of the enzyme can be obtained by the analysis of the effect of different mutations on the topology of the PES of the wild-type enzyme. Changes in the pK_a values, in the reaction mechanisms, structures and barrier heights are used to define the catalytic role of the different residues. The results of the different simulations are discussed in turn.

3.2. Asp168Ala mutant

Analysis of the role of the negatively charged Asp168 has been carried out by replacing this residue by

alanine, a smaller and neutral amino acid. It has been proposed that LDHs cannot form an enzyme–cofactor–substrate complex unless the imidazole group of His195 is protonated. A negatively charged Asp168 would contribute to catalytic efficiency by raising the pK_a of His195 to ensure that there is a proton available to be transferred to the substrate from a suitably orientated positively charged imidazolium group. Table 2 shows the pK_a values of key residues in the mutant enzyme. The expected severe drop in the pK_a value of His195 upon deletion of the negative charge of the neighbour residue number 168 is not observed, which is in agreement with experimental observations (Clarke *et al.* 1988). The pK_a values of His195 and Glu199 are decreased to 7.3 and 4.2 in the mutant, respectively. This means that, at pH 7, His195 is still protonated while the glutamate becomes unprotonated. When modelling the mutant we consequently removed a proton from the carboxylate group of Glu199. It is the presence of this new negative charge in the surroundings of His195 that prevents a drastic shift of the His195 pK_a towards lower values in the mutant. The small shift in the His195 pK_a means that a positively charged imidazolium is slightly less favourable in the mutant than in the wild type due to the loss of the strong electrostatic interaction with Asp168, but the loss of this interaction is, to some extent, compensated by the negative charge of the ionized Glu199. Consequently, the PES of the chemical reaction does not change dramatically as a result of this mutation. The resulting PES is depicted in figure 3 and corresponds to a concerted but asynchronous proton and hydride transfer, as in the wild-type case. However, in this new PES, the degree of hydride and proton transfer reached at the TS is quite different from the wild-type TS. The position of the TS on the Asp168Ala mutant PES appears at less advanced values of the hydride transfer coordinate (R_1) and at a more advanced values of the proton transfer coordinate (R_2). Table 1 provides some key distances of the stationary structures obtained for the Asp168Ala mutated LDH-catalysed reaction. According to equations (2.1) and (2.2) and values reported in table 1, the hydride transfer coordinate in the wild-type TS is 0.12 Å while it is -0.01 Å in the mutant. The opposite trend is observed in the evolution of the proton transfer coordinate: -0.48 Å in the wild-type enzyme and -0.37 Å in the Asp168Ala mutant. These changes can be observed in figure 4, where we have overlapped the transition structures obtained in both enzymatic environments. The energetic consequence is that the potential energy barrier associated with the reaction in the Asp168Ala mutant is increased with respect to the wild-type enzyme (24 and 21 kcal mol⁻¹, respectively). This result is in agreement with experimental observations (Clarke *et al.* 1988). The potential energy barrier increase (3 kcal mol⁻¹) is consistent with the 45-fold decrease in the k_{cat} at 25°C (Clarke *et al.* 1988), which can be translated to an increase of approximately 2.3 kcal mol⁻¹ in the activation free energy. Obviously, both quantities cannot be compared directly but the fact that they are of the same order of magnitude gives us confidence in our results.

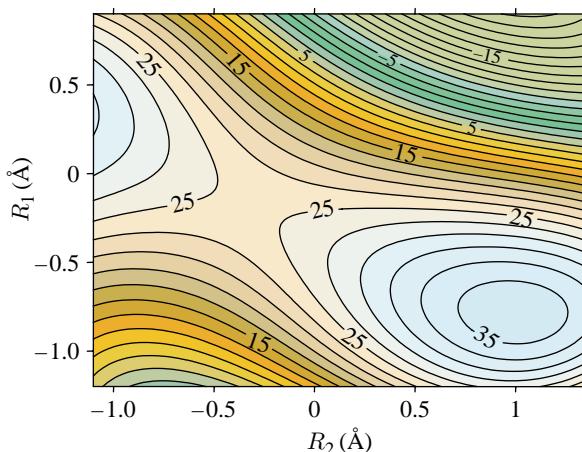


Figure 3. QM/MM energy contours (kcal mol^{-1}) for pyruvate to lactate transformation in the Asp168Ala mutated LDH-catalysed reaction. Energies have been computed at the AM1/MM level adding MP2/6-31G(d,p) corrections as a two-dimensional spline function of R_1 and R_2 coordinates as explained in the text.

The changes observed in both the hydride and the proton transfer can be rationalized on the basis of the displacement of the negative charge from Asp168 in the native enzyme to Glu199 in the mutant. This second residue is more distant from NADH than Asp168. The distance from the closer oxygen atom of the carboxylate group of Glu199 to the carbon donor atom of NADH in the reactant state is 7.9 Å for Asp168 (wild-type enzyme) and 9.4 Å for Glu199 (mutant enzyme). This displacement of a negative charge moving away from the substrate clearly assists the hydride transfer from NADH to pyruvate. The net effect on the proton transfer is somewhat trickier, although it can be explained on the same basis. As shown by the pK_a calculations, the displacement of the negative charge from Asp168 to Glu199, which is more distant from His195, raises the acidity of this residue and thus one would expect an easier proton transfer to the substrate. However, the displacement of the negative charge also increases the distance to the substrate, a negatively charged pyruvate molecule. Effectively, the average distance from the carboxylate oxygen atoms of Asp168 (wild type) to the proton acceptor atom is 6.9 Å, while the distance from the oxygen atoms of Glu199 carboxylate group (mutant) is 7.6 Å. Thus, when the negative charge is displaced, we have two opposed tendencies: a more acidic histidine and a less basic pyruvate carbonyl group because the deprotonated form is stabilized upon mutation moving away the negative charge. The balance between these two effects results in a higher barrier for the proton transfer and the displacement of the transition structure to a more advanced value of the reaction coordinate.

4. CONCLUSIONS

The reduction of pyruvate to lactate catalysed by LDH has been studied by means of hybrid QM/MM simulations incorporating high-level corrections. A

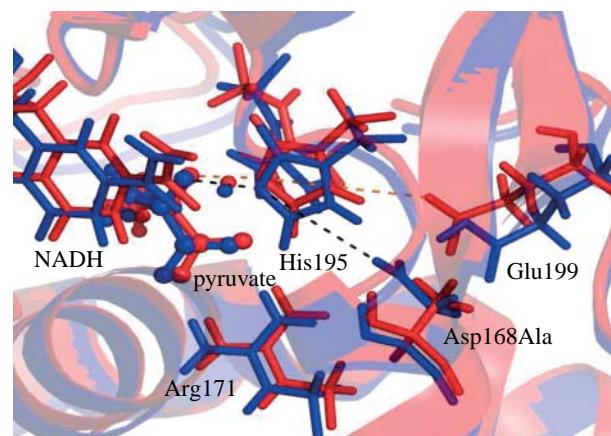


Figure 4. Superposition of the TS structures corresponding to the wild-type LDH (red) and Asp168Ala mutant (blue). Dashed lines are depicted from the proton acceptor atom (O_{Pyr}) to the proton donor atom (N_{His195}), and from this one to the carboxylate group of Glu199 and Asp168, respectively.

very flexible molecular model consisting of the full tetramer of the enzyme together with the cofactor NADH, the substrate and solvent water molecules has allowed us to mimic the effects of site-directed mutagenesis that was previously performed experimentally. The PESs obtained for a single mutation, Asp168Ala, and the one corresponding to the native enzyme, have been used to trace the possible reaction pathways and to locate and characterize the structures corresponding to the stationary points. Analysis of the reaction mechanisms and the properties of the stationary structures is a very powerful tool for determining the roles of the key residues on the vacuole formed in the active site of the enzyme. Furthermore, it has been observed that there is an important shift in pK_a values of key ionizable residues from aqueous solution to the enzyme, and also from native enzyme to the mutant, with consequent implications for catalysis.

Results show that the chemical steps can be described as concerted but asynchronous hydride and proton transfers. The electrostatic characteristics of the enzymatic environment affect the degree of proton and hydride transfer attained in the TS. Thus, when Asp168 is mutated to alanine, the pK_a of the protonated His195 is slightly decreased. Moreover, a dramatic pK_a shift is observed after mutation in Glu199, becoming unprotonated at physiological pH. The presence of a now unprotonated Glu199 can partly compensate the loss of the negative charge of Asp168 and then prevents a drastic shift in the His195 pK_a . A double mutation of Asp168 and Glu199 would most probably render an inactive enzyme where His195 residues were found in a deprotonated form at neutral pH.

The net effect on the catalytic rate constant is an increase in the potential energy barrier of the chemical process from the wild-type enzyme to the mutant. This can be explained by considering that while the hydride transfer is assisted the proton transfer to the substrate is more difficult. These effects can be rationalized on the basis of the changes in the electrostatic characteristics of the enzyme active site. In particular, there is an

important effect of mutation on the protonation state of Glu199 and, as a consequence, on electrostatic interactions with the transferring proton. We believe that this example illustrates the possibilities of chemical modelling in the prediction of catalytic properties of newly designed proteins.

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