Theoretical site-directed mutagenesis. The Asp168Ala mutant of L-Lactate Dehydrogenase

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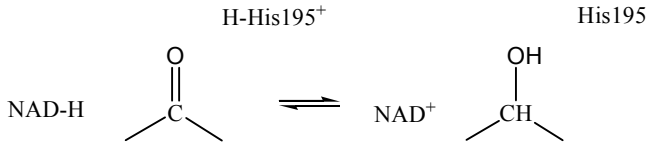
ABSTRACT: The reduction of pyruvate to lactate catalyzed by the L-Lactate dehydrogenase has been studied in this paper by means of hybrid Quantum Mechanical / Molecular Mechanical simulations. A very flexible molecular model consisting on the full tetramer of the enzyme, together with the cofactor NADH, the substrate and solvent water molecules has allowed to theoretically mimic site directed mutagenesis studies, most of them previously experimentally performed. The potential energy surfaces obtained for every single mutation, compared with 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. The analysis of the results has been a very powerful tool to conclude about the role of key residues on the vacuole formed in the active site of the enzyme. Our results are in very good agreement with the previous conclusions derived from site directed mutagenesis. This strategy can be extrapolated to other enzyme systems thus opening the door of a very promising methodology that, in combination with the appropriate experimental technique, will enable us to describe enzyme catalysis phenomenon and the particular role of the residues that form the protein. This knowledge placed us in a privileged position to modify the activity of enzymes and to propose efficient inhibitors.

INTRODUCTION

L-Lactate dehydrogenase (LDH) is a highly stereospecific metabolic enzyme which catalyses the interconversion of pyruvate and L-lactate using the NADH/NAD+ pair as redox cofactor. Enzymes like LDH, involved in 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.[1]

The chemical step of LDH, in the pyruvate to L-lactate direction (see Scheme I), 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 residue (the His195 residue).[2]



Scheme I

The whole catalytic process goes through several stages: substrate binding, unimolecular rearrangement consisting on a closure of a surface loop (99-110) down over the active site, pyruvate reduction, reverse protein conformational change and products release.[1] As shown by Dunn et al.[3] and by Waldman et al.,[4] closing of the 99-110 loop enforces isolation of the active site center from bulk solvent which is necessary for the reaction to proceed at a noticeable rate. In the wild-type enzyme this conformational rearrangement is in fact the rate limiting step.[3]

During the last decades, the advent of protein engineering has enabled us to define the role of specific residues on the active site by substituting them by other aminoacids presenting differences in charge or/and size.[5-10] Site-directed mutagenesis has been proved to be a good tool not only to change the rate limiting step of the full process, but also to demonstrate the feasibility of applying rational protein engineering to optimize and control the stereoselectivity of this particular enzyme when used in asymmetric synthesis.[10]

In this sense, one of the most relevant experiments on the ionised residues that are part of the vacuole formed by the 99-110 loop was that carried out by Holbrook and Ingram,[5] who prepared one of the first variants of LDH by site-directed mutagenesis, in which the histidine at position 195 was removed, thus demonstrating its role as the proton donor/acceptor in the reaction. The influence of Asp168 on the reaction was evaluated by substituting the residue to Asn and Ala.[6] This study supported the hypothesis that the Asp168 contributes to catalytic efficiency by raising the pK_a of His195 thus favouring the double protonation state of this histidine and by anchoring it to the most favourable orientation for enhancing its acid/base catalysis proton donating and accepting properties. It has been shown that LDHs cannot form the enzyme-cofactor-substrate ternary complex unless the imidazole group of His195 is protonated. The effect of replacing the negative charged Asp168 residue by neutral ones is to destabilize this ternary complex and, in contrast to the wild-type enzyme, the rate of catalysis becomes limited by the chemical step.[6] Another important mutation was the Arg171Lys substitution that affected the catalytic properties of the enzyme by weakening the substrate binding.[7] The Arg109Gln mutation demonstrated the role of this arginine in polarizing the pyruvate carbonyl group in the ground state and stabilizing the transition state.[8] This polarisation on the carbon-oxygen bond implies the development of a positive charge on the C2 thus facilitating the hydride transfer.

Holbrook and co-workers studied the importance of the electrostatic charge balance in the active site vacuole of NAD+-L-Lactate dehydrogenase by introducing an aspartic acid at position 140 instead of the wild-type asparagine.[9] They demonstrated that Asn140Asp mutant enzyme has the same k_{cat} as the native one at low pH, but at higher pH the Km of the pyruvate increases. Experimental results and molecular modelling showed that the presence of an anion on the position of the Asn140 raises the pK_a of His195 to greater than 10, thus facilitating the opening of the loop and the entry of solvent molecules to the vacuole.[9]

Recent mutations on other residues as Ala245Lys resulted in a qualitative change in the reaction mechanism by increasing the rigidity of the loop, thus provoking a relative acceleration of the rearrangement of the enzyme-NADH-pyruvate complex making the hydride transfer as the first slow step.[1]

The chemical reaction catalyzed by the LDH has been also extensively studied by several theoretical methods, either in gas phase [11, 12] and more recently by means of hybrid quantum mechanical / molecular mechanics methods (QM/MM).[13-17]

Nevertheless, although the sequential mechanism is more likely, different computational groups' proposals differ in the order in which the proton and hydride ions are transferred. Thus, some found a mechanism in which the hydride transfer preceded proton transfer in a stepwise manner,[12, 13] while other studies [14, 15, 18] predicted a concerted but asynchronous reaction in which the proton transfer was in a very advanced stage of the reaction. A very flexible QM/MM treatment [17] found a megadimensional energy hypersurface that allowed tracing across the two distinct mechanism pathways. The difference in the timing of the hydride transfer and the proton transfer is a matter of interest in mechanistic enzymology but, as it was pointed out in this latter study, unfortunately the results at this point did not allow to make a definitive statement as to which mechanism is preferred. Finally, we have recently demonstrated the dependence of the chemical reaction mechanism of LDH on the protonation state of the ionisable residues of the protein and on the level of theory employed to describe the quantum region on a QM/MM.[19] Corrections at MP2/6-31G(d,p) level of the semiempirical AM1 description QM region rendered a PES that describes an asynchronous concerted reaction pathway in which HT precedes PT with a remarkable reduction on the effective enthalpy barrier, much more in accordance with the expected value for this biological reaction.[19]

In this paper we carry out an exhaustive analysis of the LDH catalyzed reaction by obtaining different potential energy surfaces of the native LDH as well as the Asp168Ala mutant. Our results are compared with experimental studies already carried out in this system in order to test our methodology and to explain the predicted role of the ionized residues presented in the active site of the enzyme. Finally, we suggest new mutations that could be experimentally carried out which would definitely corroborate the proposed mechanism and the particular role of key residues on the enzyme active site.

COMPUTATIONAL METHODS

The QM/MM energy hypersurfaces were obtained using the CHARMM29b1 program.[20] 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.[21] Once the oxamate was replaced by pyruvate,

CHARMM29b1 was also used to add hydrogens to all titratable residues at a state complementary to pH 7. Due to the fact that standard pK_a values of ionisable groups can be shifted by local protein environments,[19] an accurate assignment of the protonation states of all these residues was carried out by recalculating the standard pK_a values of the aminoacids using the "cluster method" [22, 23] as implemented by Field and co-workers.[24] 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,[19] a non-accurate protonation state of only a few residues of the protein can render erroneous PESs and thus erroneous reaction mechanisms or/and activation barriers. After having properly added the hydrogen atoms, the system was placed in a cavity deleted from a pre-formed 24 Å radius spheres of TIP3P [25] 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 3 times by randomly rotating the water spheres to solvate potential cavities of a 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.[18, 26] Once the entire system was built, those atoms 24 Å away from the centre of carbonyl C and O atoms of the pyruvate were kept frozen during the subsequent dynamics and optimizations. The resulting model was a pseudo-sphere of 22141 atoms of enzyme, co-factor, substrate and solvent, 15154 of them kept frozen. The entire chemical system was divided into a QM region, treated by the AM1 semiempirical MO method,[27] and a MM region comprising the rest of the protein (CHARMM24 potentials) and the water molecules. Generalized hybrid orbital (GHO)[28, 29] method 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, the pyruvate, Arginines 109 and 171 and the His195. 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.

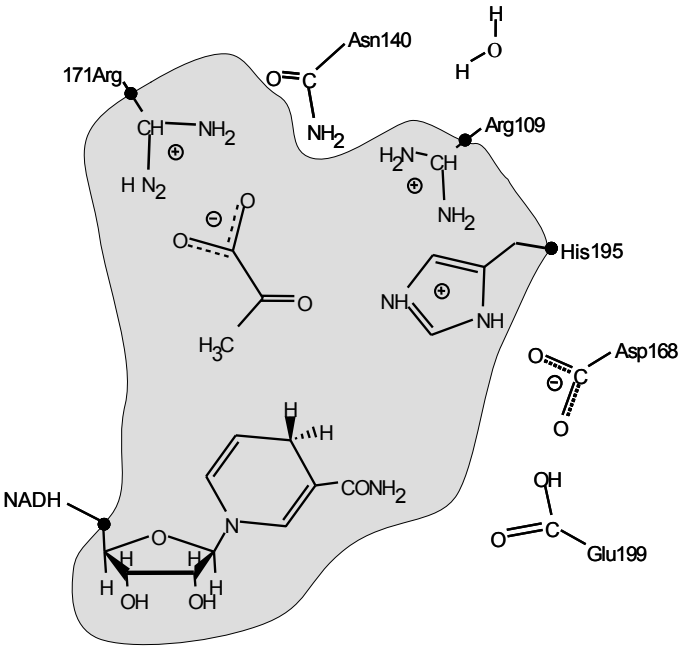


Figure 1. Schematic covalent structure for cofactor (NADH) substrate (pyruvate) and some key residues of the active site. GHO are indicated as " • ", dividing the quantum region (shaded area) and the classical region

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. Once the full system was relaxed, the same conditions were applied to all mutated and native LDH systems, slightly varying the number of MM atoms from one model to other, depending on the aminoacid substitution.

The PESs were obtained using two antisymmetric combinations of the distances between the hydrogen atoms with their donor (r_{DH}) and acceptor (r_{AH}) atoms. The reaction coordinate R_1 for the hydride transfer is defined as the difference in the distances of the bonds between the transferring hydride-ion and the donor (C_{nic}) and the acceptor atoms (C_{pyr}, see Equation 1). For the proton transfer a reaction coordinate R_2 is defined as the difference in the distance of the bonds between the transferring hydrogen atom and the donor (N_{his}) and the acceptor (O_{pyr}) atoms (Equation 2)

$$R_1 = r_{C_{nic}H_1} - r_{C_{pyr}H_1}$$ Equation 1

$$R_2 = r_{N_{his}H_2} - r_{O_{pyr}H_2}$$ Equation 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 2-D spline function of R_1 and R_2 coordinates.[19]

The CHARMM29b1 program was employed to carry out the grid scanning. Once the PESs were obtained, localised approximate transition state (TS) structures were refined using the recently modified version of the GRACE software.[16] A partial-rational-function-operator/adopted-basis-Newton-Raphson method was employed, utilising a Hessian matrix of order 156 x 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 52 atoms in the subset was less than 0.1 kcal/mol Å-1 in the optimised structure, while on the remaining atoms (ca. 3000) it was less than 0.005 kcal/mol $Å^{-1}$. Finally, the intrinsic reaction coordinate (IRC)[30] path was traced from the TSs in order to demonstrate conclusively that the reported structure was indeed the TS for the correct chemical step.

RESULTS AND DISCUSSION

Wild-type

The PES obtained for the interconversion of pyruvate into lactate in the active site of the wild-type LDH enzyme is depicted on Figure 2, the energies for minima and saddle points and selected QM/MM optimized interatomic distances of the different stationary structures located on the PES are listed in Table 1.

Table 1: Selected geometrical parameters of the different stationary point structures on the QM/MM PES of the native type LDH and the mutant and potential energy barriers (in kcal·mol^{-1}).

	Native LDH			Asp168Ala		
	Pyruvate	TS	Lactate	Pyruvate	TS	Lactate
$C_{nic}\cdots H_1$	1.13	1.43	2.35	1.13	1.37	2.42
$H_1\cdots C_{pyr}$	2.33	1.31	1.13	2.45	1.39	1.13
$N_{His195}\cdots H_2$	1.05	1.09	1.93	1.05	1.13	2.20
$H_2\cdots O_{pyr}$	1.78	1.57	0.99	1.78	1.49	0.97
O_{pyr}-HHArg109	2.13	2.08	2.15	2.13	2.06	2.10
O_{pyr}-HDAsn140	2.11	2.01	3.00	2.85	2.56	3.13
O_{pyr}-NE2His195	2.72	2.61	2.77	2.75	2.58	3.00
$\Delta E^{\ddagger}$	21			24		

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 relative total energies are provided in Table 1, resulting in a potential energy barrier of 21 kcal mol^{-1} for the reaction.

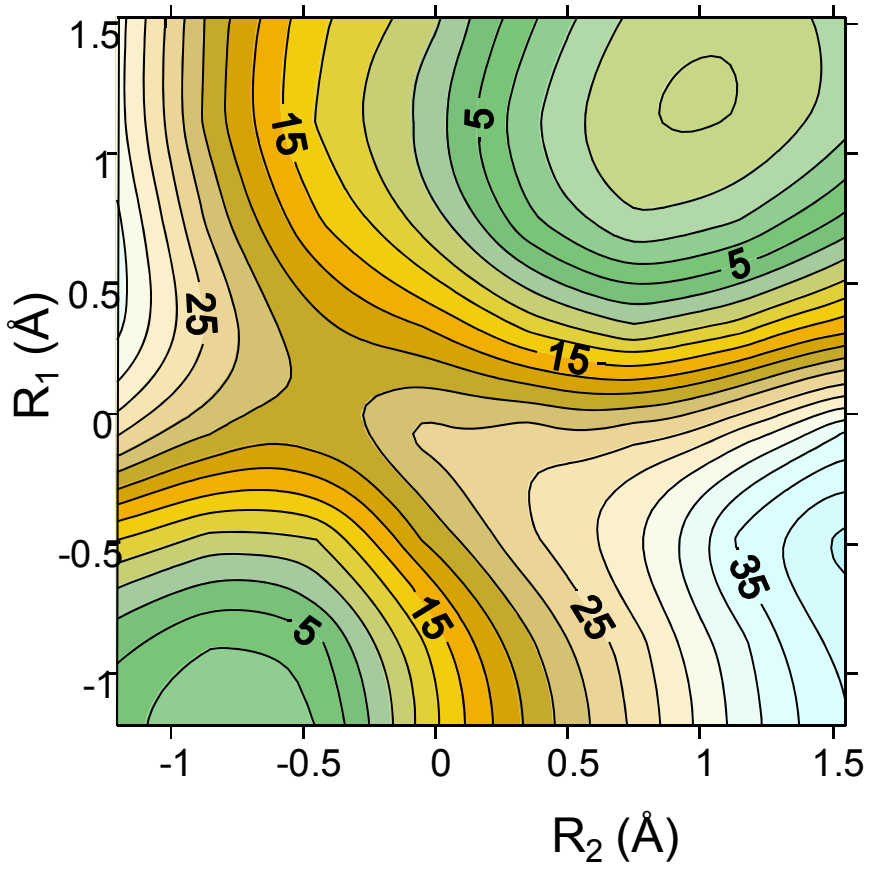


Figure 2. QM/MM contours (kcal·mol^{-1}) for pyruvate to lactate in the wild-type LDH-catalyzed reaction.

From the analysis of the substrate-enzyme interactions listed on Table 2, 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.[8] 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)[21] 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 we demonstrated in our previous paper,[19] this local environment has important consequences in the acidity of the ionisable 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 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 showed, favours the protonation state of His195.

As mentioned above, a deeper insight in the role of the different aminoacids 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 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.

Table 3: pK_a values of the different ionisable aminoacids in aqueous solution and calculated for the native type LDH and the 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

Asp168Ala Mutant

Analysis of the role of the negatively charged Asp168 has been carried out by replacing this residue by the smaller, neutral alanine. It has been proposed that LDHs cannot form an enzyme-cofactor-substrate complex unless the imidazole group of His195 was 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 3 shows the pK_a in the mutant enzyme. The expected severe drop in the pK_a value of His198 upon deletion of the negative charge of the neighbor residue

number 168 is not observed, in agreement with experimental observations.[6] In the mutant the pK_a values of His195 and Glu199 are decreased to 7.3 and 4.2, respectively. This means that a pH 7 the first one is still protonated while the glutamate becomes unprotonated. When modeling 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 hisitidine pK_a towards lower values in the mutant. The lower value of the His195 pK_a means that a positively charged imidazolium is slightly less favorable 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 ionised 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 now quite different to the wild-type TS. By comparison with the wild-type PES, the net energetic effect of this mutation is to facilitate the hydride transfer process from NADH to the substrate while the proton transfer from His195 to pyruvate is slightly destabilized. Then, the position of the TS on the PES appears at more advanced values of the proton transfer coordinate (R_1) and a less advanced values of the hydride transfer coordinate (R_1).

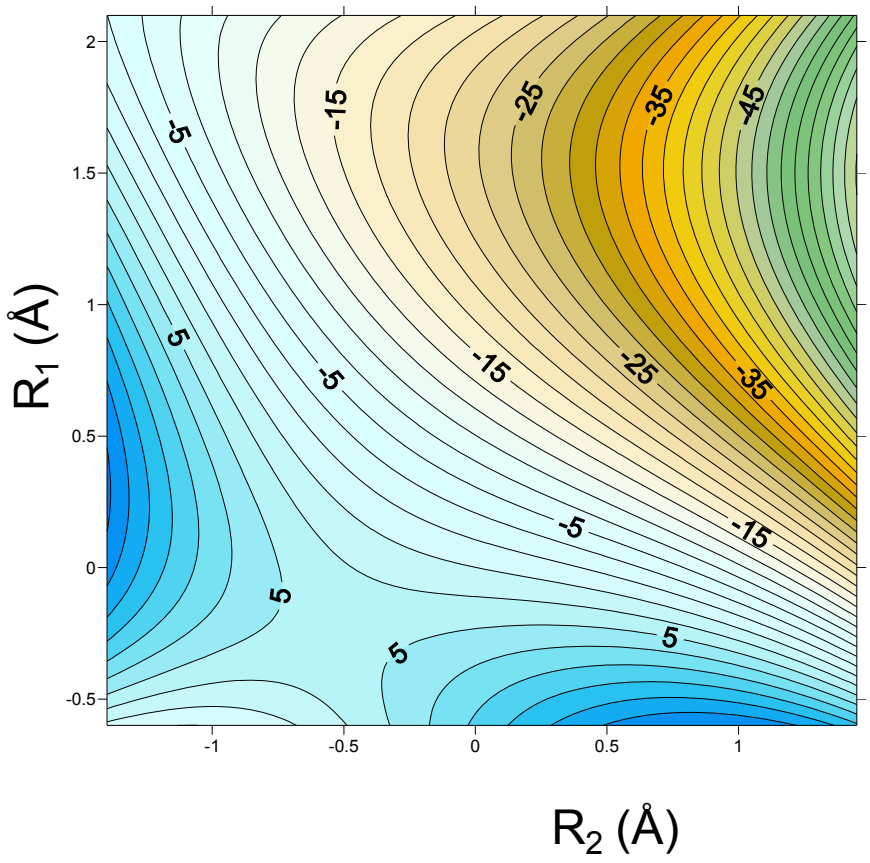


Figure 2. QM/MM contours (kcal·mol^{-1}) for pyruvate to lactate transformation in the Asp168Ala mutated LDH-catalyzed reaction.

Table 1 provides some key distances of the stationary structures obtained using as starting points for the search selected structures of the PES. According to Eqs 1 and 2 the hydride transfer coordinate in the wild-type TS is 0.12 Å and -0.02 Å in the mutant. trend is just the opposite in the proton transfer coordinate -0.48 Å in the wild-type enzyme and -0.36 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 to 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.[6] The potential energy barrier increase (3 kcal·mol^{-1}) is also consistent with the 45 fold decrease in the k_{cat} at 25ºC,[6] which can be translated to an increase of about 2.3 kcal·mol^{-1} in the activation free energy. Obviously both quantities cannot be compared directly but the fact that they are in the same order of magnitude gives as confidence in our results.

The changes observed both in 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 to the carbon donor atom of NADH in the reactant state is 7.9 Å for Asp168 (wild-type enzyme) and 9.4 for Glu199 (mutant enzyme). This displacement of a negative charge in the active site clearly assists the hydride transfer from NADH to pyruvate. The net effect on the proton transfer is somewhat trickier. As shown by the pK_a calculations, the displacement of the negative charge from Asp168 to Glu199, that 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 proton transfer to the substrate is hindered upon mutation. The reason is found in the relative position of Glu199 with respect to the direction of the proton transfer. The vector traced from the proton donor atom to the proton acceptor and to the carboxylate group of Glu199 form an angle close to 180º (see Figure 4) and then the electric field created by the negative charge of this reside hinders the displacement of a positive charge from the donor to the acceptor. While Asp168 is closer to His195 than Glu199, the relative positioning with respect to the substrate is quite different and in this case the angle formed by the respective position vector is far from coliniarity (see Figure 4).

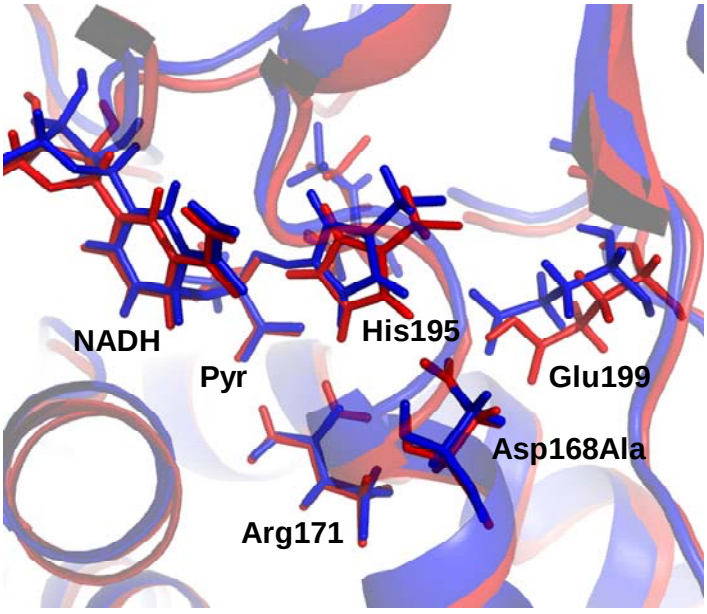


Figure 4. Superposition of the TS structures corresponding to the wild-type LDH (red) and Asp168Ala mutant (blue).

CONCLUSIONS

The reduction of pyruvate to lactate catalyzed by the LDH has been studied by means of hybrid QM/MM simulations. A very flexible molecular model consisting on the full tetramer of the enzyme, together with the cofactor NADH, the substrate and solvent water molecules has allowed us to mimic computationally the effects of site directed mutagenesis studies, most of which have previously been performed experimentally. The PESs obtained for a single mutation, compared with 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, some of the substitutions have dramatic effects on pKa values of ionisable residues, with consequent implications for catalysis.

In the particular case of LDH we have shown that the chemical step can be described as a concerted but asynchronous hydride and proton transfer. The electrostatic characteriscts of the enzymatic environment affects the degree of proton and hydride transfer attained in the transition state. When Asp168 is mutated to Alanine the pK_a of the protonated His165 is slightly decreased. This can modify the ratio of enzyme-substrate complex found in the adequate protonation state for catalysis. In addition, when a negative charge on residue 168 vanishes the pK_a of Glu199 shifs, becoming unprotonated at physiological pH. The net effect on the catalytic rate constant is an increase of the potential energy barrier of the chemical process because while the hydride transfer is assited 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. We beleive that this example illustrates the possibilities of chemical modelling in the prediction of catalytic properties of newly designed proteins.

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