Sampling the Conformational Energy Landscape of a Hyperthermophilic Protein by Engineering Key Substitutions

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Abstract

Proteins exist as a dynamic ensemble of interconverting substates, which defines their conformational energy landscapes. Recent work has indicated that mutations that shift the balance between conformational substates (CSs) are one of the main mechanisms by which proteins evolve new functions. In the present study, we probe this assertion by examining phenotypic protein adaptation to extreme conditions, using the allosteric tetrameric lactate dehydrogenase (LDH) from the hyperthermophilic bacterium Thermus thermophilus (Tt) as a model enzyme. In the presence of fructose 1, 6 bisphosphate (FBP), allosteric LDHs catalyze the conversion of pyruvate to lactate with concomitant oxidation of nicotinamide adenine dinucleotide, reduced form (NADH). The catalysis involves a structural transition between a low-affinity inactive “T-state” and a high-affinity active “R-state” with bound FBP. During this structural transition, two important residues undergo changes in their side chain conformations. These are R171 and H188, which are involved in substrate and FBP binding, respectively. We designed two mutants of Tt-LDH with one (“1-Mut”) and five (“5-Mut”) mutations distant from the active site and characterized their catalytic, dynamical, and structural properties. In 1-Mut Tt-LDH, without FBP, the $K_m$ for pyruvate is reduced compared with that of the wild type, which is consistent with a complete shifting of the CS equilibrium of H188 to that observed in the R-state. By contrast, the CS populations of R171, $k_{cat}$, and protein stability are little changed. In 5-Mut Tt-LDH, without FBP, $K_m$ for pyruvate approaches the values it has with FBP and becomes almost temperature independent, $k_{cat}$ increases substantially, and the CS populations of R171 shift toward those of the R-state. These changes are accompanied by a decrease in protein stability at higher temperature, which is consistent with an increased flexibility at lower temperature. Together, these results show that the thermal properties of an enzyme can be strongly modified by only a few or even a single mutation, which serve to alter the equilibrium and, hence, the relative populations of functionally important native-state CSs, without changing the nature of the CSs themselves. They also provide insights into the types of mutational pathways by which protein adaptation to temperature is achieved.

Key words: protein adaptation, protein conformational energy landscape, lactate dehydrogenase, hyperthermophilic.

Introduction

Many proteins achieve their function via a shuffling between a large number of conformational substates (CSs) on their potential energy landscapes; therefore, their activities depend as much on their dynamics as on the structures encoded in their sequences (Frauenfelder et al. 2009; Ramanathan et al. 2011). It has been proposed that the evolutionary trajectory of a protein is governed by the effect of mutations on interconversions between these CSs (Fields and Somero 1998; Schrank et al. 2009). Yet, how neutral, deleterious, and advantageous mutations influence the transitions between CSs is the subject of intense debate (Bloom and Arnold 2009), and, in particular, whether adaptive mutations follow or precede neutral ones (Jackson et al. 2009). The Neo-Darwinian hypothesis argues that adaptive mutations are fixed first in the sequence and then other mutations (neutrals or global suppressors) accumulate, whereas the concurrent view posits that it is the accumulation of neutral mutations that yields a protein with a strong potential for evolvability, after which adaptive substitutions occur under a given selective pressure (Bershtein et al. 2008; Patthy 2008; Sokine and Tawfik 2010).

By comparing sequences from orthologous proteins with different properties, it is usually impossible to distinguish adaptive mutations from neutral ones and analyze their respective influences on protein behavior. A combination of experimental approaches assessing the catalytic, dynamic, and structural properties of a protein is therefore...
termine the nature of this transition (Iwata and Ohta 1993; active “R-state.” Crystallographic studies have helped to de-
tween a low-affinity inactive “T-state” and a high-affinity phosphate (FBP) to induce the structural transition be-
are allosteric and require activation by fructose 1, 6 bis-
mal adaptation of a model enzyme, lactate dehydrogenase
lography, and molecular simulation to investigate the ther-
we describe a combined use of enzymology, X-ray crystal-
ture experimentally demonstrates that the T- and R-states
in equilibrium, in agreement with the concerted MWC model of allostery (Monod et al. 1965). Later, similar observa-
tions were reported for Lactobacillus casei LDH (Arai et al. 2010), thereby supporting our results.

Comparison of the Dr-LDH and Tt-LDH structures indi-
cated that, apart from the mobile cap, structural rearrange-
ments within the catalytic pocket were limited to a change
in the side chain conformation of the substrate-binding res-
due, R171. In Tt-LDH, R171 can adopt two different con-
formations, being either outside or inside the active site in
the APO and HOLO structures, respectively. We denote
these conformations as Tt-R171-CS-out and Tt-R171-CS-in
(see fig. 1). By contrast, in the APO structure of Dr-LDH,
R171 is located within the catalytic site, thereby mimicking
the conformation R171 adopts in the HOLO-state of
Tt-LDH (Tt-R171-CS-in; fig. 1). An analysis of the structural
effects of the amino acid substitutions between the Tt-
and Dr-LDH sequences led us to hypothesize that only a few of
them, located in key positions, are responsible for shifting
the CS equilibrium of R171 in Dr-LDH toward the function-
ally productive conformation (CS-in) that is observed in
the HOLO structure of Tt-LDH and, furthermore, that it
was these that may have been involved in the adaptation
of Dr-LDH to mesophilic conditions (Coquelle et al. 2007).

In the present work, we test this hypothesis by reproduc-
ing an adaptation of Tt-LDH from hyperthermophilic to
mesophilic conditions. To achieve this, we designed two
variants of Tt-LDH with one and five mutations, respec-
tively, and compared their structural, functional, and dy-
amical properties with those of the wild-type enzyme.
Our results demonstrate that only a small number of mu-
tations, remote from the catalytic R171, are sufficient to
shift this residue’s CS sampling from the inactive T-state
(CS-out) toward that of the active R-state (CS-in). In addition,
the mutations were shown to shift the CS sampling of
another important residue that is involved in the regula-
tion of the catalytic properties of LDH, H188.

Fig. 1. Close-up views of LDH from Thermus thermophilus (Tt) and Deinococcus radiodurans (Dr) in their APO and HOLO states. The ribbon colors are yellow for the APO state and gray for the HOLO state. CS-out and CS-in are the CSs observed for R171, whereas those for H188 are CS1 and CS2. Their positions in Wt Tt-LDH are highlighted by green and red circles for the APO and HOLO states, respectively. Comparison of the colored circles in the right panel indicates that the CSs of H188 and R171 in APO Dr-LDH correspond to intermediate conformations. The letters in parentheses indicate monomer numbering.
Escherichia coli

Materials and Methods

Strains and Plasmids

The complete 1-Mut and 5-Mut Tt-LDH genes were made using chemical DNA synthesis by GeneCust Europe (http://www.genecust.com). The coding sequences were cloned in the overexpression plasmid pET 11a and transformed in the Escherichia coli strain Bl21 DE3.

Protein Expression, Purification, and Biochemical Measurements

The expression and purification of Tt-LDH mutants were done according to the protocols developed for the wild-type enzyme (Coquelle et al. 2007). As previously reported, thermal stability measurements of each LDH were carried out on a Jobin Yvon CD6 CD spectropolarimeter using a quartz cuvette with a path length of 0.1 cm (Coquelle et al. 2007). Spectra were recorded after 30 min of incubation at various temperatures on protein samples diluted at a concentration of 0.1 mg/ml in a 50 mM Tris–HCl buffered at pH 7. The unfolding process was checked to be irreversible. The kinetic parameters were determined at various temperatures using a thermostated Beckman spectrophotometer with protocols described previously (Coquelle et al. 2007). Three replicates of ten pyruvate concentrations were used to determine K_m for each LDH. The pH measurement was made up at room temperature and used without further pH adjustment. This is without any strong consequences on enzyme activity because the limited shift toward lower pH at higher temperatures favors the protonated state of the catalytic histidine residue H195. We note that the use of phosphate buffer, which is commonly used to overcome the temperature dependence of pH, cannot be used because it mimics the allosteric activator FBP.

Crystallization, Data Collection, Model Building, and Refinement

5-Mut Tt-LDH was crystallized at 10 mg/ml by the hanging-drop vapor-diffusion method, following the mixing of the protein and the mother liquor solutions at a 1:1 ratio. The mother liquor solution was 0.1 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer pH 9, 18–20% polyethylene glycol 6000, 2 M LiCl, and 500 μM ADP. Crystals grew within a week at 4 °C.

Prior to data collection, the crystal was washed with a mother liquor solution containing 18% glycerol, then mounted in a nylon loop and flash cooled in a nitrogen gas stream at 100 K. Diffraction data (2.9 Å) were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble), on the ID14-EH1 beam line, using λ = 0.933 Å wavelength with an ADSC Quantum 4 detector. Data were processed and scaled with XDS and XSCALE (Kabsch 1993), respectively. The structure was then solved using the PHENIX suite of crystallographic tools (Adams et al. 2010). In summary, an initial solution model was determined by molecular replacement with PHASER (Read 2001) using as a starting model, the APO Tt-LDH structure (PDB entry 2V6M) from which all waters were removed. The model was then refined by iterative cycles of model building with COOT (Emsley and Cowtan 2004) and reciprocal-space refinement with PHENIX. Reciprocal-space refinement included restrained positional refinement as well as B-factors and TLS refinements. Noncrystallographic symmetry between the four monomers in the tetramer, as well as Ramachandran and secondary structure restraints, was used, so as to compensate for the relatively low resolution of the structure.

Two alternate conformations were modeled R171 side chain in each of the four monomers. The initial mF_o-DF_c difference electron density map featured strong negative peaks on R171 side chains in the four monomers (below —4.5σ) and positive peaks around them (above 3.5σ); likewise, the initial 2mF_o-DF_c electron density map featured extra density starting from their Cβ when displayed at 0.8σ. The initial mF_o-DF_c map was not straightforwardly interpretable given the shape and extent of the difference density peaks. As automated procedures for alternate-conformations assignment are ineffectual at 2.8 Å resolution, various models were manually generated, reciprocal space refined, and the resulting mF_o-DF_c difference maps compared. The model agreeing the best with the electron density maps featured three R171 conformers in monomers A and B and two in monomers C and D. However, owing to the limited resolution of the structure, we favored a model in which only two R171 conformers (50% occupancy each) are present in each monomer, and where the extra density corresponding to the possible third conformer is accounted for by waters. The final mF_o-DF_c difference map is featureless in this region when displayed at 3σ.

A similar, comparative-modeling procedure was used to attribute other residues alternate conformations, notably those of H188. The 5-Mut Tt-LDH structure has been deposited in the PDB under accession code 3ZZN.

The structure of 1-Mut Tt-LDH was solved and deposited in the PDB independently of this work (PDB accession code 2XXE). The PDB header indicates that the protein was crystallized by the hanging-drop vapor-diffusion method, following the mixing of the protein and the mother liquor solutions at a 1:1 ratio. The mother liquor solution was 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer pH 6, 2.4 M sodium formate. Crystals grew within a week at 20 °C. Inspection of the structure and electron density maps of 1-Mut Tt-LDH (recalculated from the structure factors amplitudes deposited in the PDB) indicate that an alternate conformation could have been modeled for R171, which would have merely correspond to the Tt-R171-int conformer.

Figures representing the structures were produced using PyMOL (Delano 2002).

Residue Numbering

LDH structures were described according to the nomenclature proposed for dehydrogenases (Eventoff et al. 1977). With this numbering, important active site residues are labeled R109, D168, R171, and H195. Mutations are indicated...
by a one-letter amino acid code following the residue number (e.g., E299A), whereas monomers are indicated by an uppercase letter in parentheses following the amino acid code (e.g., R171 (A)).

Molecular Dynamics Simulations
Starting structures for the APO Wt LDH were generated from a 2.2 Å resolution crystal structure of \( T. thermophilus \) LDH, PDB entry code 2V6M (Coquelle et al. 2007). For the various \( Tt \)-LDH mutants, we used the crystal structures of the 1-Mut (PDB accession code 2XXE) and 5-Mut (PDB accession code 3ZZN) \( Tt \)-LDHs reported in this study.

We included all residues in the complete tetrameric protein. Hydrogens were constructed with ideal stereochemistry. Protonation states of histidines were assigned by visual inspection. Additional pK\(_s\) were computed with the PROPKA program (Li et al. 2005) to verify the assignment of protonation states. Orientations of His, Asn, and Gln side chains in the active site were taken from the crystal structure and verified by visual inspection.

In addition to crystal waters, solvation was performed by overlaying the protein with a 116 Å cubic box of water and removing overlapping water molecules. The final model contained 1,240 protein residues and around 43,000 waters, including 513 crystal waters. Periodic boundary conditions were assumed, meaning that the entire 116 Å box was replicated periodically in all directions.

All long-range electrostatic interactions were computed with the particle mesh Ewald method. The appropriate number of potassium counterions was included in the wild-type and mutant LDH simulation systems to render them neutral. Molecular dynamics (MD) was performed at constant temperature and pressure control with Berendsen’s algorithm. The complexes were simulated for 25 ns each following 200 ps of thermalization.

The CHARMM27 force field (MacKerell et al. 1998) was used for the protein and a slightly modified TIP3P model for water (Jorgensen et al. 1983). All MD simulations were performed with the NAMD program (Phillips et al. 2005), version 2.7.

Quantum Chemical/Molecular Mechanical Simulations
Starting structures for the ternary complex between LDH, NADH, and pyruvate were generated from a 2.1 Å resolution crystal structure of \( T. thermophilus \) LDH with bound NADH and oxamate, PDB entry code 2V7P (Coquelle et al. 2007). Nitrogen of oxamate was converted into an sp\(^3\)-hybridized carbon to produce the pyruvate ligand. We considered protein residues within a 40 Å sphere centered on the pyruvate ligand of monomer A of the LDH tetramer. Hydrogens were constructed with ideal stereochemistry. Protonation states of histidines were assigned by visual inspection. Additional pK\(_s\) were computed with the PROPKA program (Li et al. 2005) to verify the assignment of protonation states. E199 was taken as neutral, since in a previous study, this residue was predicted to be protonated (Ferrer et al. 2005), and the PROPKA pK\(_s\) prediction for this residue was 9.3. To reduce the formal charge of the system, ten chloride counterions were included. The complex was equilibrated for 5 ns following 200 ps of thermalization.

For the quantum chemical (QC)/molecular mechanical (MM) calculations, the system was partitioned between QC and MM regions. The dihydronicotinamide group, ribose ring of NADH, pyruvate, and the side chain of H195 were included in the QC region, which had 50 atoms, and the MM region contained 9,226 atoms. The CHARMM27 force field was employed for the MM region, whereas the AM1 semiempirical method was used for the QC region. All geometry optimization and reaction path calculations were performed with this semiempirical QC/MM potential. These were followed by single-point calculations on the optimized structures with a density functional theory (DFT) QC/MM potential employing the B3LYP functional and a large 6-311G basis set with polarization and diffuse functions.

Finally, Poisson calculations on the optimized reaction path structures were also performed to estimate the contribution from the region outside the 40 Å sphere, which was treated as a uniform dielectric continuum, with a dielectric constant of 80. The CHARMM program (Brooks et al. 2009) was employed, and the Poisson equation was solved using a finite-difference two-step grid-focusing procedure, with spacings between grid points on the coarse and fine grids of 0.8 and 0.4 Å, respectively. Charges for the QC atoms were obtained by a standard DFT electrostatic potential fitting procedure, whereas those for the MM atoms were taken from the CHARMM force field.

The QC/MM calculations were performed with the pDynamo software (Field 2007, 2008), along with its interface to the ORCA program (Neese 2008), which was employed for the DFT calculations. No truncation or cut-off was employed to calculate nonbonded interactions. Reaction paths were optimized with the nudged elastic band (NEB) method (Creuuet and Field 2003; Creuuet et al. 2005; Galvan and Field 2008) implemented in pDynamo. The utility of NEB is that it does not require a predefined set of reaction coordinate variables and makes no assumptions as to how the reaction proceeds. The only bias in the calculation comes from the structures employed in the starting guess for the NEB pathway.

Results
Mutation Strategy
Our working hypothesis was that there exist “hot spots” in a protein’s structure, within which mutations can induce drastic effects on the protein’s dynamics and, therefore, on the sampling of its conformational energy landscape. In previous work, we analyzed the differences in thermal properties of various LDHs, including Dr-LDH and \( Tt \)-LDH (Coquelle et al. 2007). We found that all conservative and most nonconservative substitutions had little influence on Dr and \( Tt \)-LDH properties due to the formation of compensating stabilizing and destabilizing interactions. By contrast, a series of 21 nonconservative mutations were identified, which we hypothesized were more likely to play a role in
thermal adaptation. These are localized in five distinct areas, each of which features ionic networks involved in the stabilization of the hyperthermophilic enzyme (see N1–N5 in supplementary fig. S1, Supplementary Material online). In these areas, the local structural organization of the APO-state of Dr-LDH resembles that of the Tt-LDH HOLO-state, implying that the substitutions have shifted the equilibrium between these two states in the mesophilic LDH compared with its hyperthermophilic counterpart (Coquelle et al. 2007).

As a first step, we produced a penta-mutant of Tt-LDH (referred to as “5-Mut” throughout the manuscript) featuring a mutation in each of the five ionic networks, N1–N5. The mutations in N1–N4 are intrachain and therefore do not directly affect intersubunit interactions. That in N5 is interchain and centered around the (A/B)·R218:(D/C)·E299 ionic interaction. It was introduced to reduce ionic contacts between adjacent domain-swapped dimers in the functional tetramer. Charged residues were mutated to alanine so as to disrupt ionic interactions as directly as possible, namely R151A, E279A, E313A, and E299A in N2, N3, N4, and N5, respectively. The R79W mutation, in N1, represents a special case that corresponds to the substitution that exists in Dr compared with Tt (Coquelle et al. 2007). This substitution implies both the loss of an ionic interaction and the creation of an unfavorable energetic situation in which a bulky hydrophobic residue is surrounded by two negatively charged residues (Thomas and Elcock 2006).

Because this situation cannot be mimicked by an alanine substitution, we decided to reproduce this local effect.

After a first round of analyses with 5-Mut Tt-LDH was performed (see following subsections), the N5 network was identified as the major player in the thermal adaptation of the protein. This led us to design a single-point mutant, featuring a mutation in the N5 network only (referred to as “1-Mut” throughout the manuscript). To further test our hot spots hypothesis, we perturbed the opposite end of the (A/B)·R218:(D/C)·E299 ionic interaction by mutating into an alanine R216, which is one of the residues adjacent to R218 (see figs. 1 and 3), instead of E299.

**Thermal Stability and Enzymatic Activity of Mutants**

The thermal stabilities of Tt-LDH mutants were assessed using circular dichroism (CD) spectroscopy. The spectra of each variant and the wild-type (Wt) Tt-LDH were strictly superimposable (fig. 2, top left), indicating that the secondary structure contents of the folded forms in solution were insensitive to mutation. The small inset shows the thermal transition of each protein, recorded by taking the residual molar ellipticity at 220 nm after incubation at various temperatures.

**Fig. 2.** Thermal dependencies of enzymatic parameters and stability of the 1-Mut (squares), 5-Mut (triangles), and Wt (black dots) Tt-LDHs. Top left: CD spectrum of the folded mutant and Wt Tt-LDHs taken at 50 °C. Small inset: thermal transition curves, with the small arrow indicating the point at which the 5-Mut starts to unfold. Top right: \( K_m \) values in the presence and absence of FBP. Bottom left: \( k_{cat} \) values without FBP. Bottom right: catalytic efficiency.
temperatures. The melting temperatures ($T_m^{1/2}$) of 1-Mut and 5-Mut Tt-LDHs are 5 and 20 °C lower than that of Wt Tt-LDH, respectively.

The enzymatic activities of Wt, 1-Mut, and 5-Mut Tt-LDH were measured in the presence and absence of the allosteric activator FBP. As mentioned earlier, the latter induces a noticeable increase in LDH affinity for pyruvate, a fact which has been proposed to stem from a structural transition between a low-affinity inactive T-state and a high-affinity active R-state (Iwata and Ohta 1993).

In the presence of FBP, the affinities of Tt-LDH variants for pyruvate ($K_m^{Pyr}$) are independent of temperature and more or less identical for the three enzymes (around 50 μM; fig. 2, top right). Thus, the introduced mutations have no noticeable effect on the catalytic properties of the enzyme in the presence of FBP, which implies that the R-states of all the enzyme variants are the same.

In the absence of FBP, however, the affinities of the two variants for pyruvate display different temperature dependencies (fig. 2, top right). Without FBP, the affinity of Wt Tt-LDH for pyruvate is ≈4-fold higher at 80 °C than at 30 °C (i.e., $K_m^{Pyr}$ is ≈4-fold lower), although still 2-fold lower than in the presence of FBP, at any temperature, we tested. This suggests that thermal energy can, to a certain extent, compensate for the absence of the allosteric activator. That $K_m^{Pyr}$ for Wt Tt-LDH at 30 °C in the absence of FBP is only ≈eight times higher than that at 80 °C in the presence of FBP is also in agreement with our previous proposal that the T- and R-states of Tt-LDH are in equilibrium, which we here show can be shifted toward the R-state with increasing temperature.

The behaviors of the 5-Mut Tt-LDH and Wt Tt-LDH are strikingly different in the absence of FBP. In the 30–70 °C range, the $K_m^{Pyr}$ values of 5-Mut Tt-LDH show only a slight dependence on temperature and are significantly less than those for Wt Tt-LDH. They are also only, at most, 2-fold higher than those determined in the presence of FBP. At temperatures higher than 70 °C, the $K_m^{Pyr}$ values of 5-Mut Tt-LDH in the absence of FBP increase sharply, which our CD spectroscopic data indicate is due to thermal denaturation. The fact that, in the absence of FBP, no increase in $K_m^{Pyr}$ value is observed at 80 °C suggests that FBP plays a role in the stabilization of the functional tetramer at higher temperatures. The stabilizing effect of FBP has also been observed using the LDH from Bacillus stearothermophilus (Cameron et al. 1994). As for the 1-Mut Tt-LDH, it displays a behavior intermediate between the Wt and 5-Mut Tt-LDHs. Its $K_m^{Pyr}$ values in the absence of FBP are lower than those of Wt Tt-LDH and display a less pronounced dependence with temperature.

The temperature dependencies of $k_{cat}$ for the three Tt-LDH variants in the absence of FBP are reported in figure 2, bottom left. Over the temperature range within which the 5-Mut Tt-LDH is stable, its $k_{cat}$ values are more or less twice those of Wt Tt-LDH, whereas the 1-Mut Tt-LDH again displays an intermediate behavior to the other two forms, with $k_{cat}$ values 15–20% higher than those of Wt Tt-LDH. The $k_{cat}/K_m^{Pyr}$ ratio (fig. 2, bottom right), which measures an enzyme’s catalytic efficiency, shows that the single mutation has induced a slightly more efficient LDH, compared with the Wt form, mainly by increasing the affinity for pyruvate without strong effects on its conformational stability.

In the case of the 5-Mut Tt-LDH, there is a strong increase in catalytic efficiency at lower temperatures compared with the Wt. However, this increased activity is accompanied by a loss of thermal stability. Together, these results suggest that the mutations introduced in the Tt-LDH mutants have induced a shift in the CS equilibrium toward the R-state, such that the latter dominates even at lower temperatures and/or in the absence of FBP.

### Table 1. Data Collection and Refinement Statistics.

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<tr>
<th>S-Mut Thermus thermophilus LDH</th>
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<tr>
<td>PDB accession code</td>
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<td>Beamline</td>
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<td>Temperature (K) of data collection</td>
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* Values in parentheses are for the highest resolution shells.

b $R_{merge} = \sum_{hkl} |\sum_{i} (I_{obs} - I_{calc})| / \sum_{hkl} \sum_{i} I_{calc}$

Structural Comparison between Wt, 1-Mut, and 5-Mut Tt-LDH

We solved the crystals structure of 5-Mut (PDB accession code: 3Z2N) Tt-LDH at 2.9 Å resolution. Information and details concerning data collection and refinement statistics are given in table 1. The structure of 1-Mut Tt-LDH was solved and deposited in the PDB independently of this work (PDB accession code 2XGE). To facilitate the discussion, we highlight below the structural differences and similarities between the 1-Mut and 5-Mut structures and those of the APO and HOLO forms of Wt Tt-LDH determined previously (Coquelle et al. 2007).

Although the two mutants of Tt-LDH crystallized in different space groups, their overall structures are close (root mean squared deviation [RMSD] between 1-Mut and 5-Mut Tt-LDH is 0.921 Å) and very similar to that of Wt Tt-LDH (PDB accession code: 2V6M; RMSDs between 1-Mut and Wt Tt-LDH and between 5-Mut and Wt Tt-LDH are 0.404 and 0.807 Å, respectively). It is notable that the
conformations of the strictly conserved catalytic residues D168 and H195 are almost identical in the three Tt-LDH variants, indicating that the effects of the introduced mutations on the APO enzyme structures are quite subtle.

In 5-Mut Tt-LDH, the mutations introduced in N1, N2, N3, and N4 (R79W, R151A, E279A, and E313A, respectively) are accompanied by local rearrangements in the side chain conformations of the residues formerly involved in ionic pairs in Wt Tt-LDH. Overall, the total number of ionic contacts decreases, which accounts for the mutant’s reduced thermostability. The cases for N3–N5 are illustrated in supplementary figure S2 (Supplementary Material online). In N3 and N4 of the mutant, the distances and angles of residues R312 and of residues R316 indicate H-bonds between their side chain nitrogen and the main chain carbonyl oxygen of A279, instead of the ion pairs with E279 and E313 observed with the wild type. As already mentioned, the mutation in N5, E299A, is the only one that affects intermolecular interactions. As anticipated, it causes the disruption of a double ionic interaction with R181 and R216, although there are no other noticeable effects on the local structure when compared with APO Wt Tt-LDH. Interestingly, the same changes are observed in the HOLO Wt Tt-LDH, leading to the suggestion that they are implicated in the allosteric T- to R-state transition (Coquelle et al. 2007).

R171 is the substrate-binding residue whose side chain points outside (Tt-R171-CS-out) and inside (Tt-R171-CS-in) the catalytic site in the APO and HOLO WT Tt-LDH structures, respectively. It is remote from all mutations sites. In the 5-Mut Tt-LDH, the side chain of R171 displays two conformations: Tt-R171-CS-out and Tt-R171-CS-int, which is an intermediate conformation between Tt-R171-CS-in and Tt-R171-CS-out (see fig. 3). In the refined model of 5-Mut Tt-LDH, the occupancies of Tt-R171-CS-out and Tt-R171-CS-int were set to 50% in each monomer.

We also found significant variability in the side chain conformations of three other important residues, R56, H68, and H188, none of which are in direct contact with the mutation sites. The side chain of R56 helps to stabilize a small loop that is part of the NADH-binding site. In monomer D, it adopts the conformation reported for the HOLO Wt Tt-LDH structure, whereas in the other three monomers, it displays two alternative conformations. The first is similar to that in the APO Wt Tt-LDH structure, whereas the second corresponds to one intermediate between the conformations observed in the APO and HOLO Wt Tt-LDH structures.

H68 and R171 are neighboring residues with coordinated side chain conformations. When H68 is in the conformation observed in the APO structure, it prevents the side chain of R171 from accessing the active site and adopting the HOLO Wt Tt-LDH conformation that binds the substrate analog, oxamate. In all monomers of the 5-Mut Tt-LDH, H68 adopts a conformation that is in between those observed in the APO and HOLO Wt structures (see fig. 3).

H188 is a residue whose side chain was shown to undergo a 180° rotation upon FBP binding and therefore suggested to play a role in the allosteric activation of LDHs (Cameron et al. 1994; Iwata et al. 1994). In all monomers, its position is best modeled as an equally populated mixture of the two conformers observed in the APO and HOLO Wt structures (see fig. 3).

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The R218A mutation introduced in 1-Mut Tt-LDH perturbs the N5 network from the opposite end of the R216:E299 ionic interaction to that of 5-Mut Tt-LDH and results in the total suppression of ionic interactions.
between the two domain-swapped dimers that form the functional tetramer (fig. 4, small panel). The local structure of the N5 network region is not drastically affected, but what effects there are propagate to the middle of the kinked alpha-helix $\alpha1G/\alpha2G$ (residues 224–233) that, interestingly, undergoes a similar tilting to the one observed in the HOLO Wt $\sigma$Tt-LDH structure (fig. 4, large panel). At the hinge region between helix $\alpha1G$ and $\alpha2G$, the CSs of residues R233 and Y236 are drastically shifted from their positions observable in the APO Wt $\sigma$Tt-LDH (data not shown). The side chain of H188 adopts, in all monomers, the conformation found in the HOLO Wt $\sigma$Tt-LDH structure (fig. 3) and, it is again worth noting, that this is the conformation attained upon binding of the allosteric activator, FBP. The side chain of R56 exhibits the conformation it has in the APO Wt $\sigma$Tt-LDH structure in monomers A and C and that observed in the HOLO Wt $\sigma$Tt-LDH structure in monomers B and D (not shown). Likewise, the side chain of H68 displays the same conformation as in the HOLO Wt $\sigma$Tt-LDH structure in monomers B and D, whereas monomers A and C feature the conformation exhibited in the APO Wt $\sigma$Tt-LDH. As for R171, its conformational sampling is less diverse in 1-Mut compared with 5-Mut $\sigma$Tt-LDH, with $\sigma$Tt-R171-CS-out being observed in monomers A, B, and D (fig. 3) and a conformation close to $\sigma$Tt-R171-CS-int in monomer C. We note, however, that careful inspection of the residual mFo-DFc electron density map, calculated using the structure and structure factors amplitudes deposited in the PDB, revealed positive electron density peaks in the vicinity of the R171 side chain, suggesting that a low occupancy conformer could be present, possibly corresponding to $\sigma$Tt-R171-CS-int.

The above crystallographic analysis of the APO $\sigma$Tt-LDH variants demonstrates that mutations can have a major impact on the CS sampling of distant residues, in agreement with the conclusions from our biochemical experiments.

**Fig. 4.** Top panels: pairwise ribbon drawing superposition between Wt (green), 5-Mut (olive green), and 1-Mut (lime green) $\sigma$Tt-LDHs. The red arrows in the top right panel show the small change in the conformation of the $\alpha1G/\alpha2G$ helix induced by the 1-Mut $\sigma$Tt-LDH mutation. The black arrows in the top panels indicate the directions, A and B, of the close-up views that follow. Central panels: close-ups of the N5 network between monomers A (green) and D (yellow). Bottom panels: slices through the APO $\sigma$Tt-LDH active site. The surface and ribbon drawings of monomers A and B are colored in green and blue, respectively, and R171 is colored in red. The bottom panels indicate that the catalytic site is most accessible to the lateral chain of R171 in APO 1-Mut $\sigma$Tt-LDH.
The residues affected include the substrate-binding R171 and H188, which are involved in FBP binding and which adopt a conformation akin to that of the HOLO structure of Tt-LDH. On the other hand, the location of the catalytic H195, which is important for hydride transfer, was not impacted by the mutations of either Tt-LDH variant (see supplementary fig. S3, Supplementary Material online).

Molecular Simulations

To study the dynamics of the APO Wt and mutant Tt-LDHs, we performed MD simulations of 25-ns duration for each structure. In all cases, the simulation system included a complete protein solvated in a large box of water. The simulations were performed at temperatures of 50 and 70 °C. Figure 5 presents the root mean square fluctuations (RMSFs) computed for the proteins’ Cα atoms from the last 20 ns of the equilibrated MD simulations and averaged over the four monomers of the tetrameric protein. Overall, the RMSFs agree well with the experimentally measured values (derived from the crystallographic temperature factors) with correlations between the two of over 70% for all structures.

We find that the biggest RMSFs for all structures and all temperatures are observed for residues 100–112. This region contains the mobile surface loop, which is held open by electrostatic repulsion in APO LDH (Wilks et al. 1988) and caps the active site upon substrate binding. This loop was not completely resolved in the experimental structure of APO Wt Tt-LDH nor in that of 1-Mut Tt-LDH. In 5-Mut Tt-LDH, however, its structure was fully determined.

At 50 °C, the 5-Mut Tt-LDH has significantly higher RMSFs than the APO Wt in most parts of the sequence, except for the mobile loop region. Conversely, the 1-Mut Tt-LDH has very similar RMSF values to the APO Wt, apart from the mobile loop, whose fluctuations are smaller, and a region that includes the helix α1G/α2G, whose fluctuations are larger. The latter region is important as it delineates, together with helices α2F and βH (supplementary fig. S4, Supplementary Material online), the tunnel through which the side chain of R171 slides during the structural rearrangement between the APO solvent–exposed conformation (Tt-R171-CS-out) and that observed in the ternary complex, in which R171 is positioned inside the binding cavity and interacts with the pyruvate substrate (Tt-R171-CS-in). During the MD simulations, we did not observe a change in conformation of R171 between the two CSs, indicating a reasonably high-energy barrier to R171 rotation in the three Tt-LDHs. Nevertheless, the higher mobility of the residues in this region suggests that this transition is likely to be facilitated in the mutant structures because of the enhancement of the tunnel breathing motions that are needed to make the tunnel wide enough to accommodate the arginine side chain.

Interestingly, the RMSFs from the simulations at 70 °C are very similar for all structures, including in the region around the helix α1G/α2G. This correlates well with the experimental measurements since, at this temperature, the turn over and $K_{n}^{Pyr}$ values (fig. 2) of all three enzymes in the absence of FBP are more nearly equal.

To study the effect of the mutations on the catalytic reaction, we used a hybrid QC/MM method, full details of which are given in the Materials and Methods section. In short, for these calculations, the dihydronicotinamide group, ribose ring of NADH, pyruvate, and the side chain of H195 were treated quantum chemically, whereas the rest of the system was treated with an MM model. We employed the NEB algorithm to compute reaction paths.

The computed activation and reaction energies for the catalytic reaction are very similar in all Tt-LDH structures. The activation energies are 18.6, 18.4, and 18.1 kcal/mol and the reaction energies are −4.7, −5.6, and −4.8 kcal/mol for the Wt, 5-Mut, and 1-Mut LDHs, respectively. This confirms the idea that the mutations considered here have little direct effect on the chemical reaction and is consistent with the facts that all mutations are distant from the catalytic center and do not greatly perturb the protein structure.

The computed activation barrier of 18.6 kcal/mol for Wt Tt-LDH is similar to the activation energy calculated for another thermoresistant LDH (Ferrer et al. 2006). The reaction mechanism is also consistent with previous studies, with hydride transfer from the dihydronicotinamide ring of NADH to pyruvate preceding proton transfer from protonated H195 to pyruvate (Ranganathan and Gready 1997; Ferrer et al. 2005). No intermediate was formed after hydride transfer, and the reaction proceeded directly to the product state.
Discussion

Does the thermal adaptation of a protein follow a stepwise or a direct adaptation pathway? This is an important question that has been addressed by several studies. Many biochemical experiments indicate that the trade-off between stability and activity dictates thermal adaptation of naturally occurring enzymes (Hochachka and Somero 2002; Siddiqui and Cavicchioli 2006). However, a number of directed-evolution studies (Giver et al. 1998; Gershenson et al. 2000) as well as investigations using fusion proteins (Mingardon et al. 2011) have shown that thermal stability and the shift in the temperature optima for activity are not necessarily interlinked. They also suggest that there is no inherent physical constraint to achieving highly stable and very active enzyme at low temperatures, although this is of less biological relevance (reviewed in Arnold et al. 2001). Other works have demonstrated that small local changes outside the active site can alter steady-state catalytic parameters without any major change in fold (Aghajari et al. 1998; Feller and Gerday 2003; Bae and Phillips 2006). A pioneering comparative analysis in this area was carried out using LDH as a model enzyme without any knowledge of the thermally adapted LDH structure (Fields and Somero 1998; Fields and Houseman 2004). It was hypothesized that adaptation toward low temperatures arose from a modification of the interconversion rate between the binding and nonbinding CSs of the native state and that the change in equilibrium between these substates was due to adaptive substitutions located in regions that control the structural reorganization associated with catalysis.

In a follow-up study in our laboratory, crystallographic analysis of thermally adapted LDHs from various sources allowed a direct observation of local substitution effects (Coquelle et al. 2007). The conformational diversity of the structures of Wt Tt-LDH without FBP—especially of the crucial residues R171 and H188—attested that the low-affinity T- (CS-out) and active R- (CS-in) states are in equilibrium, independent of allosteric effectors, as proposed by the MWC model of allostery (Monod et al. 1965). In addition, it was shown that most of these substitutions have pleiotropic effects that modify both activation enthalpy and entropy and promote, in the low temperature adapted LDH, small local structural changes that mimic those observed in the HOLO state of an LDH adapted to higher temperature. This structural information provided an indirect static observation of adaptive conformational change.

The observation of shifts between CSs of active site residues within a crystal structure is very rare (Henzler-Wildman et al. 2007; Fraser et al. 2009; Jackson et al. 2009; Carroll et al. 2011; Sanson et al. 2011). The present work demonstrates directly that a few mutations distal to the active site can modify the CS populations of residues that are important in catalysis. The magnitude of a mutation’s effect depends strongly on its location with respect to the hinge area of the enzyme, which links the secondary structure elements that display the largest deviations upon substrate binding (see supplementary fig. S4, Supplementary Material online). The single mutation in 1-Mut Tt-LDH has a strong impact on the CS sampling of residues that control catalytic activity, notably H188, whereas it only has a small influence on protein stability. This is in agreement with studies that show that important evolutionary changes can be selected very easily. However, this single mutation was insufficient by itself to increase pyruvate affinity by shifting the CS sampling of R171 toward that of the R-state. Instead, the four extra mutations in 5-Mut Tt-LDH were required, although they also led to a decrease in the conformational stability of the enzyme at high temperatures. An ideal mutant Tt-LDH, conforming to the framework of a trade-off between stability and activity, would display, compared with Wt Tt-LDH: 1) an increase of its turn over at temperatures below its maximal stability and 2) an equivalent (or higher) catalytic efficiency when it starts to unfold (70 °C), as seen for Dr-LDH (Coquelle et al. 2007). The 5-Mut Tt-LDH displays only the first property, which implies that other compensatory mutations are required to improve its enzymatic properties in highly destabilizing conditions, as predicted by a mechanistic model of protein evolution (Soskine and Tawfik 2010).

Recent work on the effect of mutations has shown that the initial mutation strongly drives the pathway of protein evolution (Salverda et al. 2011) and that the coevolution of groups of moving residues is important for facilitating protein conformational changes (Jeon et al. 2011). Our results provide hints of these processes, although a full understanding of the thermal adaptation of allosteric enzymes, such as LDH, requires an in-depth analysis of their evolutionary trajectories so as to determine the exact order of substitutions and their effects. We are currently pursuing this approach in our laboratory using ancestral sequence reconstruction.

A hypothesis stating that mutations that change the conformational energy landscape are the main mechanism by which protein evolves new functions has recently emerged (Tokuniki and Tawfik 2009). Crystallographic and biochemical work on the evolution of phosphotriesterases suggest that enhancements in catalytic efficiency are mainly due to a few mutations that reorganize the CSs of important active site residues in conformations that are suitable for chemical reaction (Jackson et al. 2009). This observation has been supported by two more recent studies of other enzymes using site-directed mutagenesis. In one, it was confirmed that small local modifications in the allosteric enzyme, adenylate kinase, can induce conformational change in a distant-binding site (Schrank et al. 2009). Likewise, a similar approach, in conjunction with MD simulations, showed that changes in the conformational fluctuations of an arginine within the catalytic sites of mesophilic and thermophilic acylphosphatases were responsible for thermal adaptation (Lam et al. 2011). Our work is in accord with these studies as the resampling of important residues within the catalytic site of the mutant LDHs confirms the key role that dynamical properties play in protein adaptation. It also shows that the relative populations of CSs can be modulated by very few or even a single mutation,
leading to marked changes in properties but without changing the CSSs themselves.

We conclude by noting that we could not have drawn our conclusions based solely on the crystallographic data. X-ray crystallography is the most widely used method for macromolecular structure determination, but it lacks the ability to provide direct dynamical information due to the inherently time-averaged nature of the crystallographic structures. In addition, an increased sampling of the conformational energy landscape leads to a marked loss of resolution, as illustrated here by the 0.6–0.8 Å decrease observed between the Wt and mutant Tt-LDH structures. As a result, it was necessary to complement the structural data with that coming from other techniques, although the structures that we obtained were essential as a starting point for the theoretical simulations and for the interpretation of the results of the kinetic experiments. Our study, therefore, illustrates the potency of a combined use of experimental and theoretical approaches for assessing, in a single study, the structural, functional, and dynamical aspects of protein evolution.

Supplementary Material

Supplementary figures S1–S4 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org).

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References


