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Redesign of the coenzyme specificity in L-Lactate dehydrogenase from *Bacillus stearothermophilus* using site-directed mutagenesis and media engineering

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L-Lactate dehydrogenase (LDH) from *Bacillus stearothermophilus* is a redox enzyme which has a strong preference for NADH over NADPH as coenzyme. To exclude NADPH from the coenzyme-binding pocket, LDH contains a conserved aspartate residue at position 52. However, this residue is probably not solely responsible for the NADH specificity. In this report we examine the possibilities of altering the coenzyme specificity of LDH by introducing a range of different point mutations in the coenzyme-binding domain. Furthermore, after choosing the mutant with the highest selectivity for NADPH, we also investigated the possibility of further altering the coenzyme specificity by adding an organic solvent to the reaction mixture. The LDH mutant, I51K:D52S, exhibited a 56-fold increased specificity to NADPH over the wild-type LDH in a reaction mixture containing 15% methanol. Furthermore, the NADPH turnover number of this mutant was increased almost fourfold as compared with wild-type LDH. To explain the altered coenzyme specificity exhibited by the D52SI51K double mutant, molecular dynamics simulations were performed.

Keywords: coenzyme specificity/lactate dehydrogenase/methanol/NADPH/site-directed mutagenesis

Introduction

L-Lactate dehydrogenase (LDH) from *Bacillus stearothermophilus* (EC 1.1.1.27) is an NAD(H)-dependent enzyme which catalyses the inter-conversion of lactate and pyruvate in the last step of the anaerobic glycolytic pathway (Piontek *et al*., 1990). L-Lactate dehydrogenase is ubiquitous, and structurally related enzymes are found in both prokaryotic and eukaryotic organisms (Barstow *et al*., 1986). Like almost all other redox enzymes involved in the catabolic processes of the cell, LDH uses the coenzyme NAD(H) and is unable to accept NADP(H) (Feeney *et al*., 1990).

The coenzyme-binding domains in the dehydrogenase enzyme family have been thoroughly investigated, revealing a conserved β1-αA-β2 motif of the Rossman fold (Rossman *et al*., 1974; Lesk, 1995). The ability of the dehydrogenases to discriminate against NADP(H) lies in the amino acid sequence of this β1-αA-β2 motif. An aspartate residue at position 52 in the polypeptide chain sterically hinders the 2′-phosphate group of the adenine ribose ring to bind into the coenzyme pocket (Scrutton *et al*., 1990; Mittl *et al*., 1994). The negative charge of this aspartate group also repels the negative 2′-phosphate group (Feeney *et al*., 1990).

Feeney *et al*. (1990) attempted to change the coenzyme specificity of *Bacillus stearothermophilus* LDH from NADH to NADPH. The researchers replaced the aspartate 52 with a serine residue, rendering an enzyme that also accepts NADPH. However, the shift in catalytic specificity towards NADPH was due to more discrimination *against* NADH than selection *for* NADPH. Chen *et al*. (1994) also demonstrated this approach for alcohol dehydrogenase from *Drosophila*. The researchers increased the cofactor specificity for $NADP^+$ by exchanging an aspartate and an arginine residue in the coenzyme-binding pocket. The most elegant approach to alter the NAD to NADP specificity was achieved by Chen *et al*. (1996). In this study, the enzyme isopropylmalate dehydrogenase was switched from a 100-fold preference for NAD to a 1000-fold preference for NADP by replacing a β-turn with an α-helix and a loop, and substituting four additional amino acids. Other methods to change the coenzyme specificity described in the literature are bio-imprinting (Johansson *et al*., 1995) and using different organic solvents (Arnold, 1993).

In this report, we describe the introduction of point mutations in the coenzyme-binding domain of the thermostable LDH from *Bacillus stearothermophilus* in an attempt to alter the coenzyme specificity. This enzyme was chosen because its primary structure is known (Barstow *et al*., 1986) and the three-dimensional structure has been solved at high resolution using X-ray crystallography (Piontek *et al*., 1990). To facilitate purification, an affinity tail consisting of six histidine residues was introduced at the N-terminus (Carlsson *et al*., 1993). Different single- or double-point mutations were introduced in the coenzyme-binding domain, concentrating on the critical Asp52 and Ile51 residues. The different mutants constructed were D52S, D52G and I51K:D52S. In order to investigate the mechanisms underlying coenzyme binding and the possibility of changing the coenzyme specificity, we characterized the catalytic properties of these LDH mutations. L-Lactate dehydrogenase activity can be regulated by fructose-1,6-bisphosphate (FBP). Since it is well known that FBP substantially favours NADPH over NADH utilization, we focused our study of LDH without addition of the regulator (Feeney *et al*., 1990). Moreover, we also investigated the possibility to further influence the catalytic properties by adding the organic solvent methanol to the reaction mixture. We further studied the structural features of I51K:D52S, the mutant exhibiting the highest NADPH preference and turnover, using a molecular dynamics simulation approach.

Materials and methods

Bacterial strains, phages and plasmids

Escherichia coli strain TG 1 [(*lac-pro*), *supE*, *thi1*, *hsdD5*/*F′traD36*, *proA*$^+$*B*$^+$, *laci*, *lacZ*, *M15* (*ung*$^+$, *dut*$^+$)] was used in all cloning procedures and expression studies. Phage M13mp18 and plasmid pUC18 were obtained from Pharmacia LKB and Amersham, respectively. The plasmid pLDH41 (Barstow *et al*., 1986), containing the *Bacillus stearothermophilus lct* gene, was a generous gift from Prof. Tony Atkinson.

Chemicals and enzymes

The restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from BRL. NADH, NADPH and pyruvate used for enzyme characterization studies were of more than 98% purity (Boehringer Mannheim) (Metzger and Hollenberg, 1995). Ni-NTA agarose was purchased from Qiagen, Sweden. All other chemicals were of analytical grade and commercially available.

Nucleic acid manipulations and sequencing

Oligonucleotides used as PCR primers, mutagenesis primers and primers to construct a histidine linker were purchased from the Biomolecular unit (University of Lund, Sweden). The following oligonucleotides were used: His1, 5′-AAT TCT CAC CAT CAC CAT CAC CAT G-3′; His2, 5′-GA TCC ATG GTG ATG GTG ATG GTG AG-3′; LDH1, 5′-ATA ATA GGA TCC AAT GCA ATG CAA TGA AAA ACA ACG ACG GTG GAG CCC-3′; LDH2, 5′-AAG CTT CTG CAG GCC TCA TCG CGT AAA AGC ACG GGC TAG C-3′; NH3320, 5′-GCA ATC ATC GTA ATC GCC-3′; and to generate the mutants, D52S, 5′-GCT TTC ATT CGC ACT GAT GAG CAC G-3′; D52G, 5′-CTT TCA TTC GCA CCG ATG AGC ACG A-3′; and I51K:D52S, 5′-CTT GCT TTC ATT CGC AG TTT GAG CAC GAT CTC ATC-3′. To obtain a DNA linker encoding six histidine residues, the chemically synthesized oligonucleotides His1 and His2 were mixed in equal amounts, heated to 65°C for 10 min, and then slowly cooled to room temperature. Plasmids were prepared using the Wizard™ miniprep DNA purification system from Promega, and point mutations were introduced in the *lct* gene by utilizing the Sculptor™ *in vitro* mutagenesis system from Amersham and the oligonucleotides listed above. PCR was performed according to Saki *et al*. (1988) using PCR primers LDH1 and LDH2. All other cloning procedures and restriction enzyme digests were performed as described by Sambrook *et al*. (1990). The first 250 bases of final constructions and mutants were sequenced on an ABI 373 sequencer according to the supplier's manual using the 18 bp primer NH3320.

Cell culture growth and enzyme purification

The bacterial cultures harboring the desired plasmid were grown on a rotary shaker in Luria broth supplemented with ampicillin (100 μg/ml). Isopropyl-β-D-thiogalactopyranoside was added (0.2 mM) when the optical density at 550 nm reached 0.2. After growing overnight, the cells were harvested by centrifugation (5000 *g*, 10 min). The cells were resuspended in 50 mM sodium phosphate, 0.5 M sodium chloride buffer at pH 7.0. The cell suspension was sonicated 10 × 20 s on wet ice with a W380 Heat System Ultrasonic (output = 7). To obtain a clear lysate, the sonicated suspension was centrifuged (10 000 *g*, 30 min). The lysate was heated at 65°C for 20 min, then centrifuged (10 000 *g*, 20 min) to pellet denatured proteins, and the final protein extract was decanted.

The native and mutated enzymes were purified using the his_6 affinity tail and immobilized metal affinity chromatography (IMAC). Batches were prepared by pouring 300 μl Ni-NTA agarose gel into 1.5 ml microtubes. The gel was washed four times in a 50 mM sodium phosphate, 0.5 M NaCl pH 8.0 buffer. Heat treated protein extracts (65°C, 20 min) in a 50 mM sodium phosphate pH 8.0 buffer containing 0.5 M NaCl were loaded onto the gel, and the batches were incubated for 60 min on a rocking table at room temperature. Unbound proteins were removed by washing the gels four times with 1 ml 50 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole pH 8.0 buffer. Proteins bound to the metal groups of the gel surface were eluted with 1 ml 100 mM Mes buffer at pH 6.5 containing 125 mM imidazole. The protein extracts, heat treated extracts and eluted fractions were analyzed on 12% polyacrylamide slab gels using a Tris-glycine pH 8.3, discontinuous buffer system as described by Laemmli (1970). The protein concentrations were determined by Bradford's method using bovine serum albumin as the standard (Bradford, 1976).

Kinetic analyses

All kinetic parameters were measured in 100 mM Mes pH 6.5 buffer at 25°C. Kinetic parameters were determined by monitoring the oxidation of the nicotinamide coenzymes at 340 nm in 10 mm cuvettes mounted in a Hitachi U-3200 spectrophotometer. Rates were calculated using a molar extinction coefficient for NAD(P)H of 6200 M^{-1} cm^{-1}. The data were fitted to Eady–Hofstee plots ($r^2 > 0.9$) using Cricket Graph III from Computer Associate Incorporate.

Molecular dynamics simulations

Molecular dynamics simulations were performed using the program package Amber 4.1 (Pearlman *et al*., 1995). The potential function of this program contains a standard harmonic potential for bond and angle deformation, a truncated trigonometric series ($n = 1$–3) for dihedral angles, a Coulombic term for the electrostatic interactions, and a 6-12 Lennard-Jones potential for the van der Waals interactions.

Two systems were investigated: the native enzyme with NAD^+ and the mutant with $NADP^+$. The starting coordinates were taken from the Brookhaven Protein Databank file 2LDB (lactate dehydrogenase from *Bacillus stearothermophilus* at 3.0 Å resolution) (Piontek *et al*., 1990). All crystal water molecules were retained in the calculation, but a sulfate ion was removed. The mutant was constructed by simple model building.

All Asp and Glu residues were treated as anions and all Lys and Arg residues as cations. The total charges of NAD^+ and $NADP^+$ were assumed to be –1 and –3, respectively. The protonation status of the histidine residues was determined by examination of the hydrogen bonding structure: His264 was found to have a proton on ND1, His67 and 316 on NE2, and His155, 186, 193 and 315 on both nitrogens. The positions of the hydrogen atoms were determined using the Amber program protonate.

Partial charges and force-field parameters were taken from the standard Amber 4.1 all amino94 and parm94 files (Cornell *et al*., 1995). The parameters for NAD^+ were taken from Ryde (1995), except that the partial charges on the adenine and ribose moieties were updated with the Amber 4.1 charges (Cornell *et al*., 1995). Parameters for $NADP^+$ were the same as for NAD^+, except that the H2′A atom was replaced by a PO_3^2 group. The partial charges of the latter group were estimated by quantum chemical electrostatic potential charges (about 30 000 point charges; CHelpG radii) using the RESP scheme (Cornell *et al*., 1993) on the ribose moiety (truncated at C1 and C4; AM1 geometry) and 6-31G* basis sets. This gave the following charges: C2′A, –0.0300; O2′A, –0.6735; P2′A, +1.4657 and OP2, –0.9635 (the charges on the other atoms were not changed).

All atoms in the residues with at least one atom within 2.0 nm from the O2′A atom in the coenzyme were included in the calculations: residues 22–40, 47–67, 76–79, 82–86, 91–98, 107–127, 129, 132–140, 142–144, 146–147, 150, 159–161, 165, 193, 237, 240–241, 244–253, 255–256, 259 and 331

in the same subunit; residues 30, 33–34, 37, 57, 61, 64 and 237–250 from the neighboring subunit. Crystal water molecules 336–338, 340–341, 352, 372–375 and 382–385 from the same subunit; and crystal water molecules 372–373, 382–383 and 385 from the neighboring subunit—in total, 2240 atoms for the mutant (total charge –6). In addition, a spherical water cap centred at the O2′A atom of the coenzyme with a 2.0 nm radius was included (471 molecules); they were kept within the sphere using a soft force constant (6.3 kJ/mol/Å^2).

The following simulation protocol was used for both systems. First, a 1000-step molecular mechanic minimization without Shake was carried out, followed by a 15 ps molecular dynamics simulation, keeping all heavy atoms fixed, except the solvent water molecules (and the mutated groups). Then a 30 ps molecular dynamics equilibration was run, where all atoms in all residues with at least one atom within 1.5 nm from the O2′A atom in the coenzyme (and all solvent water molecules) were allowed to move (a total of 2622 atoms). In one simulation of the mutant, this equilibration was preceded by a 15 ps molecular dynamics simulation, in which the CE atom in Lys51 and the adenine moiety of $NADP^+$ were also fixed. Finally, a 100 ps molecular dynamics production run was carried out, and the coordinates were sampled every 1.5 ps. For figures, molecular mechanical minimizations were run until the root-mean-squared gradient was below 0.004 kJ/mol/Å. Simulation parameters are gathered in Table I.

Results

Cloning and expression of LDH(his)$_6$

Vector pUC18, digested with *Eco*I and *Bam*HI, was subjected to agarose gel electrophoresis and the 2.8 kbp fragments were excised and purified. A linker encoding six histidine residues, obtained by hybridizing oligonucleotides His1 and His2, was ligated into the digested vector, transformed into *E.coli* TG 1, and plated onto plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). White colonies were picked and screened at DNA level using a unique *Nco*I site in the (his)$_6$ linker. The constructed vector, pHis, was digested with *Bam*HI and *Pst*I, and the DNA fragment was purified. The *lct* gene encoding LDH was cloned from plasmid pLDH 41 utilizing PCR and primers LDH1 and LDH2. In order to facilitate the cloning, the PCR primers were designed containing a unique restriction enzyme site, the 5′ primer holding a *Bam*HI site, and the 3′ primer a *Pst*I site. After amplifying and purifying the *lct* gene, the fragments were digested with these enzymes. The digested *lct* fragments were ligated to pHis and transformed into *E.coli* TG 1.

Table I. Simulation parameter[a]

Temperature (temp0)	300 K
Time step (dt)	1.5 fs
Number of time steps between the updates of the non-bonded pair list (nsnb)	50
Cut-off radius for non-bonded interactions (cut)	12 Å
Bond lengths constrained with Shake (ntc = ntf = 3)	
Temperature scaling using the Berendsen algorithm (ntt = 1)	
Time constant for temperature bath coupling (tautp)	0.2
Dielectric function (dielec; idiel = 1)	ε = 1.0
Scaling factor for 1,4 non-bonded interactions (scnb)	2.0
Scaling factor for 1,4 electrostatic interactions (scee)	1.2

[a]Words in parentheses are the corresponding AMBER keywords.

Site-directed mutagenesis of the coenzyme binding motif

Plasmid pHLDH was digested with *Pst*I and *Eco*RI, and the *lct* gene was purified and inserted into the phage vector M13mp18 yielding M13HLDH. Single-stranded DNA was obtained from *E.coli* infected with phage M13HLDH. Oligonucleotide linkers, complementary to the sense strand, were prepared in order to generate the desired point mutations. The linkers were designed to destroy a *Cla*I site in this region to facilitate screening for the desired mutants. M13HLDH-positive mutants were digested with *Pst*I/*Eco*RI, and the fragments were cloned back into pHis. The positive mutants were sequenced confirming that the mutations were correctly positioned (Table II).

Kinetic analyses of wild-type and mutant LDH in aqueous and organic reaction mixtures

Native and mutated LDH was purified using IMAC. As shown in Figure 1, the (his)$_6$ affinity tail facilitated easy purification of LDH, thus rendering preparations that were at least 98% pure. The steady-state catalytic properties of both the native and mutated enzymes were measured using both NADH and NADPH as coenzymes. The catalytic efficiencies were determined by the quota K_{cat}/K_m, where K_m refers to the Michaelis–Menten constant for the coenzyme. Moreover, the preference of NADPH over NADH was evaluated by the quota $[K_{cat}/K_m(\text{NADPH})]/[K_{cat}/K_m(\text{NADH})]$.

The kinetic values of wild-type and mutated LDH assayed in both aqueous and methanol-containing reaction mixtures are compiled in Table III. By changing Ile51 to Lys and Asp52 to Ser, a mutant LDH with some interesting kinetic features was generated. This mutant exhibited a 3.9-fold increase in

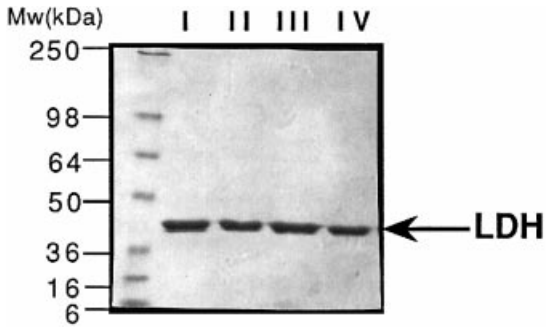

Fig. 1. A 12% SDS–PAGE gel of *B.stearothermophilus* LDH after IMAC purification. Lane I contains purified wild-type LDH enzyme, lanes II–IV contain the purified mutants in the other D52S, D52G and I51K:D52S.

Table II. Aligned primary sequences surrounding the coenzyme-binding pocket in wild-type and mutated enzymes

	1	11	21	31	41	51
Wild-type LDH	ITNSHHHHHH	GDNAMKNNGG	ARVVVIGAGF	VGASYVFALM	NQGIADEIVL	IDANESKAIG
D52G	----------	----------	----------	----------	----------	-G--------
D52S	----------	----------	----------	----------	----------	-S--------
I51K:D52S	----------	----------	----------	----------	----------	KS--------

Table III. Kinetic parameters of wild-type and mutated enzymes using both NADPH and NADH with water (A) or 15% methanol (B) as solvent

Enzyme	NADPH			NADH			Preference (a/b)
	K_m (μM)	k_{cat} (s^{-1})	(a) k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	(b) k_{cat}/K_m (μM^{-1} s^{-1})	
(A) Water							
Wild-type	290	41	0.14	63	240	3.8	0.04
D52G	740	10	0.01	550	12	0.02	0.50
D52S	260	16	0.06	480	44	0.09	0.67
I51K:D52S	710	160	0.23	1020	160	0.16	1.4
(B) 15% methanol							
Wild-type	120	46	0.38	32	270	8.4	0.04
D52G	520	8	0.02	220	3.5	0.02	1.0
D52S	270	18	0.07	400	31	0.08	0.9
I51K:D52S	760	170	0.22	1080	110	0.10	2.2

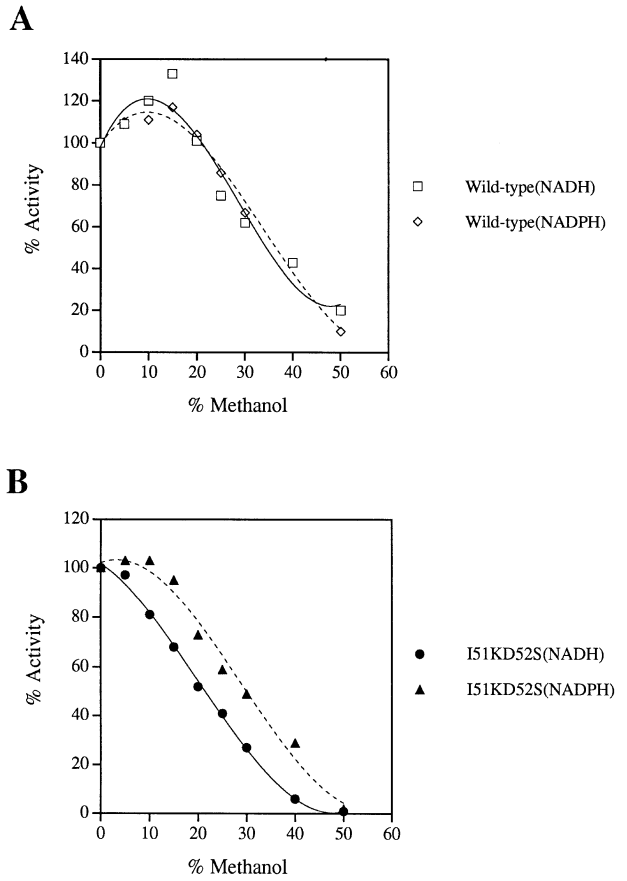

Figure 2. The effect of methanol on the enzymatic activity of native and mutated *B.stearothermophilus* LDH using both NADH and NADPH as coenzyme. The activities are given as percentage activity of the reaction without methanol for (**A**) wild-type LDH and (**B**) the I51K:D52S mutated enzyme.

NADPH turnover compared with wild-type LDH. The K_m value for NADH was also increased generating a mutant LDH having a 21-fold improved preference for NADPH over NADH (0.82/0.04).

Activity measurements in various concentrations of methanol

To assess the influence of an organic solvent, the activities of wild-type LDH and the I51K:D52S mutant were measured in a wide range of methanol concentrations (0–50%). The activities are given as percentage activity relative to the enzymatic activity without adding methanol to the reaction mixture. As shown in Figure 2A, the maximal activity of wild-type LDH

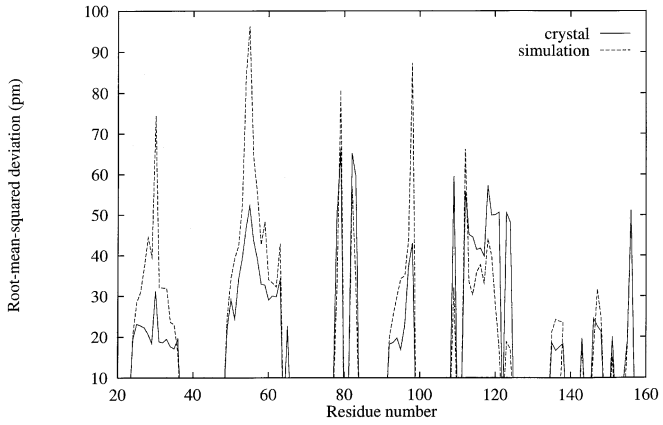

Fig. 3. The average r.m.s. deviation per residue in the crystal structure compared with the simulation of the native enzyme. Only the residues that were free to move in the simulation are included. For convenience, residues 246–248, 251 and 255 have been given numbers 146–155, and the coenzyme is residue number 156. The temperature B-factors in the PDB file 2LDB were used for the crystal (Piontek *et al.*, 1990).

was achieved by adding 15% methanol when utilizing either NADH or NADPH as a coenzyme. This is in contrast to the I51K:D52S mutant which exhibits the maximal NADH activity *without* the addition of methanol, and the highest NADPH activity *with* the addition of 15% methanol (Figure 2B). Another distinct difference between the wild-type and I51K:D52S LDH was the relative activity using NADPH compared with NADH. The relative activity of the I51K:D52S mutant was higher over the whole range of methanol concentrations, while the native enzyme exhibited comparable relative activities at methanol concentrations above 20% (Figure 2). By adding 15% methanol to the reaction mixture, the NADPH specificity could therefore be further increased (Table III B).

Molecular dynamics simulations

Molecular dynamics simulations were performed to get some information on the structure of one of the mutants, I51K:D52S. First a molecular dynamics was run on the native enzyme in order to check the method. The results show that the structure of the native enzyme is not significantly changed during the 145 ps simulation. The average difference between the crystal structure and the average molecular dynamics structure is 0.043 nm for backbone atoms and 0.087 nm for side-chain atoms (only atoms allowed to move are included in the averages). In Figure 3, the average r.m.s. displacement for the

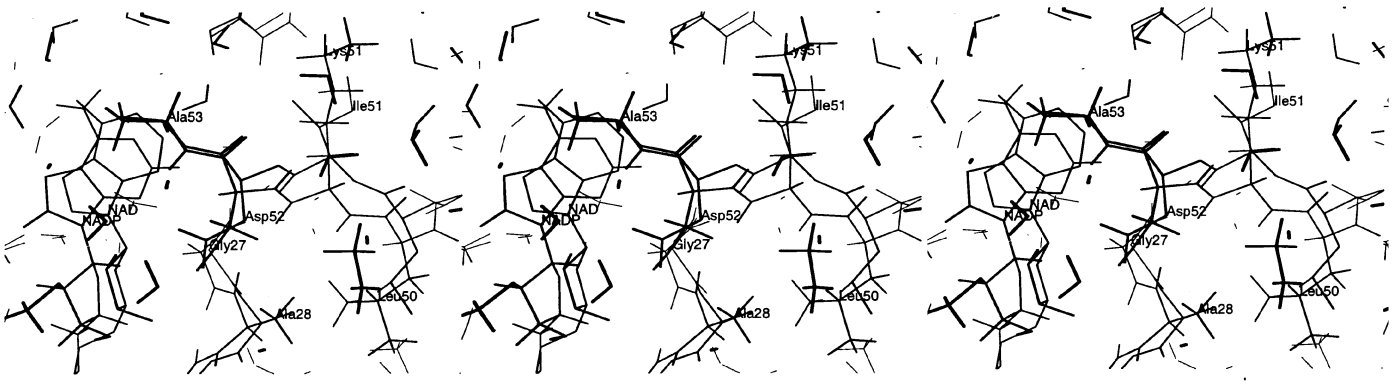

Fig. 4. The positions of the mutated residues in the simulation of the mutant I51K:D52S. The mutant structure was obtained with a molecular mechanical minimization after all the molecular dynamics simulations.

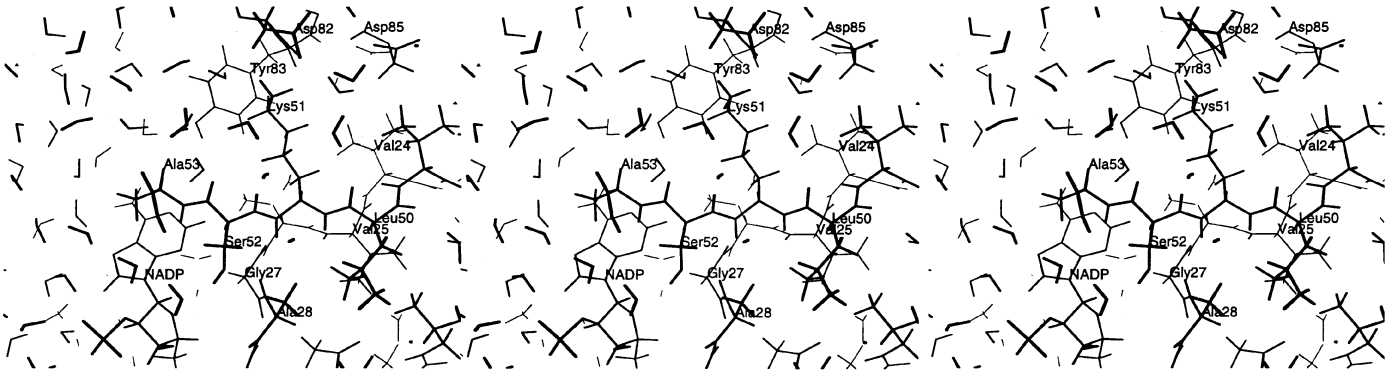

Fig. 5. Comparison between the crystal structure (without hydrogens) and the simulated structure of the mutant I51K:D52S. The mutant structure was obtained with a molecular mechanical minimization after all the molecular dynamics simulations.

atoms in each residue is compared with the average temperature factor for each residue. The magnitude is similar, and the trends in the temperature factors are well reproduced. Altogether, these results indicate that the molecular dynamics protocol behaves well, and therefore can be applied safely to the mutant enzyme.

A starting structure for the mutant enzyme was constructed by simple model building. Asp52 was converted to Ser by removing the O^{δ} atoms and changing the C^{γ} atom to an oxygen. The hydroxide hydrogen atom was directed against the O3′ atom of the A-ribose in the coenzyme (0.26 pm distance). Val51 was similarly converted to Lys, keeping the C^{β} and the $C^{\gamma 2}$ atoms, and the Lys side chain was built towards O2′A in the coenzyme. Finally, the HO2A atom of the coenzyme was replaced by a $–PO_3^{2–}$ group, turned so that one of the oxygen atoms was only 0.37 nm from the N^{ζ} atom of Lys51. This could be done without moving any other atom in the enzyme, but the N^{ζ} atom makes two mildly repulsive contacts with surrounding amino acids (Lys51 lies mainly on the surface of the protein).

During the simulations, the structure of the mutant changes significantly in two aspects. First, the whole side chain of Lys swings through the solvent from the position near the coenzyme to the neighborhood of Asp82 (1.5 nm from the coenzyme). As can be seen in Figure 4, the terminal amino group of Lys51 makes hydrogen bonds to O and O^{δ} of Asp82, to water molecules, and in some simulations, also to O^{δ} of Asp85. This behavior was observed even if the protein was allowed to relax (to remove short contacts) before the Lys51 was allowed to move.

Secondly, the 2′-phosphate group moves out into the solution so that it does not interact with the protein (HO3′ makes a strong internal hydrogen bond with the phosphate group). To some extent, the adenine and A-ribose moieties of $NADP^+$ follow this motion (0.05–0.1 nm). In the crystal structure, the adenine moiety makes only one polar interaction with the protein, a hydrogen bond between N3 and H of Ala53 (0.18 pm); the two N7 atoms are solvated. The A-ribose moiety, however, is strongly bound to the enzyme by a hydrogen bond between O^{δ} of Asp52 and HO3′ (0.17 nm). This bond is present all the time through the simulation of the native enzyme, and it is stabilized by a hydrogen bond between the other O^{δ} atom and H of Asn54 (0.16 nm; also present throughout the simulation). The other hydroxyl groups of the A-ribose are solvated, O3′ by a crystal water molecule. In the mutant, Asp52 is replaced by Ser, and the strong hydrogen bond disappears. As can be seen in Figure 5, the serine residue is too short to make a hydrogen bond with (H)O3′; instead it binds through a bridging of water molecules. Ser52 stays near the starting position throughout the simulation, while the ribose moiety is slightly tilted out into the solution, so that the O^{γ}–O3′A distance increases from 0.35 to 0.47 nm.

Discussion

The simulations explain several aspects of the kinetic characteristics displayed by the I51K/D52S double mutant of LDH. First, they show that the binding of the adenine end (but not the nicotinamide end) of the coenzyme is slightly destabilized (one strong hydrogen bond has been removed). Since the

dissociation of the coenzyme is the rate-limiting step in the reaction of lactate dehydrogenase (Holbrook *et al*., 1975) this explains why K_{cat} is increased. Second, the fact that Lys51 does not interact with the 2′ phosphate of NADPH explains why the K_m(NADPH) of the mutant is not improved. If the terminal amine group of Lys51 interacted directly with the phosphate group, a large decrease in the K_m(NADPH) value would have been expected since the Coulombic interaction between two charged groups in water solution at hydrogen bond distance amounts to at least 20 kJ/mol or a 1000-fold decrease in the K_m value. When Lys51 resides in the position observed in the simulations, the interaction with the coenzyme would be expected to be negligible, except perhaps from the effect of increasing the total charge of the protein with one unit. However, the simulations give no explanation to the kinetic differences between the present mutant and the single Asp52 to Ser mutant (Feeney *et al*., 1990). Probably, they can be attributed to subtle changes in the protein structure due to the Ile51 to Lys substitution.

The simulations also gave some interesting suggestions for other mutations of NAD^+-binding enzymes. Apparently, the substitution of Asp52 to Ser does not preserve the hydrogen bond between the protein and HO3′. Probably, Asn would provide a better alternative. It is long enough to reach the coenzyme; hence, it may form a hydrogen bond to O3′A with the amide hydrogens, and it may still form the hydrogen bond to H of Asn54 that fix the group is in the right direction. Perhaps, an Asn54 to His mutation would give rise to favorable interactions between the enzyme and the 2′-phosphate group of the coenzyme. Although it is uncertain whether the electrostatic interactions between a possibly positively charged histidine residue and the phosphate group is large enough to compensate for the loss in rotation freedom of the phosphate and histidine group.

The addition of methanol to the reaction mixture had different effects on wild-type and each of the mutated enzymes. A reduction of K_m(NAD(P)H) was observed in wild-type LDH and the mutants D52G and D52S, whereas the K_m(NAD(P)H) of mutant I51K:D52S was unaffected. The decrease in K_m(NAD(P)H) was accompanied by a concomitant reduction in k_{cat} (Table III). The solvent, in this case methanol, plays an important role in the strength of noncovalent interactions, such as electrostatic and hydrogen bonding, which all occur within the coenzyme-binding pocket. The dielectricity constant (ε_r) of water and methanol are 80 and 33, respectively (Reichardt, 1988). Thus by adding 15% methanol to the reaction mixture, the degree of ion association is increased. Even though these solvent effects probably do not influence ‘first layer’ interactions, i.e. direct interactions between the unique cofactor and amino acid residues, they could influence second layer interactions, i.e. interactions between the first layer and enzyme residues, or ‘long range’ interactions.

In conclusion, by introducing two point mutations, I51K:D52S, in the coenzyme-binding pocket of LDH, we have created a thermostable dehydrogenase with altered coenzyme specificity and reasonable high turnover number, which is extremely easy to produce and purify in large quantities. Potential applications for this enzyme in the future could be the regeneration of the expensive NADPH in enzyme reactors, since only a few thermostable enzymes are capable of oxidizing NADPH.

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