Journal of Molecular Catalysis B: Enzymatic 77 (2012) 111–118

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# Kinetics and docking studies on the effect of chemical modification of NADH for redox reactions with dehydrogenases

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ARTICLE INFO

*Article history:*
Received 21 August 2011
Received in revised form
20 December 2011
Accepted 19 January 2012
Available online 28 January 2012

*Keywords:*
8-(6-Aminohexyl)-amino-NADH
Molecular docking
Alcohol dehydrogenase
Lactate dehydrogenase

ABSTRACT

Cofactor analogs promise important applications in biosynthesis. The effect of chemical modification on the reactivity of NADH for redox reactions catalyzed by dehydrogenases was examined in this work. Compared with the native NADH, kinetics and molecular docking studies with 8-(6-aminohexyl)-amino-NADH showed that its binding with alcohol dehydrogenase (ADH) was not much affected or even enhanced by a factor of 4.9-fold with lactate dehydrogenase (LDH), but complicated the binding of substrates to the enzymes. For ADH, the Michaelis constant for acetaldehyde decreased from 0.47 to 0.048 mM, while that of sodium pyruvate with LDH increased to 0.81 from 0.18 mM. On the other hand, the modified coenzyme showed a 19.3-fold decrease in turnover number ($k_{cat}$) with ADH, while a slight increase with LDH. Molecular docking analysis showed that the hexanediamine arm on the modified coenzyme generated an extra hydrogen bond at the active site of ADH, as well as additional hydrophobic interactions with both ADH and LDH. It appeared that the apparently decreased reactivity of modified cofactor with ADH was caused mainly by the enhanced stability of ternary coenzyme–enzyme–substrate complex, while in the case of LDH, the reduced substrate binding as a result of the chemical modification of NADH led to a slight increase in the overall reaction reactivity.



## 1. Introduction

Site-directed mutagenesis of the key amino acids in active sites of coenzyme-dependent oxidoreductases has been proven effectively in manipulating enzyme activity and specificity by affecting the binding of coenzyme to corresponding enzymes [1–3]. Generally speaking, enzyme–coenzyme binding is one of the most critical steps in controlling the overall efficiency of redox transformation reactions [4]. In this regard, changing the structure of coenzyme NAD(H) through chemical modification will potentially provide an alternative strategy to genetic modification of enzyme since the complexation of enzyme–coenzyme and subsequent steps in reaction mechanism might be changed accordingly [4]. Within 1970s to 1980s, a great variety of chemical reaction routes were developed for coenzyme modification, and related works were comprehensively reviewed by Bückmann and Carrea [5]. One of the main objectives of coenzyme modification was to facilitate affinity purification of coenzyme-dependent oxidoreductases [6–10], while little attention was paid to retaining activity of the modified coenzyme. The other important application of modified cofactor was aiming at sustainable enzymatic biosynthesis. Cofactor-dependent enzymatic reactions have a wide spectrum of applications in organic synthesis especially chiral materials [11–14]. For continuous production in membrane reactors [5,15,16] or with semi-permeable microcapsules containing multienzymes and coenzyme [17,18], chemically modified cofactors were usually required. For this application, coenzyme derivatives with good activity have been a considerably challenging subject [19–22].

It has been demonstrated that both position of modification (N-1, $N^6$ or C-8 of the adenine ring) and property of substitution groups (charge, hydrophobicity or hydrophilicity) have profound effects on the activity of coenzyme derivatives [5]. Hendle et al. [4] prepared a series of coenzyme derivatives by functionalizing the adenine moiety with small molecules possessing different charges such as propane sultone and 2,3-epoxypropyl-trimethyl-ammoninum chloride. The catalytic activity measured with lactate dehydrogenase isoenzyme $H_4$ showed that $N^6$-postion substituted $NAD^+$ derivatives were 25- to 250-fold more active than N-1 derivatives, and the positive charged substitution was more active than negative charged one, yet all derivatives were less active compared with the native $NAD^+$. Bückmann and Carrea [5] sumarrized the activity of functionalized coenzyme derivativs with repect to several dehydrogenases, in most cases, a decrease of $V_{max}$ is accomponied by an increase of $K_m$.

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doi:10.1016/j.molcatb.2012.01.018

Mechanism of coenzyme-dependent oxidoreductases catalyzed reaction is complicated that generally involves the binding of coenzyme to enzyme, the formation of coenzyme–enzyme–substrate ternary, the hydride transfers, as well as the dissociation of product and oxidized or reduced coenzyme. Analysis on the apparent Michaelis–Menten constants $V_{max}$ and $K_m$ of enzymes with native and modified coenzyme therefore usually could not elucidate the effect of modification of coenzyme on the individual step in the reaction mechanism [23,24]. The development in modern computational simulation tools provides opportunities to study the molecular mechanism interpreting relationship between the activity and structure of coenzyme derivatives. Hendle et al. [4] used AMBER, a docking tool based on energy minimization theory, to study molecular mechanics affecting the recognition of modified coenzyme by lactate dehydrogenase. It was found that though $N^6$-(3-sulfopropy1)-NAD (6SP-$NAD^+$) had the lowest interaction energy with lactate dehydrogenase and one third of the $K_m$ of native $NAD^+$, but its turn over number $k_{cat}$ was only one fourth of the value of $NAD^+$. AMBER program was also applied to studying the relationship between activity of $NAD^+$ derivatives modified in the nicotinamide group and their geometries in ternary complex with alcohol dehydrogenase [23,24]. The 'out-of-plane' rotation of the side chain of the pyridinium ring in geometry of $NAD^+$ derivatives in dehydrogenase was considered decisive for its activity. In all above studies, the effect of chemical modification of coenzyme on its activity was only analyzed from the point of recognization and binding of coenzyme to the active sites in respective dehydrogenases. The lack of comprehensive analysis on the effect of structural change in coenzyme on substrate binding and other subsequent steps in reaction mechanism makes the explanation of effect resulting from coenzyme modification still rather speculative.

From above considerations, full kinetic studies incorporating with molecular docking analysis by taking exact reaction mechanism into account will provide further insight into relationship between the structure of coenzyme derivatives and its activity with respective dehydrogenase. Therefore, in the present study 8-(6-aminohexyl)-amino-NADH ($C^8$-HAD-NADH), a coenzyme derivative suitable for further macromolecularization or linking to solid matrix [5,7], was prepared by substituting the C-8 of adenine of NADH with 1,6-hexanediamine. Alcohol dehydrogenase and lactate dehydrogenase, both have wide range of applications in biosynthesis were selected as model dehydrogenases to comprehensively study the effect of modification of coenzyme on its catalytic activity by kinetics study and molecular docking study at the same time. Auto Docking based on Larmarckian Genetic Algorithm (LGA) that has been successfully used to study the binding of small ligand to macromolecular was applied for the molecular docking study [25–28].

## 2. Material and methods

### 2.1. Materials

Alcohol dehydrogenase (ADH) from yeast (EC: 1.1.1.1, lyophilized powder with a protein content of 90%), cofactor $NAD^+$ and NADH were purchased from Sigma Chemical Co. (St. Louis, USA). Lactate dehydrogenase (LDH) from *Staphylococcus* sp. (EC: 1.1.1.28, lyophilized powder) was obtained from Amano Enzyme Inc. (Nagoya, Japan). Sodium pyruvate was purchased from Acros Organics (Gell, Belgium). DEAE-Sepharose FF was purchased from GE Healthcare (Uppsala, Sweden). All other reagents including alcohol, acetaldehyde, sodium carbonate, sodium bicarbonate, liquid bromine, dimethyl sulfoxide (DMSO), 1,6-hexanediamine, and carbon tetrachloride were all of analytical grade.

### 2.2. Synthesis of 8-(6-aminohexyl)-amino-NADH

8-(6-Aminohexyl)-amino-NADH ($C^8$-HAD-NADH) were synthesized following a procedure modified from that reported by Lee et al. [7]. The synthesis reaction was started from $NAD^+$ due to the fact that $NAD^+$ is more stable in acid aqueous solution but labile in base solution, while NADH is subject to serious decomposition in acid [29]. Typically, $NAD^+$ (100 mg) was dissolved in 5 ml sodium acetate buffer (pH 4.0, 0.1 M) to which liquid bromine was added in four portions (25 μl each) at intervals of 30 min under magnetic stirring at room temperature. The unreacted bromine was removed by repeated extraction with carbon tetrachloride until neither phase changed color. Br-NADH has been found to be more thermally stable than Br-$NAD^+$ [30], therefore Br-$NAD^+$ was enzymatically reduced to Br-NADH by ADH prior to the next displacement reaction. The reduction of Br-$NAD^+$ was verified by the increase in absorbance at 340 nm using USB2000 UV–Vis spectrophotometer (Ocean Optics Inc., Dunedin, FL USA). Once the reduction was complete, the ratio of the absorbance at 340–260 nm was approximately 0.33 [7]. Then the mixture was dried using a rotary evaporator.

To displace the bromine at the C-8 position of adenine with 1,6-hexanediamine, 100 mg Br-NADH was dissolved in a 10 ml DMSO solvent containing 5 g 1,6-dianminohexane. The reaction was carried out at 70 °C, and the completion of the displacement of Br with 1,6-dianminohexane was verified by the shifting of wavelength of maximum absorbance from 263 nm to 280 nm, usually the reaction lasted for 4 h [7]. To purify the $C^8$-HAD-NADH, the 20-fold diluted reaction mixture was loaded to DEAE-Sepharose FF column (i.d., 1.0 cm × 13 cm) that was pre-equilibrated with 1 M sodium carbonate and water, 50 mM sodium carbonate buffer (pH 9.3) was used to elute the product from the column. Fractions with maximal absorbance at both 280 and 340 nm were pooled and dried on vacuum rotary evaporator at 40 °C, from which $C^8$-HAD-NADH was obtained as white powder. The overall yield of $C^8$-HAD-NADH was estimated about 60% based on the initial $NAD^+$ amount. The chemical modification on the C-8 position of NADH was confirmed by $^1$H NMR and MALDI-TOF MS analysis of native NADH and $C^8$-HAD-NADH. NADH, $^1$H NMR ($\delta$ ppm): 8.53 (s, $C_2$-adenine proton), 8.29 (s, $C_8$-adenine proton), MS: 664.4 (Mt 664.4); $C^8$-HAD-NADH, $^1$H NMR ($\delta$ ppm): 8.04 (s, $C_2$-adenine proton), 1.34-1.69 (m, the hexyl protons); MS: 780.5 (Mt 780.5).

### 2.3. Kinetic characterization of native and modified NADH

The activity of native and modified NADH was evaluated by two enzymatic reduction reactions, which are ADH catalyzed reduction of acetaldehyde to ethanol and LDH catalyzed reduction of sodium pyruvate to lactic acid. Both reactions were conducted at 25 °C in 200 mM Tris–HCl buffer (pH 8.0), their reaction rates were acquired by measuring the decrease in absorbance at 340 nm ($\varepsilon^{NADH} = 6.22\ mM^{-1}\ cm^{-1}$). For kinetic studies, initial velocities were measured with substrates concentrations being varied at each of four coenzyme concentrations as follows: 0.04, 0.06, 0.08, and 0.1 mM. To determine the kinetic data of ADH with NADH or $C^8$-HAD-NADH as coenzyme, the acetaldehyde concentration ranged from 0.1 to 0.25 mM or 0.04 to 0.1 mM, respectively. To determine the kinetic data of LDH, the sodium pyruvate concentration ranged from 0.1 to 0.4 mM or 1 to 4 mM by using NADH or $C^8$-HAD-NADH as coenzyme, respectively.

### 2.4. Modeling of coenzyme binding to enzyme by AutoDock

The structures of ADH and LDH (PDB codes 2HCY and 1J49, respectively) were directly taken from the Protein Data Bank (PDB), http://www.rcsb.org/pdb. The crystal structure of ADH

$$E \overset{NADH}{\rightleftharpoons} E{:}NADH \overset{S}{\rightleftharpoons} E{:}NADH{:}S \overset{H^+}{\rightleftharpoons} E{:}NAD^+{:}P \rightleftharpoons E{:}NAD^+ + P \rightleftharpoons E + NAD^+ + P$$

**Scheme 1.** Mechanism for reduction reaction of ADH and LDH. E, ADH or LDH; S, acetaldehyde or sodium pyruvate; P, ethanol or lactic acid.

is in the formation of enzyme–coenzyme binary complex with nicotinamide-8-iodo-adenine-dinucleotide (NAI), a derivative of $NAD^+$ with hydrogen atom at C-8 position substituted by iodine atom, co-crystallized at the active site. While in the structure of LDH obtained from PDB, $NAD^+$ is co-crystallized. Auto Docking based on Larmarckian Genetic Algorithm (LGA) [31] provided by The Scripps Research Institute [AutoDock, Copyright©1989–2008, La Jolla, CA, USA] was used for modeling the coenzyme binding to enzyme. During calculation, cofactors were retrieved from each enzyme by AutoDock tools v1.4.5 (ADT), with which the missing polar hydrogen atoms were then generated and unnecessary waters in pdb files were removed from ADH and LDH. At the same time, Kollman partial atomic charges to the enzymes Gasteiger charges to coenzyme (NADH and $C^8$-HAD-NADH) was assigned.

ADH is a tetramer containing four monomers with identical conformation. For docking study, the "A" monomer was chosen as the target. The active site was determined within a cubic box centered on the retrieved co-crystallized NAI (co-ordinates: $X = 79.850$; $Y = -15.929$; $Z = 43.022$) with the edges of 2.0 nm, thereby ensuring that the active region was wholly covered. Conformational changes of the active-site amino acids as a result of the rather small modification of the nicotinamide moiety are neglected in our calculations [24]. Considering the active sites of enzyme as rigid molecule while coenzyme (NADH and $C^8$-HAD-NADH) as being flexible, the coenzyme was docked into the active sites of ADH by genetic algorithm followed by a local search procedure, also known as Larmarckian Genetic Algorithm (LGA) [31], with the number of docking runs 20, the maximum number of generations 25,000,000 and the maximum number of energy evaluations 270,000. Molecular graphics were generated using PyMOL [32].

The docking study for LDH follows the same procedure as for ADH, the "A" chain of the LDH homodimer was chosen as the target. The retrieved cofactor $NAD^+$ co-crystallized with LDH was used to define the active site, centered at coordinates: $X = 57.125$; $Y = 37.012$; $Z = 114.019$.

## 3. Results

### 3.1. Reaction kinetics of ADH and LDH with NADH and $C^8$-HAD-NADH

The effect of modification on the coenzyme activity was examined by conducting reaction experiments separately with ADH from yeast and LDH from *Staphylococcus* sp. As we started with NADH, reduction reactions were the focus in this work with acetaldehyde and sodium pyruvate as substrate for ADH and LDH, respectively. It is generally accepted that both reduction reactions follow a compulsory-order mechanism (Scheme 1), in which NADH must bind to ADH or LDH prior to the binding of acetaldehyde or sodium pyruvate, then the reaction goes through an intermediate ternary complex, and finally the product and $NAD^+$ dissociate from enzyme consequently [33–35]. The reaction mechanism of ADH and LDH is presented in Scheme 1; and their reaction kinetic mechanism obeys the following rate equation [36],

$$v = \frac{k_{cat}[E][A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

where $v$ is initial velocity; $k_{cat}$ is the turnover number; [E], [A] and [B] are the molar concentrations of enzyme, coenzyme and substrate, respectively; $K_a$ and $K_b$ are Michaelis constants for coenzyme and substrate, respectively; and $K_{ia}$ is the apparent dissociation constant of the enzyme–coenzyme complex. By fitting the kinetics experimental data to Eq. (1), kinetics parameters including $k_{cat}$, $K_a$, $K_b$ and $K_{ia}$ could be derived from double-reciprocal plots.

To demonstrate the determination process of kinetic parameters, primary and secondary double-reciprocal plots of the initial-rate measurement results of ADH obtained with acetaldehyde as substrate and $C^8$-HAD-NADH as coenzyme are shown in Fig. 1A and B. Both plots are linear within experimental error over the ranges of substrate and coenzyme concentration used here (acetaldehyde 0.04–0.1 mM; N-NADH 0.02–0.1 mM). The kinetic coefficients in Eq. (1) could be derived from the intercepts and slopes of Fig. 1B. The estimated values for the kinetic parameters of ADH catalyzed acetaldehyde reduction and LDH catalyzed sodium pyruvate reduction obtained with either NADH or $C^8$-HAD-NADH are listed in Table 1.

The comparison of dissociation constants ($K_{ia}$) of enzyme–coenzyme complex showed that $C^8$-HAD-NADH could bind to both ADH and LDH even more favorable than native NADH did as indicated by a decreased $K_{ia}$ values. This does not match what were expected for the modified cofactor, as the modification was occurred at the adenine moiety of coenzyme, which is essential for the recognition by enzyme [4]. It was expected that the

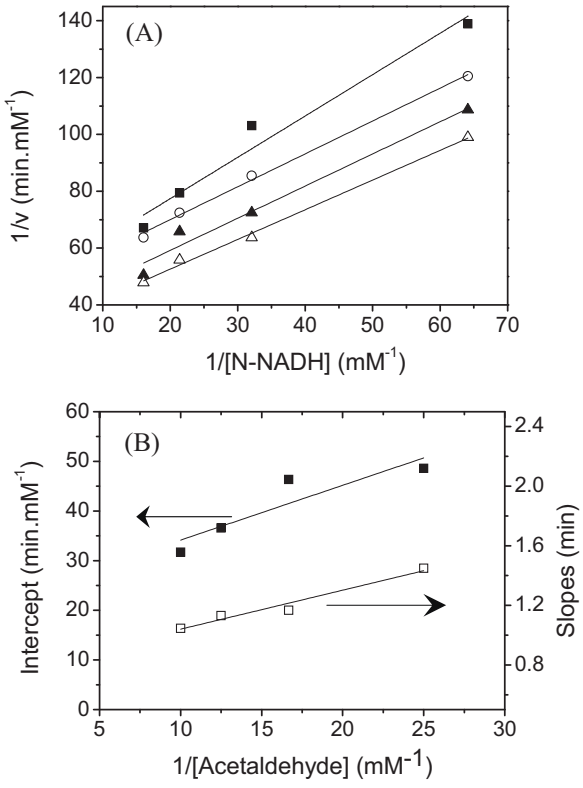


**Fig. 1.** Determination of kinetic coefficients of acetaldehyde-ADH reactions using $C^8$-HAD-NADH as cofactor. (A) Primary Lineweaver–Burk plots showing variation of the reciprocal of the initial reaction rate with the reciprocal of the $C^8$-HAD-NADH concentration for several constant acetaldehyde concentrations. The acetaldehyde concentrations (mM) were: (■) 0.04; (○) 0.06; (▲) 0.08; and (△) 0.1. Each data point for reciprocal plots represents an average of triplicate measurements with standard error less than 5%. (B) Secondary plot showing the variation of the intercepts (■) and slops (□) obtained from the Lineweaver–Burk plots in (A) with the reciprocal of the acetaldehyde concentration.

**Table 1**
Kinetic parameters for acetaldehyde-ADH reaction and pyruvate-LDH reaction determined at 25 ± 1 °C with $C^8$-HAD-NADH or NADH as coenzyme.

| Parameter | ADH from baker yeast [a] | | LDH from *Staphylococcus* sp.[b] | |
|---|---|---|---|---|
| | NADH | $C^8$-HAD-NADH | NADH | $C^8$-HAD-NADH |
| $K_a$ (μM) | 81.1 | 33.7 | 88.9 | $1.48 \times 10^2$ |
| $K_b$ (μM) | $4.71 \times 10^2$ | 47.8 | $1.78 \times 10^2$ | $8.11 \times 10^2$ |
| $K_{ia}$ (μM) | 25.6 | 23.7 | $1.40 \times 10^2$ | 28.8 |
| $k_{cat}$ ($s^{-1}$) | $2.49 \times 10^3$ | $1.29 \times 10^2$ | $1.34 \times 10^2$ | $1.76 \times 10^2$ |
| $k_{cat}/K_a$ ($mM^{-1}\ s^{-1}$) | $3.11 \times 10^4$ | $3.83 \times 10^3$ | $1.51 \times 10^3$ | $1.19 \times 10^3$ |
| $k_{cat}/K_b$ ($mM^{-1}\ s^{-1}$) | $5.29 \times 10^3$ | $2.70 \times 10^3$ | $7.53 \times 10^2$ | 21.7 |

[a] Obtained under conditions where the concentrations of coenzymes (NADH or $C^8$-HAD-NADH) were varied in the range of 0.02–0.1 mM, and concentrations of acetaldehyde were varied in the range of 0.1–0.25 mM or 0.04–0.1 mM for each NADH or $C^8$-HAD-NADH concentration.
[b] Obtained under conditions where the concentrations of coenzymes (NADH or $C^8$-HAD-NADH) were varied in the range of 0.02–0.1 mM, and concentrations of sodium pyruvate were varied in the range of 0.1–0.4 mM or 1–4 mM for each NADH or $C^8$-HAD-NADH concentration.

hexanediamine tail will affect the formation of coenzyme-enzyme binary complex.

The comparison of Michaelis–Menten constants ($K_b$) of substrates of ADH and LDH indicated that the chemical modification of NADH affected the affinity of substrates to ADH and LDH differently. With the modified NADH, the $K_b$ for acetaldehyde is 10 times lower, while the $K_b$ for sodium pyruvate is 4.6 times higher than values obtained with native NADH.

The changes in overall catalysis efficiency ($k_{cat}$) showed that the modification of NADH affected ADH and LDH reaction systems differently. As for ADH, the $k_{cat}$ value obtained with $C^8$-HAD-NADH was 19.3-fold lower than that with native NADH, which mainly accounted for the decreased specificity constant $k_{cat}/K_m$ for both cofactor and acetaldehyde (8-fold and 2-fold decrease in comparison with the respective $k_{cat}/K_m$ values obtained with the native coenzyme). For LDH, although $k_{cat}$ value increase slightly from 134 $s^{-1}$ with NADH to 176 $s^{-1}$ with $C^8$-HAD-NADH, the high Michaelis constants values for both coenzyme and sodium pyruvate obtained with the modified NADH led to 1.3-fold and 34.7-fold decrease in their specificity constants, $k_{cat}/K_m$.

Since the changes in $K_{ia}$ indicate that the binding of modified cofactors to the active sites of enzymes were not much affected (for ADH) or even 4.9-fold more favored over the native cofactor with LDH, the lower catalytic efficiency of $C^8$-HAD-NADH seems to be inconsistent. It was speculated that modification of coenzyme have more profound negative effects on the subsequent steps involved in the reaction mechanism (Scheme 1), while the exact mechanism will be further analyzed.

### 3.2. Docking studies of $C^8$-HAD-NADH with ADH and LDH

Molecular docking study provided a useful tool to analyze the catalytic mechanism at a molecular level by modeling the most preference position of ligands to macromolecule at minimum binding energy [25–28]. In this present study, AutoDock 4.0 was used to generate the docking profile of coenzyme into the active sites of ADH and LDH, respectively.

ADH is composed of four identical subunits, each subunit has 347 amino acids, one coenzyme-binding site, and one firmly bound zinc atom which is essential for catalysis [37–39]. LDH is a homodimer, each subunit consists of a single polypeptide chain with 332 amino acid residues [40]. By retrieving NAI or $NAD^+$ from the enzyme–coenzyme binary complex and docking NADH into the active site of ADH or LDH, the AutoDock program successfully reproduced the binding pattern of NADH to the respective dehydrogenases. Because there are many degrees of freedom in the conformation of modified coenzyme, we used a piecewise approach to dock the modified coenzyme to dehydrogenases. That is, the unmodified moiety of NADH was firstly docked into the active site of enzymes and restrained to the conformation same as in the previous simulation, and then hexanediamine moiety was added by allowing its rotation freely so that the most preferred binding conformation could be obtained through Larmarckian Genetic Algorithm protocol.

Fig. 2A shows the binding profile of the $C^8$-HAD-NADH to ADH. By comparing with the profile of NADH molecule to ADH, it was found that the NADH moiety of $C^8$-HAD-NADH interacts with ADH through exactly the same hydrogen bonds with neighboring residues at the active site as native NADH does. These specific hydrogen bonds include the interactions of pyridine and adenine rings with amino acids Val-268, Ser-293, Val-295 and Ser-248; the oxygen atoms of nicotinamide-ribose with the main-chain nitrogen atoms of Met-270 and the side-chain of His-48 and Thr-45; the hydroxyl groups of adenine-ribose with the residues Asp-201 and Lys-206; as well as the oxygen atom of the pyrophosphate group with main-chain nitrogen atom of Gly-181, Leu-182 and the side-chain of His-44 and Arg-340. Additionally, the terminal amino group of the hexanediamine generated one extra hydrogen bond to main-chain oxygen atom of Glu-333 at measured distance of 3.04 Å. The LIGPLOT program [41] presented the detailed binding pattern of the adenine moiety of $C^8$-HAD-NADH to ADH, in which all the hydrogen-bond and hydrophobic contacts between $C^8$-HAD-NADH and ADH at the active sites were illustrated (Fig. 2B).

Fig. 3A presents the crystal structure of NADH–LDH complex and the docking mode of $C^8$-HAD-NADH to LDH, it was shown that $C^8$-HAD-NADH possessed the same docking conformation as NADH did in the crystal structure. The acylamide group of the nicotinamide moiety and amino group of adenine formed hydrogen bonds with the neighboring amino acid residues Val-234, Phe-300 and Asn-213. Hydrogen bonds were also formed between oxygen atoms of pyrophosphate and main-chain residues His-156, Ile-157, as well as the oxygen atoms of two riboses and carbonyl atoms of side-chain residues Asp-176 and Ser-235. Apparently, the LIGPLOT presentation showed that the hexanediamine in $C^8$-HAD-NADH generated extra hydrophobic interactions with surrounding residues Pro-208 compared to native cofactor (Fig. 3B).

The free energy of binding for coenzymes complex with the ADH and LDH was calculated by AutoDock, and the results were listed in Table 2. By analyzing the changes in docking free energy and apparent dissociation constants of enzyme–coenzyme complex ($k_{ia}$) caused by modification on NADH, it was indicated that the results were well consistent with each other. The more negative the free energy of binding, the smaller apparent dissociation constant values of $K_{ia}$; the bigger change in $K_{ia}$ value, the more apparent change in free energy of binding. However, more accurate correlation between $K_{ia}$ and free energy of binding was not observed. This could be explained as follows. The docking results are related exclusively to the ligand–receptor interactions according to the parameterization of program's internal score function, while the experimentally determined kinetics parameter $K_{ia}$ was also dependent on the experimental condition used for measurement. Moreover, the docking results obtained here took into

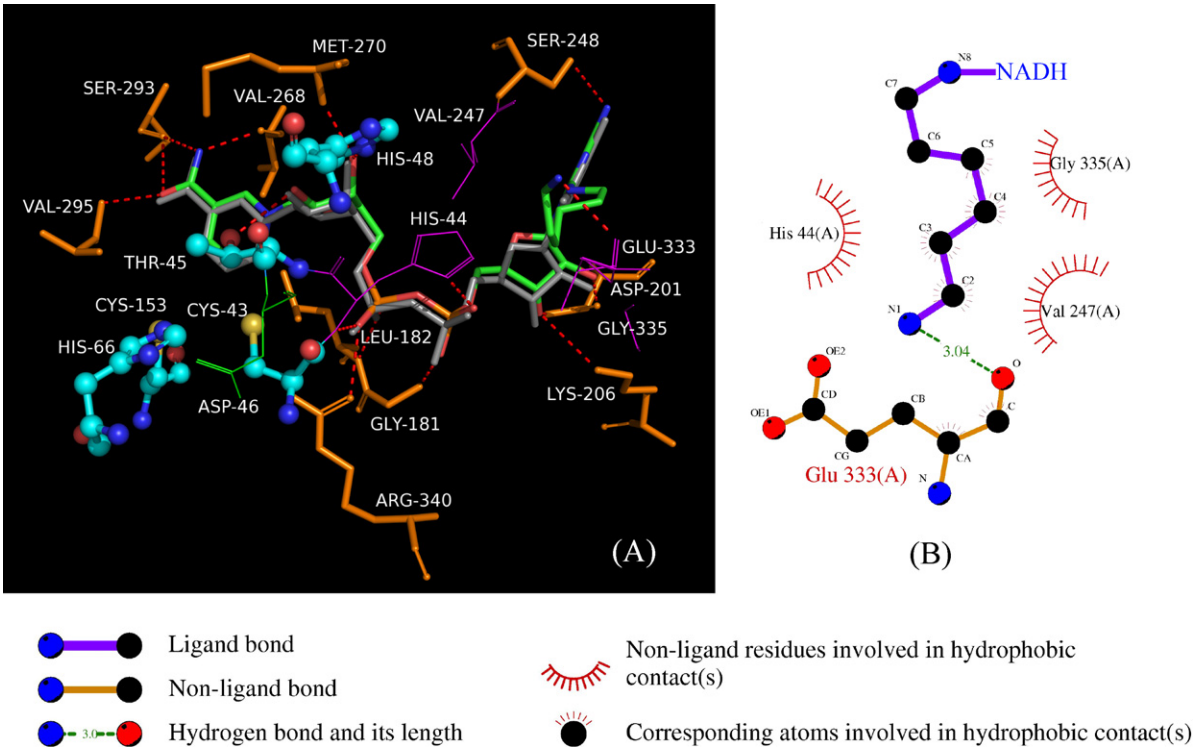


**Fig. 2.** Binding of $C^8$-HAD-NADH in ADH active center generated using Autodock and schematic depiction of interactions between ADH and the hexanediamine moiety of $C^8$-HAD-NADH. (A) Stereo picture for binding of $C^8$-HAD-NADH (docking result, in green) and NAI (X-ray crystal structure, in grey) in the ADH active center. The most important active residues associated in substrate binding and hydride transfer are presented as black and ball formats. The hydrogen bond is displayed as red dashes and the relevant residues are represented in orange stick format. The amino residues possessing extra hydrophobic (His-44, Gly-335, Val-247) and hydrogen interaction (His-44) with $C^8$-HAD-NADH were shown as magenta lines. The $Zn^{2+}$ is ligated by the sulfur atoms of cysteine residues 43 and 153, the imidazole group of His-66 and the substrate. (B) A diagram of hydrogen bonds and hydrophobic interactions between ADH and hexanediamine moiety (N1 presenting the primary amino group) of $C^8$-HAD-NADH that was generated by LIGPLOT program.

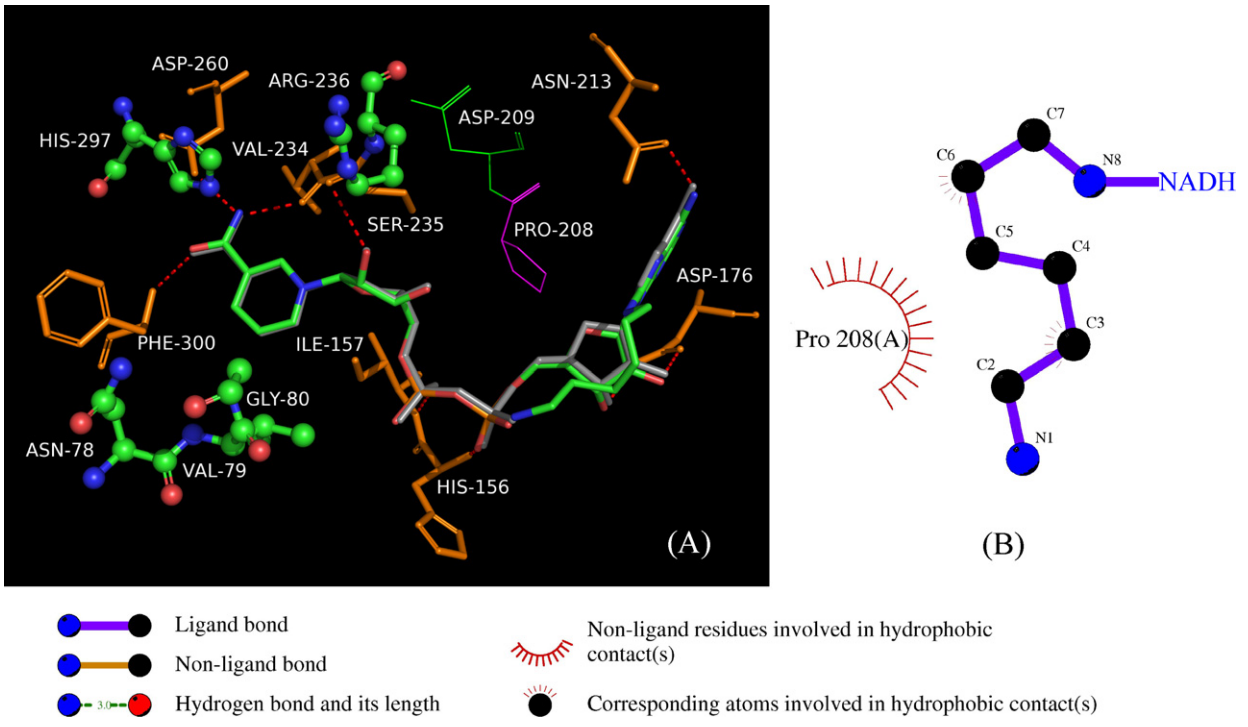


**Fig. 3.** Binding of $C^8$-HAD-NADH in LDH active center generated using Autodock and schematic depiction of interactions between LDH and the hexanediamine moiety of $C^8$-HAD-NADH. (A) Stereo picture for binding of $C^8$-HAD-NADH (docking result, green) and NAD (X-ray crystal structure, grey) in the LDH active center. The active residues associated in chemical catalysis are presented as black and ball formats, including His-297, Arg-236 and residues 78–80. The hydrogen bonds are displayed as red dashes and the relevant residues are represented in orange stick format. The residue Pro-208 which possesses extra hydrophobic interactions with $C^8$-HAD-NADH shows as magenta lines. (B) A diagram of showing the extra hydrophobic interactions between LDH and the hexanediamine moiety of $C^8$-HAD-NADH that was generated by LIGPLOT program.

**Table 2**
Comparison between the docking energy (kcal mol$^{-1}$) and apparent dissociation constant $K_{ia}$ (μM) determined by full kinetic experiment.

| Enzyme | Coenzyme | Intermolecular energy | | Total internal energy | Torsional free energy | Unbound system energy | Total energy[b] | $K_{ia}$ |
|---|---|---|---|---|---|---|---|---|
| | | vdW + H-bond + desolvation energy | Electrostatic energy | | | | | |
| ADH | NADH | −15.0 | −2.04 | −1.64 | +2.74 | −0.930 | −15.0 | 25.6 |
| | C$^8$-NADH[a] | −19.5 | −1.17 | −0.490 | +4.94 | −0.180 | −16.1 | 23.7 |
| LDH | NADH | −10.4 | −0.250 | −1.16 | +2.74 | −0.640 | −8.45 | $1.40 \times 10^2$ |
| | C$^8$-NADH | −11.0 | −1.34 | −3.14 | +4.66 | −0.710 | −10.2 | 28.8 |

[a] C$^8$-NADH represents for 8-(6-aminohexyl)-amino-NADH.
[b] Total energy = intermolecular energy + total internal energy + torsional free energy − unbound system energy.

account only the most preferred binding mode on the active sites; while for the apparent dissociation constant determined experimentally, the values may reflect the overall effect of other possible binding sites or different binding modes at the active sites. Even though, the consistence between docking energy calculated by docking study and $K_{ia}$ values from kinetic experiments provided a rational tool to understand the binding mechanism of cofactor to enzymes.

## 4. Discussions

Docking results obtained in this work indicates that C$^8$-HAD-NADH could complex with both ADH and LDH more stably by forming extra hydrogen bond (ADH) or hydrophobic interactions (both ADH and LDH) with surrounding amino acids at active sites. Nevertheless, the catalyst efficiency of enzymes ($k_{cat}$ values) with C$^8$-HAD-NADH as coenzyme changed differently for ADH and LDH systems. According to the reaction mechanism shown in Scheme 1, the binding of coenzyme to the active sites of enzymes is just one of the key steps controlling the overall reaction rate, the following steps including the substrate binding, hydride transfer, dissociation of coenzyme and product might also be affected by the modification of coenzyme.

Here firstly, we attempted to analyze the effect of modification of NADH on the substrate binding to enzyme–NADH complex. As for ADH, a zinc atom is necessarily coordinated to Cys-43, Cys-153 and one imidazole nitrogen of His-66 in the substrate binding site [37]. The other imidazole nitrogen of His-66 is hydrogen-bonded to a carboxylic group of Asp-46, which is conserved in all known zinc-dependent alcohol dehydrogenases. As shown in Fig. 2B, the modified coenzyme C$^8$-HAD-NADH can form extra hydrophobic interaction with His-44 residue, which is just next to Cys-43 and Asp-46 (Fig. 2A). We speculate that the extra hydrophobic interactions may have some positive effect on the substrate-binding residue Cys-43 and Asp-46, which indirectly led to about 10-fold decrease in $K_b$ for acetaldehyde.

As for LDH, similar analysis was made based on the effect of hexanediamine on the substrate-binding site which contains residue Arg-236, the side-chain of Asn-78, and the main chain amide groups of Gly-80 and Val-79 on a loop structure that act as bidentate

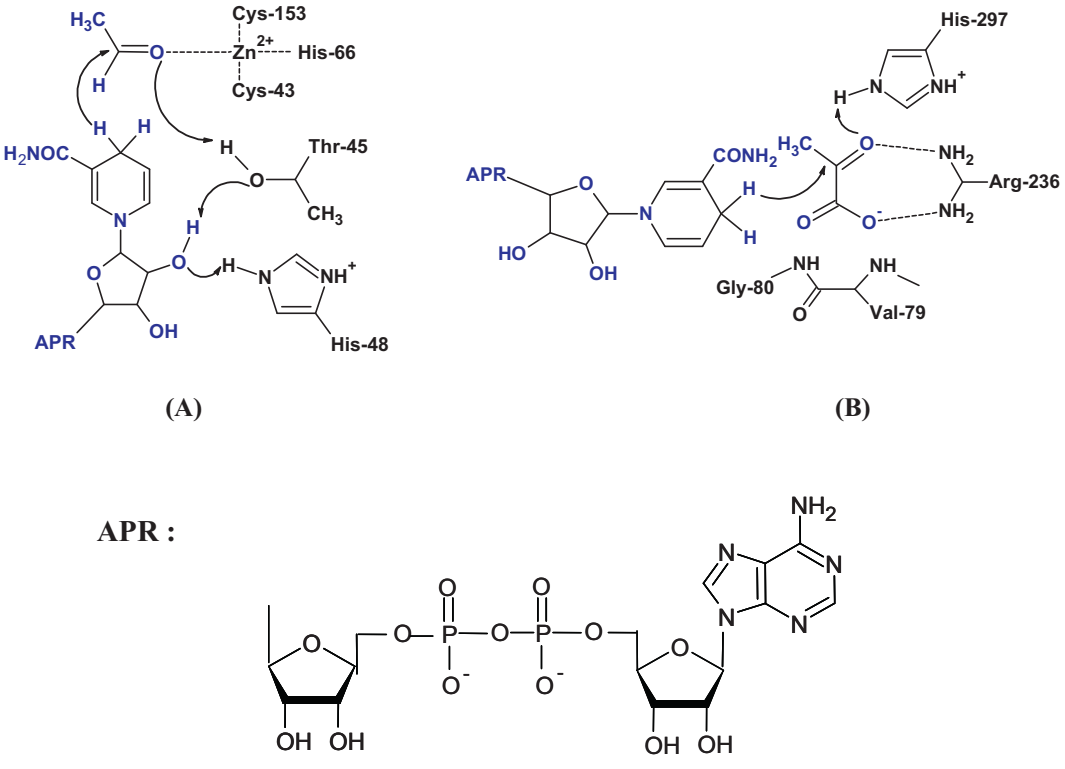


**Fig. 4.** Schematic illustration of (A) reduction of acetaldehyde by alcohol dehydrogenase and (B) reduction of sodium pyruvate by D-lactate dehydrogenase. Arrows indicate the movements of electron pairs in the catalysis.

hydrogen bond donors to the substrate carboxyl group to promote suitable substrate binding (Fig. 3A) [40]. The extra hydrophobic interaction formed between the hexanediamine of $C^8$-HAD-NADH and Pro-208 residue may affect the hydrogen bond interaction between the nearest residue Asp-209 and the substrate-binding residue Arg-236, thereafter indirectly led to about 4.5-fold increase in $K_b$ for sodium pyruvate. This reduced substrate binding as a result of the chemical modification of NADH may hamper its positive effect on the complexation of LDH and modified NADH, therefore led only to a slight increase in the overall reaction reactivity.

Site-directed mutation experiments for both ADH and LDH in the substrate-binding sites amino acid have proven that change of key residues have direct influence on substrate binding [42–45]. In our study it seemed that the coenzyme derivatives modified in the adenine moiety also have profound effect on the binding of substrate to respective dehydrogenase. These results indicate that His-44 in ADH and Pro-208 in LDH may also play an important role in the binding of substrate to the binding sites of enzymes. Further studies on the catalysis mechanism are needed, however, to confirm such assumptions.

Secondly, the effect of modification of coenzyme on the hydride transfers was analyzed as follows. The chemical step in enzymatic conversions of this two examined systems is the hydride transfers from coenzyme to acetaldehyde or sodium pyruvate, or vice versa. Fig. 4A schematically presents the acetaldehyde reduction mechanism catalyzed by ADH, which is essentially an electrophilic catalysis mechanism mediated by the active-site zinc atom. The detailed hydrogen bonded relay system in the yeast ADH was proposed and shown in Fig. 4A: His-48. . .NADH. . .Thr-45. . .$CH_3CHO$. . .$Zn^{2+}$ [37]. The LDH has a simple proton relay system (Fig. 4B): NADH. . .$CH_3COCH_2OH$. . .His-297 [40,46] In the present study, since the modification on coenzyme did not have any influence on the key amino acid residues involved in the hydride transfers, it can be reasonably postulated that the hydride-transfer step was not or little affected.

Finally, the relationship between the rate of product dissociation steps and the overall catalytic efficiency was discussed. As for acetaldehyde-ADH reaction system with modified NADH, decrease in $K_m$ values for both coenzyme and acetaldehyde favored the formation of ternary complex, while the following hydride-transfer step was little affected. Therefore, the product dissociation steps, including the dissociation of product acetaldehyde from the ternary complex and the dissociation of $NAD^+$ from the terminal enzyme–$NAD^+$ complex, may be the rate-limiting steps and the $k_{off}$ dissociation values must be low enough to result in the lower $k_{cat}$ value. This assumption was supported by previous studies on acetaldehyde reduction by both yeast ADH [34] and liver ADH [47] that the dissociation of the product enzyme–$NAD^+$ complex is the rate-limiting step. Basso and coworkers [48] studied the binding of 1,$N^6$-entheno-$NAD^+$ to glutamate dehydrogenase by kinetics measurement, similar results were obtained that more stable coenzyme–enzyme complex resulted in lower enzyme activity. The authors speculated that the additional hydrogen bond and hydrophobic interactions formed between modified coenzyme and enzyme increased the energy necessary for coenzyme release, which was consistent with previous reports.

## 5. Conclusion

In conclusion, it appeared that although the binding of modified NADH with ADH was not much affected or even 4.9-fold more favored with LDH over the native cofactor, the extra hydrogen bond and more hydrophobic interactions generated between hexanediamine in modified NADH and amino acid residues associated with the substrate-binding sites affected the catalytic efficiency of ADH and LDH in different ways. As for ADH system, the decreased catalytic efficiency found with $C^8$-HAD-NADH mainly due to the more stable ternary complex of coenzyme–enzyme–substrate, which consequently led to the difficulties in the dissociation of product. While for LDH systems, the reduced substrate binding as a result of the chemical modification of NADH may hamper its positive effect on the complexation of LDH and modified NADH, therefore led only to a slight increase in the overall reaction reactivity. Again, though further detailed studies on the catalysis mechanism are needed to confirm such assumptions, the analyses on the binding model based on both kinetic experiment and molecular modeling may provide some insight into the relationship between the binding of coenzyme to respective dehydrogenase and the overall catalytic efficiency.

## Acknowledgements

The authors thank support from the National Natural Science Foundation of China (#20728607, 20706054, 20976180), and 973 Program (2009CB724705).

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