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Effects of N-Terminal Deletion Mutation on Rabbit Muscle Lactate Dehydrogenase

Yanbin Zheng[1], Shuyuan Guo[1], Zhi Guo[1], and Xicheng Wang[1,2]*

[1]Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China; fax: 8610-6277-2248; E-mail: wangxic@mail.tsinghua.edu.cn
[2]Protein Science Laboratory of the Ministry of Education, School of Life Science and Engineering, Tsinghua University, Beijing 100084, China

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Abstract—Deletion mutants of rabbit muscle lactate dehydrogenase (LDH) were constructed using polymerase chain reaction (PCR) to study the roles of N-terminal residues. The coding sequences of the first 5 (LD5) and 10 (LD10) amino acids of the N-terminus were deleted and the gene was inserted into the prokaryotic expression vector pET21b. The mutant enzymes were expressed in *E. coli* BL21/DE3 and were purified. Then their characteristics and stabilities were studied. The results showed LDH was completely inactivated when the first 10 N-terminal amino acid residues were removed, but the mutant (LD10) could have partially restored activity in the presence of structure-making ions. The removal of the first 5 and 10 N-terminal amino acid residues did not affect the aggregation state of the enzyme, that is, LD5 and LD10 were still tetramers. The stabilities of recombinant wild-type LDH (RW-LD), LD5, and LD10 were compared by incubating them at low pH, elevated temperature, and high GuHCl. The results showed that the N-terminal deletion mutants were more sensitive to denaturing environments; they were easily inactivated and unfolded. Their instability increased and their ability to refold decreased with the increased number of amino acid residues removed from the N-terminus of LDH. These results confirm that the N-terminus of LDH plays a crucial role in stabilizing the structure and in maintaining the function of the enzyme.

Key words: lactate dehydrogenase, deletion mutation, stability

Lactate dehydrogenase (EC 1.1.1.27, LDH) plays a vital role in the energy flow of higher organisms. It is a tetrameric enzyme that catalyzes the reversible dehydrogenation of lactate converting it to pyruvate. The crystal structure of LDH shows that the enzyme is a dimer of two dimers, the assembly of the tetramer depending on a long N-terminal extension [1]. The association of LDH follows a monomer–dimer–tetramer pathway, and a variety of works have been done to study this process [2-6].

It was reported that stable dimers could form after limited proteolysis with thermolysin during reconstitution, and they were inactive under standard conditions [7]. But the proteolytic dimers exhibited some catalytic activity in the presence of a stabilizing salt (e.g., $(NH_4)_2SO_4$) [8]. Further studies purified the proteolytic dimers and found that they were composed of the intact chain lacking the N-terminal 10-11 amino acid residues with heterogeneous C-terminus, varying in the range between residues 326 ± 5 [9]. But until now no work has been done to study the role of the N-terminus in LDH by means of accurate deletion mutations.

In the present investigation recombinant wild-type LDH (RW-LD) and N-terminal deletion mutants of LDH lacking the first 5 (LD5) and 10 (LD10) amino acids were constructed and purified. The effects of N-terminal deletion mutations on the stability, activity, conformation, and refolding of LDH were studied. These works show that the N-terminus plays a crucial role in maintaining the conformational stability and catalytic activity of LDH.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were prepared by the Genebase Biotechnology Company (the coding sequences are shown in the table). The plasmid pET21b and *E. coli* stain BL21/DE3 were purchased from Novagen. The TRIZOL Reagent, one-step RT-PCR with

Abbreviations: LDH) lactate dehydrogenase; SDS) sodium dodecyl sulfate; GuHCl) guanidine hydrochloride; RW-LD) wild-type LDH; LD5 and LD10) N-terminal deletion mutants of LDH lacking the first 5 and 10 amino acids, respectively.

* To whom correspondence should be addressed.

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Upstream and downstream primers for deletion mutants

Name	Sequence
P_1RW-LD	5' GGAATTC CATATG GCA GCT CTC AAG GAT C 3'
P_1LD5	5' GGAATTC CATATG CAG CTG ATT CAC AAC CTT C 3'
P_1LD10	5' GGAATTC CATATG CTT CTG AAG GAA GAA CAT G 3'
P_2	5' CGGAATTC TTA GAA CTG CAG CTC CTT TTG 3'

Platinum Taq, restriction enzymes, RNase, T_4 DNA ligase, and Taq DNA polymerase were purchased from Invitrogen (USA). Ampicillin, ethidium bromide, and agarose were purchased from Sangon Biotechnology Company. CM-cellulose (CM32) was a Pharmacia (Sweden) product. NADH, pyruvate, SDS, and GuHCl were Sigma (USA) products. All the other reagents were local products of analytical grade, used without further purification.

Generation and purification of mutant LDH. Total RNA of rabbit muscle was isolated using TRIZOL Reagent, and the *Ldhm* cDNA was obtained using a one-step RT-PCR system. The obtained fragment was digested with *Nde*I and *Eco*RI and inserted into vector pET-21b between the same sites. The ligation product was transferred into *E. coli* DH5α and selected with 50 μg/ml ampicillin pressure. The *Ldhm* gene in the recombinant plasmid was confirmed by DNA sequencing. The resulting construct containing the entire *Ldhm* gene was designated as pET21b-LDHM, which was used as a template for PCR amplification. The oligonucleotide primer pairs used to generate the variant *Ldh* gene were P_1LD5-P_2 and P_1LD10-P_2. The generated cDNAs were inserted into the expression plasmid pET21b and transformed into *E. coli* strain DH5α. The recombinant plasmids were extracted from the transformants and verified by sequence analysis. The correct recombinant plasmids were then transformed into the expression host *E. coli* stains BL21/DE3.

All transformants including RW-LD, LD5, and LD10 were cultured in LB broth containing 50 μg/ml ampicillin overnight at 37°C. Then 10 ml of the respective culture was inoculated into 300 ml respective LB broth containing 50 μg/ml ampicillin. When the respective culture concentration reached to optical density of 0.6-0.8 unit, IPTG was added to induce the expression of the foreign gene, and the transformants were cultured further overnight at 25°C. Then they were harvested by centrifugation. The respective precipitation was resuspended in cell lysis buffer (50 mM phosphate buffer, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, pH 7.0) and was broken by ultrasonic treatment.

RW-LD and mutants LD5 and LD10 were separated by fractional ammonium sulfate precipitation (30-65% saturation), redissolved, and dialyzed against 5 mM phosphate buffer containing 1 mM EDTA and 10 mM β-mercaptoethanol, pH 7.0. The active fractions were then loaded on a CM-32 column for ion-exchange chromatography with gradient elution carried out using 0-0.4 M NaCl in phosphate buffer (5 mM phosphate buffer containing 1 mM EDTA and 10 mM β-mercaptoethanol, pH 7.0). The active fractions were pooled and stored in 70% saturated ammonium sulfate solution.

Enzyme concentration and activity assay. Enzyme concentration was determined by measuring the absorbance at 280 nm using the absorption coefficient $A^{1\%}_{1cm} = 14.0$ [10]. Enzyme activity was determined at 30°C by measuring the absorbance change at 340 nm accompanying the oxidation of NADH and using the molar absorption coefficient $\varepsilon_{340} = 6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ as reported by Kornberg [11]. The reaction system contained 0.25 mM NADH, 0.7 mM sodium pyruvate, and 0.1 M phosphate buffer, pH 7.5 (standard measurement). Activity measurements of the mutants in the presence of stabilizing salt made use of 2 M ammonium sulfate. Enzyme concentration and activity were determined with a Specord 200 UV VIS analytical spectrophotometer (Jena, Germany).

Fluorescence spectral measurements and CD experiments. Intrinsic protein fluorescence spectra were measured using a Hitachi F2500 spectrofluorimeter (Japan). The fluorescence spectra were recorded in cuvette of 1 cm light path at 25°C. A Jasco 500C CD spectropolarimeter was used for CD measurements ranging from 190 to 250 nm. The path length of the sample cell was 2 mm. Four scans were successively performed to ensure a good signal-to-noise ratio.

Cross-linking experiments. The aggregation state of the mutant enzymes was determined by glutaraldehyde cross-linking coupled with SDS-PAGE as described elsewhere [12, 13]. Glutaraldehyde cross-linking was initiated by adding a small volume of glutaraldehyde (25% w/v) to the enzyme solutions at 1% (v/v) of the final mixture. After 2 min, cross-linking was quenched by the addition of solid $NaBH_4$ to give a final molar ratio of $NaBH_4$/glutaraldehyde of 10. After another 5 min incubation at room temperature, 10% (w/v) aqueous sodium deoxycholate

was added into the mixture to the final concentration of 0.02%, followed by careful addition of 50-100 μl of trichloroacetic acid (78% w/v) in order to destroy excess $NaBH_4$ and to achieve precipitate. The precipitate was separated by centrifugation and redissolved in 30 μl of 1.5 M Tris-HCl buffer (pH 8.8) containing 1% (w/v) SDS and 50 mM dithioerythritol. Then the samples were boiled for 5 min before SDS-PAGE to avoided artifacts due to cystine formation. SDS-PAGE was performed at room temperature with a 4-15% acrylamide gradient slab gel using the Tris-glycine buffer system described by Laemmli [14]. The gel was stained for protein using Coomassie blue stain (0.2% Coomassie blue, 50% methanol, and 10% acetic acid).

Comparison of the stability of RW-LD, LD5, and LD10. Denaturation of RW-LD, LD5, and LD10 was accomplished by incubation at low pH, elevated temperature, and high GuHCl concentrations. Reactivation of the mutants after 6 M GuHCl denaturation was started by dilution with 0.1 M phosphate buffer containing 10 mM β-mercaptoethanol, pH 7.0. The measurements of residual activity of the enzymes were determined in the presence of 2 M ammonium sulfate.

RESULTS

Characteristics of the mutants. *Activity of the mutants.* It was found that during the purification process the activity of the mutants decreased with the increased number of amino acid residues deleted from the N-terminus. The specific activities of the purified RW-LD, LD5, and LD10 were at around 650, 150, and 5 U/mg when determined by standard measurements. But when the mutants were dissolved in 0.1 M phosphate buffer, pH 7.5, plus 1 M $(NH_4)_2SO_4$ and 10 mM β-mercaptoethanol, and when they were determined in the presence of 2 M $(NH_4)_2SO_4$, their activities markedly increased. The activity of LD10 and LD5 increased 30- and 5-fold, respectively, but the activity of RW-LD decreased to 60% of its activity by standard measurement. The results implied the conformations of the mutant enzymes were more flexible than that of the wild-type enzyme, and their conformation could be restored in the presence of $(NH_4)_2SO_4$ [8].

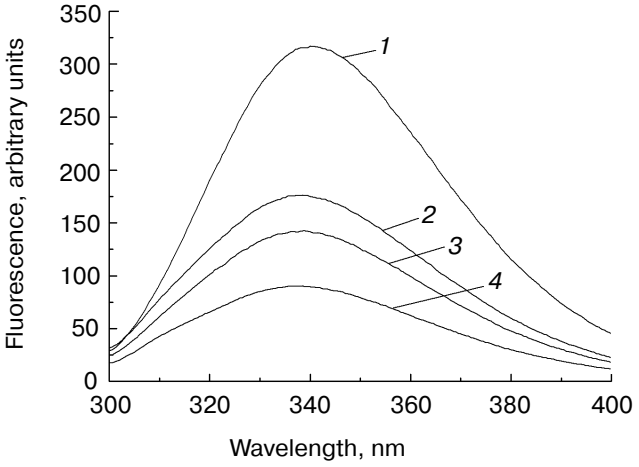

Fig. 1. Intrinsic fluorescence spectra of RW-LD (*1*), LD5 (*2*), LD10 (*3*), and LD10 in the presence of 1 M $(NH_4)_2SO_4$ (*4*). Enzyme concentration for these mutants was 0.51 μM, and the excitation wavelength was 280 nm.

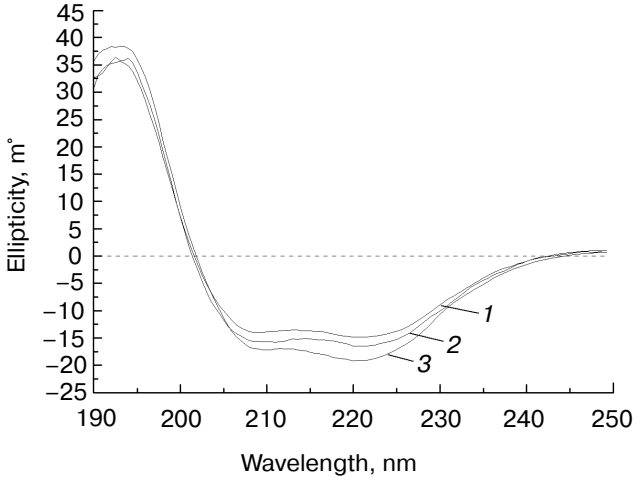

Fig. 2. CD spectra of LD10 (*1*), LD5 (*2*), and RW-LD (*3*). The concentration of the mutants was 0.51 μM.

Fluorescence and CD spectra of the mutants. The intrinsic fluorescence spectra of LD5 and LD10 were similar to that of RW-LD (Fig. 1), but the intensity of the fluorescence spectra of LD5 and LD10 decreased with the increase in the amino acid residues deleted from the N-terminus of LDH. There was a 2 nm blue shift of the peak position in the fluorescence spectrum of LD10 in the presence of $(NH_4)_2SO_4$, which indicated the conformation of LD10 became more compact in that environment [15], which might relate to its subsequent activity restoration.

The N-terminal deletion mutations had minor effects on the secondary structure in LDH. With the increased number of amino acids deleted from N-terminus of LDH, the secondary structure content in the mutants decreased to a limited degree (Fig. 2).

Aggregation state of the mutants. Cross-linking experiments were also carried out to study the aggregation state of the mutants (Fig. 3). The results also indicated that N-terminal deletion mutation did not affect the aggregation state of the mutants. The electrophoretic patterns of RW-LD, LD5, and LD10 on SDS-PAGE after cross-linking by glutaraldehyde were similar, with the main band representing the tetramer. The results indicated that the mutants mainly existed in the form of tetramer.

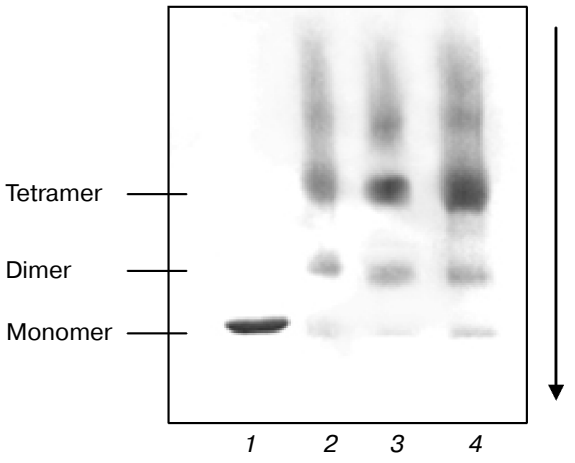

Fig. 3. Aggregation state of LDH mutants trapped by cross-linking experiments. Lanes: *1*) native LDH without cross-linking; *2*, *3*, *4*) RW-LD, LD5, and LD10 were cross-linked by glutaraldehyde before electrophoreses on a 4-15% acrylamide gradient SDS slab gel.

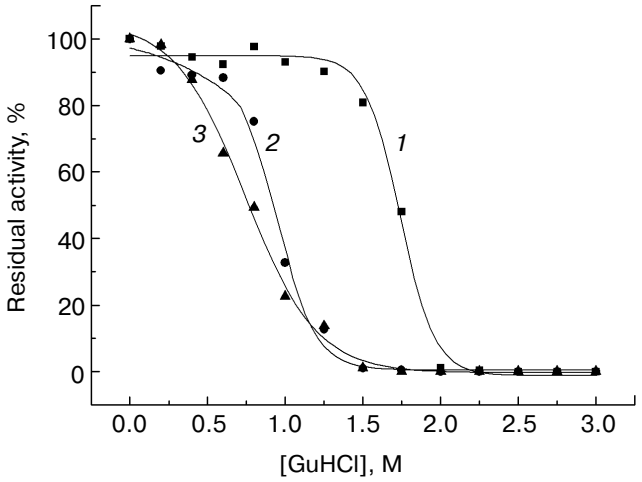

Fig. 4. Inactivation of N-terminal deletion mutants of LDH in GuHCl solutions: RW-LD (*1*), LD5 (*2*), and LD10 (*3*) were incubated in respective GuHCl solutions with different concentrations for 1 h (25°C). The residual activity was determined in the presence of 2 M $(NH_4)_2SO_4$. The concentration of the enzymes was 0.17 μM.

Stability of the mutants. To investigate the relative stability, deactivation of RW-LD, LD5, and LD10 was measured at varying GuHCl concentrations, elevated temperature, and at low pH. The enzymes were incubated under different denaturation conditions in the phosphate buffer system containing 10 mM β-mercaptoethanol and 1 M $(NH_4)_2SO_4$. Then the residual activities of the mutants were determined in the presence of 2 M $(NH_4)_2SO_4$. Figure 4 shows that inactivation of LD5 and LD10 started at lower GuHCl concentrations with low cooperativity, while RW-LD exhibited high cooperativity. The transition midpoints (50% activity after 1 h incubation) for LD10, LD5, and RW-LD were 0.77, 0.92, and 1.7 M, respectively. Similar differences in stability between the mutants and the intact enzyme were also observed in low pH-inactivation and heat-inactivation experiments. The respective transition pH values (50% activity after 1 h incubation) were 5.7 for LD10, 5.5

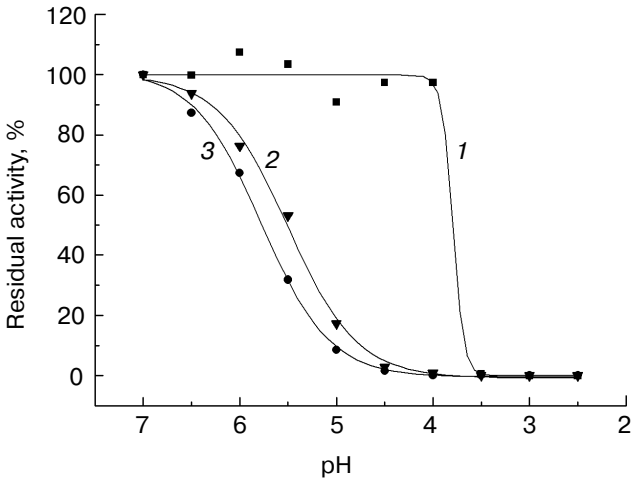

Fig. 5. Inactivation of N-terminal deletion mutants of LDH at low pH: RW-LD (*1*), LD5 (*2*), and LD10 (*3*) were incubated in respective citrate-phosphate buffer with low pH for 1 h (25°C). The residual activity was determined in the presence of 2 M $(NH_4)_2SO_4$. The concentration of the enzymes was 0.17 μM.

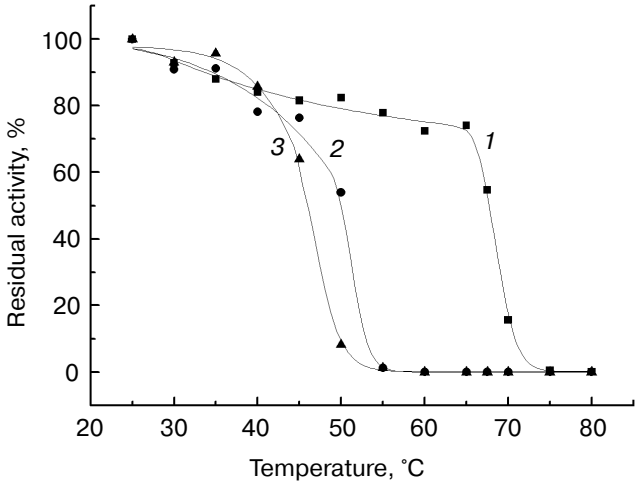

Fig. 6. Inactivation of N-terminal deletion mutants of LDH at elevated temperatures: RW-LD (*1*), LD5 (*2*), and LD10 (*3*) were incubated in phosphate buffer (pH 7.5) at different temperatures for 30 min. The residual activity was determined in the presence of 2 M $(NH_4)_2SO_4$. The concentration of the enzymes was 0.17 μM.

for LD5, and 3.8 for RW-LD (Fig. 5). The transition temperatures for LD10, LD5, and RW-LD were 46, 50, and 68°C, respectively (Fig. 6). These results showed that N-terminal deletion mutation strongly affected the stability of the mutants, increasing their susceptibility to denaturation conditions. This tendency increased with the increased number of the amino acids deleted from the N-terminus of LDH.

Unfolding and refolding of the mutants. *Unfolding of the mutants.* Unfolding equilibrium state experiments were conducted to compare the differences among RW-LD, LD5, and LD10 denatured in GuHCl solutions with different concentrations. Figure 7 shows the changes of peak position in the fluorescence spectra of the mutants and the intact enzyme in GuHCl solutions with different concentrations. With the increased deletion number of amino acids, the red shift changes in fluorescence spectra of the mutants denatured in GuHCl solutions were accelerated, which indicated that the mutants were easier to be denatured than the intact enzyme at the same GuHCl concentration. The results implied that N-terminal deletion mutation affected the structural stability of LDH, and this tendency became stronger with the increased number of deleted amino acids. But the deletion mutation had minor influence on the decrease in α-helix content in LDH mutants denatured in GuHCl solutions detected by CD spectra (data not shown), which means the deletion mutations had minor effect on the stability of secondary structure in LDH.

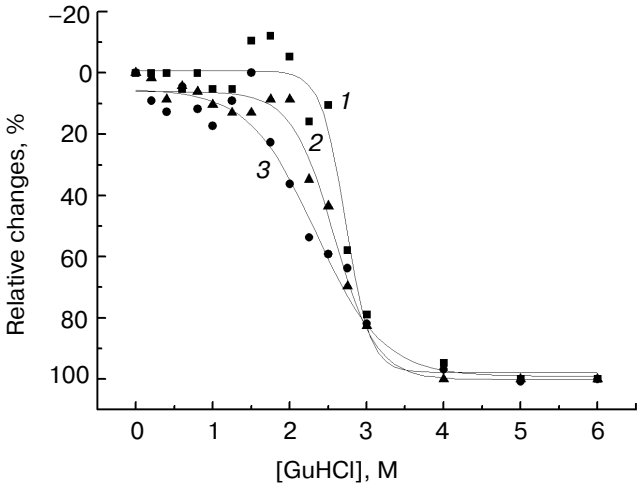

Fig. 7. Comparison of peak position in the fluorescence spectra of RW-LD (*1*), LD5 (*2*), and LD10 (*3*) after incubation in GuHCl solutions of different concentrations for 1 h at 25°C. The final concentration of the enzymes was 0.25 μM. The excitation wavelength was 280 nm, and the fluorescence spectra were measured in the range 300-400 nm. The red shifts of the fluorescence spectra for the mutants denatured in 6 M GuHCl solution were set as 100%.

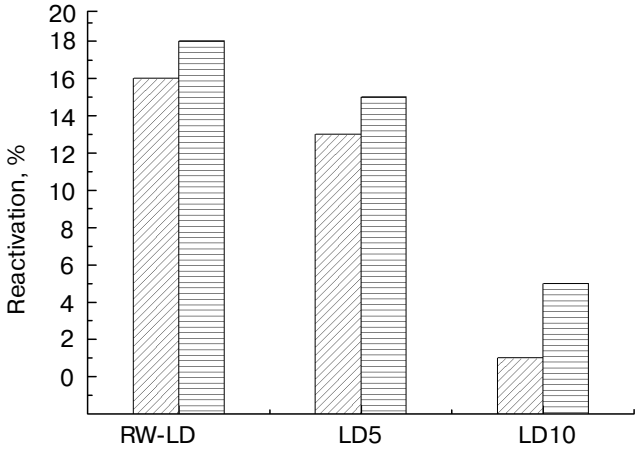

Fig. 8. Renaturation of N-terminal deletion mutants after dilution. RW-LD, LD5, and LD10 were denatured in 6 M GuHCl for 2 min, then they were diluted 100-fold in 0.1 M phosphate buffer (pH 7.5) containing 10 mM β-mercaptoethanol. After standing at 25°C for 1 h (left columns) and 12 h (right columns), their activities were determined in the presence of 2 M $(NH_4)_2SO_4$. The activity of the respective enzymes diluted 100-fold was taken as 100%.

Renaturation of the mutants. Renaturation experiments were conducted to see the effect of N-terminal deletion mutation on the reactivation ability of LDH (Fig. 8). The results show that RW-LD could regain 18% of its original activity after dilution and standing for 1 h, and its activity was restored to 20% when the renaturation time was prolonged to 12 h. The case for LD5 was similar to that of RW-LD, its activity being restored to 15 and 17% after 1- and 12-h renaturation, respectively. The renaturation ability of LD10 was much lower; it could only be restored to 3% of the original activity after 1-h renaturation and at most 7% after 12 h. The results indicated that deletions of amino acids from N-terminus of LDH influenced the renaturation of LDH, and this influence became stronger when the number of deleted amino acids reached 10.

DISCUSSION

The crystal structure of LDH showed that the tetrameric enzyme is a dimer of two dimers that is associated by the N-terminus [1]. The interaction between subunits at the R-axis interface is predominantly polar, and it is dominated by the interactions with the N-terminal arm. Residues 7 to 9 in the arm of one subunit form an antiparallel sheet with residues 301 to 304 of another subunit, and thus the interaction between subunits are strengthened [16]. So, the N-terminus of LDH plays an important role of in subunit assembly, association, and structural stability.

N-Terminal deletion mutation decreases the activity of LDH. When 10 amino acids were deleted from the N-

terminus, the enzyme was completely inactivated. But in the presence of the structure making salt $(NH_4)_2SO_4$, the deletion mutant could exhibit comparable activity, which is in accordance with previous works [8, 9]. The structures of the mutants are more flexible than that of the intact enzyme, and $(NH_4)_2SO_4$ can induce their structure to more compactness. The intrinsic fluorescence spectra and CD spectra of the mutants are similar to those of the intact enzyme, which indicate the whole structures of the mutants are similar to that of the intact enzyme. The results can be attributed to the N-terminus standing out from the main body of the subunit.

In this investigation, it was found that deletion mutations of the N-terminus markedly influence the stability of the mutant enzymes. They are easily inactivated and unfolded under weaker denaturation conditions than that needed for the intact enzyme. Renaturation experiments show that the renaturation ability of the mutants decreases with the increase in number of amino acids deleted from LDH N-terminus, which indicates that N-terminus plays a critical role in association of LDH subunits.

During the reconstitution of porcine muscle lactate dehydrogenase from its denatured polypeptide chains, Girg et al. [8] were able to show that thermolysin treatment during renaturation yields "proteolytic dimers" as stable entities. Opitz et al. [9] purified the dimers and studied their properties. They found these dimers consisted of the intact chain lacking the N-terminal 10-11 amino acid residues. But, unfortunately, the C-terminus of the dimers was heterogeneous, varying in the range of 326 ± 5 residues. In this investigation, the results are quite different; cross-linking experiments proved that the mutant (LD10) lacking 10 amino acid residues from the N-terminus of LDH is still a tetramer. Maybe the difference between the above results and ours could be attributed to the heterogeneous C-terminus of their proteolytic products and the intact C-terminus of the mutants in our investigation. Although N-terminal deletion mutation up to 10 amino acid residues has no effect on the aggregation state of LDH, it still results in the LDH being more flexible and unstable, which stresses the role of the N-terminus in stabilizing the structure of LDH.

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