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Conversion of cofactor specificities of alanine dehydrogenases by site-directed mutagenesis

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Abstract

Most alanine dehydrogenases (AlaDHs), including that of *Phormidium lapideum*, a cyanobacterium, whose X-ray structure has been determined (PlaAlaDH, PDB entry: 1PJC), are strictly NAD^+-specific. However, AlaDH from a psychrophile, *Shewanella* sp. Ac10 (SheAlaDH), exhibits dual specificity for NAD^+ and $NADP^+$. As Ile198 at the cofactor-binding site of PlaAlaDH is replaced by a corresponding arginine (Arg199) in SheAlaDH, we speculate that this arginine residue may serve as the binding site for the 2′-phosphate group of $NADP^+$ in SheAlaDH. To verify this speculation, Arg199 of SheAlaDH was replaced by isoleucine via site-directed mutagenesis, and the resulting mutant enzyme (Arg199Ile) indeed was shown to act specifically on NAD^+. On the other hand, Ile198Arg mutant of PlaAlaDH acted not only on NAD^+ but also on $NADP^+$. It was also observed that Asp197 of PlaAlaDH is conserved among various NAD^+-specific amino acid dehydrogenases, including SheAlaDH, but is replaced by hydrophobic amino acids in $NADP^+$-specific enzymes. To investigate the relevance of this residue, mutant SheAlaDHs with Asp198 replaced by either Gly, Ala, Val or Leu were obtained. The k_{cat}/K_m values for $NADP^+$ were increased from 5- to 270-folds by the mutation with Asp198Ala being the best catalyst. Thus, AlaDHs with modified coenzyme specificities can be obtained by single site-specific mutations.

Keywords: Alanine dehydrogenase; Cofactor binding site; Cofactor specificity; $NAD(P)^+$-dependent enzyme

1. Introduction

NAD^+ and $NADP^+$ are two similar redox cofactors found in biological systems. $NADP^+$ is structurally different from NAD^+ only by the presence of an additional phosphate group esterified at the 2′-hydroxyl group of its AMP moiety. It has been found that the structures of the cofactor-binding domains for both NAD^+ and $NADP^+$ in $NAD(P)^+$-dependent enzymes are very similar to each other.

Recently, based on the results of the amino acid sequence comparison between the NAD^+-specific leucine dehydrogenase (LeuDH) [L-leucine: NAD^+ oxidoreductase, deaminating, EC 1.4.1.9] from *Thermoactinomyces intermedius* (TinLeuDH) and $NADP^+$-dependent oxidoreductases, a set of amino acid residues probably determining the coenzyme specificity of TinLeuDH was assigned. Systematic replacement of these amino acid residues by other residues was carried out with the aim of switching its natural coenzyme specificity to one exhibiting preference for $NADP^+$ [1].

Alanine dehydrogenase (AlaDH) [L-alanine: NAD^+ oxidoreductase, deaminating, EC 1.4.1.1], which catalyzes the reversible oxidative deamination of L-alanine to pyruvate, has been isolated from *Bacillus* species and other bacteria [2–8]. AlaDH, an A-type stereospecific enzyme, is different from other amino acid dehydrogenases in cofactor stereospecificity [9]. Majority of AlaDHs, including that from a cyanobacterium *Phormidium lapideum*, are NAD^+-dependent enzymes. Nonetheless, AlaDH from a psychrophile, *Shewanella* sp. Ac10 (SheAlaDH), also ex-

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hibits activity towards $NADP^+$ as well [10]. Comparison of the amino acid sequence of this AlaDH with those of other NAD^+-dependent amino acid dehydrogenases reveals that an Arg residue, which is expected to be involved in binding with the 2′-phosphate group of $NADP^+$, occurs at the cofactor-binding site of SheAlaDH.

In this paper, we describe modification of cofactor specificities of AlaDHs from *P. lapideum* (PlaAlaDH) and SheAlaDH by site-directed mutagenesis.

2. Experimental

2.1. Materials

Restriction and modification enzymes were purchased from TaKaRa Biochemicals. NAD^+ and $NADP^+$ were from Oriental Yeast Co. All other chemicals were from Wako Pure Chemicals. Oligonucleotides were from Biologica and Griner (Japan). Plasmid pSheAlaDH containing the gene of SheAlaDH was prepared as previously described [10]. *Escherichia coli* MV1184 and vector plasmids pUC18 and pUC119 were used for the gene expression.

2.2. Mutagenesis

Site-directed mutations were introduced into AlaDH gene with a DNA mutagenesis kit (Amersham Biosciences) and Mutan Super Express kit (TaKaRa Biochemicals) [11]. The mutant enzymes and synthetic mutagenic primers used were: R199I, 5′-GATACTGATATCCAAAACAAC-3′; I198R, 5′-CAAATTTTTGACAGGAATGTG-3′; D198G, 5′-GTAATGCATCGATACTGCGACCCAAAACAAC-3′; D198A, 5′-GTAATGCATCGATACTGCGAGCCAAAACAAC-3′; D198V, 5′-GTAATGCATCGATACTGCGAACCAAAACAAC-3′; D198L, 5′-GTAATGCATCGATACTGCGAAGCAAAACAAC-3′. DNA sequencing to confirm mutations was performed with a DNA sequencer 370A and Genetic Analyzer 310 (Applied Biosystems).

2.3. Enzyme and protein assay

The wild-type and mutant SheAlaDHs and PlaAlaDHs were purified from the recombinant *E. coli* MV1184 cells as previously described [5,10]. AlaDH activity was assayed at 30 °C (SheAlaDH) or 45 °C (PlaAlaDH) as previously described [5] by following the reduction of $NAD(P)^+$ at 340 nm in Beckman DU650 spectrophotometer. One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADH per minute. The kinetic constants were obtained from duplicate or triplicate measurements of the initial rates at varying concentrations of $NAD(P)^+$ under a fixed concentration of L-alanine (70 mM). As an index of the coenzyme preference, activity measurements were taken at 20 mM L-alanine and 0.5 mM $NAD(P)^+$. The protein concentration was determined with a Bio-Rad protein assay kit [12].

3. Results and discussion

3.1. Prediction of the coenzyme binding site of SheAlaDH

The amino acid sequence of the NAD^+-binding site of PlaAlaDH was compared with those of other NAD^+-specific amino acid dehydrogenases including SheAlaDH [13–16] (Fig. 1). Asp197 of PlaAlaDH is conserved among various amino acid dehydrogenases, and has been shown to form hydrogen bonds with the 2′,3′-hydroxyl groups of the NAD^+ adenine ribose [17–19].

SheAlaDH is unique because it contains an arginine residue (Arg199) at the position next to the conserved Asp residue. SheAlaDH probably acts on $NADP^+$ through an ionic interaction between 2′-phosphate group of $NADP^+$ and this Arg residue.

3.2. Construction of I198R mutant AlaDH from P. lapideum

The three-dimensional structure of the cofactor-binding site of PlaAlaDH [17] indicates that the side chain of Ile198 is located at the vicinity of the adenosine moiety of NAD^+ (Fig. 2). Ile198 of PlaAlaDH is replaced by Arg in SheAlaDH (Arg199), and this Arg residue is expected to interact with the 2′-phosphate group of $NADP^+$. It is reasonable to assume that a mutant PlaAlaDH, in which Ile198 is replaced by Arg, can probably bind $NADP^+$ in the same manner like SheAlaDH. Therefore, we constructed the mutant PlaAlaDH, and determined its steady-state kinetic parameters (Table 1). As expected, the mutant enzyme acted also on

Leucine dehydrogenase, *T.intermedius* (X79068)	174	**GVGHVA**	203	**DINKENAD**
Leucine dehydrogenase, *B. sphaericus* (AB103119)	180	**GLGNVA**	203	**DVNKAAVS**
Glutamate dehydrogenase, *C. symbiosum* (Z11747)	237	**GFGNVA**	261	**GPDGYIYD**
Phenylalanine dehydrogenase, *Rhodococcus* sp. (U08381)	183	**GGGAVG**	205	**DTDTERVA**
Alanine dehydrogenase, *Shewanella* sp. (AF070715)	175	**GGGMVG**	198	**DRSIDALR**
Alanine dehydrogenase, *P. lapideum* (D37807)	174	**GGGVVG**	197	**DINVERLS**

Fig. 1. Alignment of amino acid sequences around the cofactor-binding site of AlaDHs and other amino acid dehydrogenases. Their GenBank accession numbers are shown in parentheses.

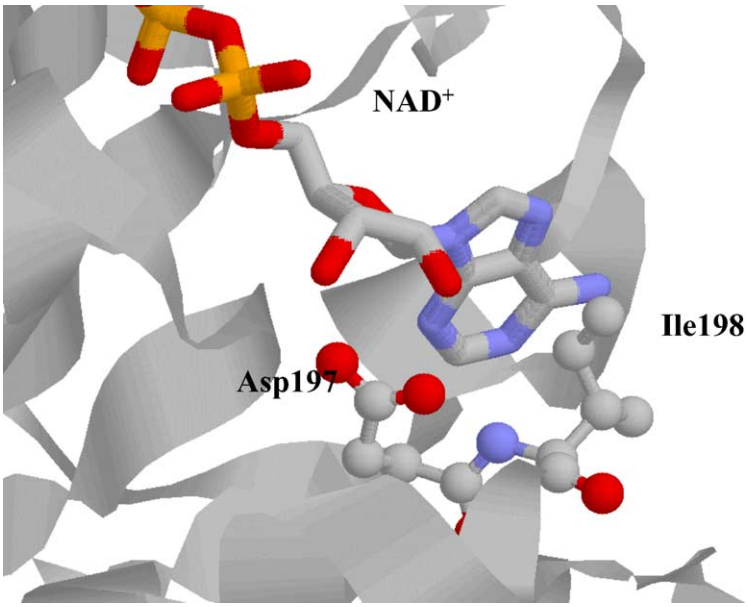

Fig. 2. Cofactor-binding site of AlaDH from *Phormidium lapideum* [17] drawn with the program RasMol [20].

$NADP^+$ in contrast to the original PlaAlaDH acting exclusively on NAD^+. The K_m and k_{cat} values of the mutant enzyme were similar to those of SheAlaDH with either NAD^+ or $NADP^+$ as a coenzyme. Thus, we successfully converted PlaAlaDH to a type of SheAlaDH with the same coenzyme specificity by a single amino acid mutation.

3.3. Construction of R199I mutant AlaDH from *Shewanella* sp. Ac10

We explored whether SheAlaDH can be converted to an enzyme similar to that of PlaAlaDH in its coenzyme specificity in the same manner as described above. A mutant SheAlaDH, in which Arg199 was replaced by Ile, was constructed and characterized as shown in Table 1. The mutant enzyme showed strict coenzyme specificity towards NAD^+ in the same manner as the wild-type PlaAlaDH. The k_{cat} value of the mutant SheAlaDH was about ten times lower than that of the wild-type PlaAlaDH with NAD^+ as a coenzyme, yet the k_{cat}/K_m values of the mutant enzyme was only three times lower than that of wild-type PlaAlaDH. Therefore, an enzyme virtually equivalent to PlaAlaDH was constructed by a simple mutation of SheAlaDH. Neither of the wild-type SheAlaDH nor the I198R mutant of PlaAlaDH acted on 3′-$NADP^+$ as a coenzyme (data not shown), and the 3′-phosphate group of 3′-$NADP^+$ is located far from Arg199 of SheAlaDH and Arg 198 of the mutant PlaAlaDH.

Table 1
Kinetic parameters of the wild type and mutants of AlaDH

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
NAD^+-binding AlaDH			
Shewanella sp. Ac10			
Wild type	0.035	34.7	991
R199I	0.010	9.50	950
Phormidium lapideum			
Wild type	0.036	95.5	2650
I198R	0.150	95.4	640
$NADP^+$-binding AlaDH			
Shewanella sp. Ac10			
Wild type	4.00	1.60	0.40
R199I	–[a]	0.00	0.00
Phormidium lapideum			
Wild type	–[a]	0.00	0.00
I198R	2.80	1.30	0.46

[a] Not measurable.

3.4. Construction of mutant AlaDHs markedly preferring $NADP^+$ as a coenzyme

The aspartic acid at residue number 198 in SheAlaDH is conserved among various NAD^+-specific enzymes, but is usually replaced by a hydrophobic residue such as Val and Ile in $NADP^+$-dependent oxidoreductases [21–23]. To construct mutant SheAlaDHs that exhibit significant preference for $NADP^+$, Asp198 was replaced by Gly, Ala, Val or Leu (Table 2). The K_m values for $NADP^+$ of the mutant enzymes were decreased 2–11 times lower than that of the wild-type enzyme. On the other hand, k_{cat} values for the mutant enzymes were increased 3–31 times higher than that of the wild-type enzyme. Consequently, k_{cat}/K_m values for the mutant enzymes were 5–270 times higher than that of the wild-type enzyme with $NADP^+$ as a coenzyme (Table 2). The mutant enzymes were much poorer catalysts than the wild-type enzyme with NAD^+ as a coenzyme: k_{cat}/K_m values for the mutant enzymes were 72–5000 times lower than the wild-type enzyme (Table 2). Thus, SheAlaDH was successfully converted to a series of new enzymes with im-

Table 2
Kinetic parameters of the wild type and mutants of *Shewanella* AlaDH

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
$NADP^+$-binding AlaDH			
Wild type	4.0	1.6	0.4
D198G	0.67	24.1	36.0
D198A	0.35	37.8	108.0
D198V	1.1	50.2	45.6
D198L	2.1	4.8	2.3
NAD^+-binding AlaDH			
Wild type	0.035	34.7	991.4
D198G	3.0	41.2	13.7
D198A	5.6	43.5	7.8
D198V	5.2	41.2	7.9
D198L	11.4	1.9	0.2

proved preference for $NADP^+$. It is interesting to note that the D198A and D198V mutants showed similar K_m and k_{cat} values not only for $NADP^+$, but also for NAD^+, despite the differences in size of their side-chains. On the basis of k_{cat}/K_m values, the D198A mutant was the best catalyst with $NADP^+$ as a coenzyme.

In the wild-type enzyme, the negative charge of Asp198 probably repels the 2′-phosphate group of $NADP^+$ by electrostatic repulsion. However, the side-chains of the altered residue at amino acid position 198 in the SheAlaDH mutant enzymes do not interact with $NADP^+$. Thus, steric hindrance by the side-chains may result in a change in the binding position of $NADP^+$.

In NAD^+-dependent leucine dehydrogenase (LeuDH) from *T. intermedius*, a set of amino acid residues that are supposed to determine its coenzyme specificity was replaced [1]. A triple mutant D203A-I204R-D210R showed the best k_{cat}/K_m value for $NADP^+$. However, we have shown here that we can easily modify coenzyme specificities of alanine dehydrogenases by single amino-acid substitutions. This example will provide us with a clue to design amino acid dehydrogenases with new cofactor specificities.

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