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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation
Unique coenzyme binding mode of hyperthermophilic archaeal NAD(P)-dependent dehydrogenases
（超好熱アーキア由来 NAD(P)依存性脱水素酵素が示す新規な補酵素結合様式）

学位論文要約：
Dissertation Summary

Introduction

Hyperthermophiles growing at temperatures around 100°C produce extremely heat-stable cell components. In particular, the enzymes from hyperthermophiles exhibit extreme stability against detergents, organic solvents, and some other chemical reagents, as well as high temperature. Therefore, these enzymes are expected to have much potential for application to industrial processes. Most hyperthermophiles belong to Archaea, the third domain of life, and attention has been paid to their unique metabolism and related enzymes which are not present in Eukarya and Bacteria.

During the course of the studies about synthetic pathways of amino acids and phospholipids in hyperthermophilic archaea, we found that the two key dehydrogenases in these pathways exhibit new variations of the cofactor binding. One is homoserine dehydrogense (HseDH) involved in the biosynthetic pathway from aspartate to homoserine, which is a common precursor for the synthesis of three amino acids, methionine, threonine and isoleucine. The other is glycerol-1-phosphate dehydrogenase (G1PDH) involved in synthesis of *sn*-glycerol-1-phosphate, which is an archaea-specific glycerophosphate backbone of phospholipids. We examined the detailed characteristics of these enzymes and determined the crystal structures, and then analyzed the factors responsible for their unique cofactor-binding mode.

Results and Discussion for HseDH

A gene encoding a HseDH was identified in the hyperthermophilic archaeon *Pyrococcus horikoshii*. The gene was overexpressed in *Escherichia coli*, and its product was purified and characterized. The expressed enzyme is the most thermostable HseDH yet described, retaining 70% of its activity even after incubation for 10 min at 95ºC. The crystal structure of the enzyme was determined at a resolution of 2.30 Å. Refinement of the structure and mass analysis showed the presence of the bound cofactor NADPH, although it has not been added during crystallization. This suggests earlier high-affinity binding of the cofactor to the protein (Fig. 1-A). To our surprise, however, NADP did not act as a cofactor with this enzyme, but as a strong inhibitor of NAD-dependent homoserine oxidation. The double-reciprocal plots of *v* versus the NAD concentration at several fixed concentrations of NADP suggest competitive inhibition. Ki value for NADP was found to be extremely low (about 5 nM). Within *Pc. horikoshii* HseDH, the C2'-phosphate group (O1X, O2X, and O3X) of the adenine ribose is tightly held at its position through interactions with the side chain and backbone N atom of Arg40, the side chain of Lys57, and via a water molecule (W4), the backbone O atom of Gly61. To reduce the number of these interactions, we constructed R40A and K57A mutants, which had striking effects on the enzymes reactivity against NADP. In particular, K57A substitution had a strongly positive effect on NADP kinetics. The mutant enzyme exhibited a $Vmax^{NADP}$ of 43.3 μmol·min^{-1}·mg^{-1}, which is about 38% of the wild-type $Vmax^{NAD}$ (114 μmol·min^{-1}·mg^{-1}). These results suggest that the inhibition by NADP is caused by its strong binding to *Pc. horikoshii* HseDH. We next determined the structure of K57A mutant at a resolution of 2.43 Å. Superposition of the Hse/NADPH-bound K57A structure onto the NADPH-bound wild-type structure showed that the NADPH molecule in the mutant structure was positioned/configured nearly identically to the NADPH molecule in the wild-type structure, except for the positioning of the C2'-phosphate group of the adenine ribose (Fig. 1-B). As described above, the C2' phosphate is tightly held in position through five surrounding hydrogen bonds in the wild-type enzyme. In K57A mutant, however, the C2'-phosphate group is rotated in a clockwise direction around C2B of NADPH by

about 30° relative to the wild-type structure. In addition, the guanidino group of Arg40 in the mutant is also rotated clockwise by about 90° around the NE atom of Arg40 relative to the wild-type structure. As a result, only one interaction between O3X of the C2’ phosphate and the NE of Arg40 was observed at the corresponding position in K57A. These observations suggest that the large number of interactions between the cofactor and the enzyme are responsible for the lack of reactivity of the enzyme towards NADP. This is the first example of an enzyme in which the very strong binding of NADP is an obstacle to its catalytic activity.

Results and Discussion for G1PDH

A gene encoding a G1PDH was identified in the hyperthermophilic archaeon *Pyrobaculum calidifontis*. The gene was overexpressed in *E. coli*, and its product was purified and characterized. In contrast to conventional G1PDHs, the expressed enzyme showed strong preference for NADH: the reaction rate with NADPH ($Vmax^{NADPH}$ = 0.42 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was only 2.4% of that with NADH ($Vmax^{NADH}$ = 17.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$). The crystal structure of the enzyme was determined at a resolution of 2.45 Å. The asymmetric unit consisted of one homohexamer. Refinement of the structure and HPLC analysis showed the presence of the bound cofactor NADPH in subunits D, E, and F, even though it was not added in the crystallization. The phosphate group at C2’ of the adenine ribose of NADPH is tightly held through the five biased hydrogen bonds with Ser40 and Thr42. However, NADP did not act as a cofactor with this enzyme, but as a strong inhibitor of NADH-dependent dihydroxyacetone phosphate (DHAP) reduction. The double-reciprocal plots of *v* versus NADH concentrations at several fixed concentrations of NADPH showed a typical competitive inhibition pattern. Ki for the NADPH was determined to be about 0.06 μM from Dixon plots. In comparison with *Methanocaldococcus jannaschii* G1PDH structure, the NADPH molecule was observed to be pushed about 2.5 Å away from the normal coenzyme binding site (Fig. 2-A). The relative positions of some residues that interact with the cofactor differ greatly in the two enzymes. By comparing the interactions around the C2’-phosphate group of the NADPH adenine ribose between the two enzymes, we found

that the phosphate group is tightly held in a position through six surrounding hydrogen bonds formed by Asn38, Thr39, and Tyr52 in *M. jannaschii* G1PDH, whereas five one-sided interactions formed by Ser40 and Thr42 were observed at the corresponding position in *Pb. calidifontis* G1PDH (Fig. 2-B). To reduce the number of biased hydrogen-bonding interactions around the C2'-phosphate group of the NADPH adenine ribose, we constructed a S40A/T42A double mutant of *Pb. calidifontis* G1PDH. Using HPLC analysis, the enzyme-bound cofactor was not detected with the purified mutant. Interestingly, the S40A/T42A double mutant enzyme acquired about 27 times higher reactivity ($Vmax^{NADPH}$ = 11.3 μmol·min^{-1}·mg^{-1}) than the wild-type enzyme with NADPH. Additionally, the mutant enzyme retained a reactivity for NADH ($Vmax^{NADH}$ = 16.9 μmol·min^{-1}·mg^{-1}), which is about 93% of the wild-type $Vmax^{NADH}$. These observations suggest that the biased interactions around the C2'-phosphate group by Ser40 and Thr42 make NADPH binding insufficient for catalysis. Our finding suggests a new variation on a structural basis for cofactor preference.

Conclusion

NAD(P)-dependent dehydrogenases differ according to their coenzyme preference: some prefer NAD, others NADP, and still others exhibit dual cofactor specificity. Because NAD differs from NADP only in the C2'-phosphate group of the adenine ribose, the amino acid residues interacting with this region are thought to be responsible for the cofactor specificity of the enzymes. The primary determinant of NAD specificity is the presence of an Asp or Glu residue, which forms hydrogen bonds with both the C2'-and C3'-hydroxyl groups of the NAD adenine ribose and occupies the space that would be occupied by the C2'-phosphate group of the NADP adenine ribose. In NADP-dependent enzymes, on the other hand, this residue is usually replaced by a smaller residue such as Gly, Ala or Ser, accompanied by one or more positively charged residues, Lys and/or Arg, which form a binding pocket for the C2'-phosphate group. In some enzymes with dual cofactor specificity, replacement of the hydrogen bonds associated with each cofactor binding has been observed.

The molecular basis for the cofactor preference observed in HseDH from *Pc.*

horikoshii and G1PDH from *Pb. calidifontis* greatly differs from those of the conventional NAD(P)-dependent dehydrogenases. The present study indicates that the molecular details underlying cofactor preference in NAD(P)-dependent dehydrogenases are more complex than expected, and the cofactor specificity cannot be predicted even from the structural information in the absence of biochemical data.

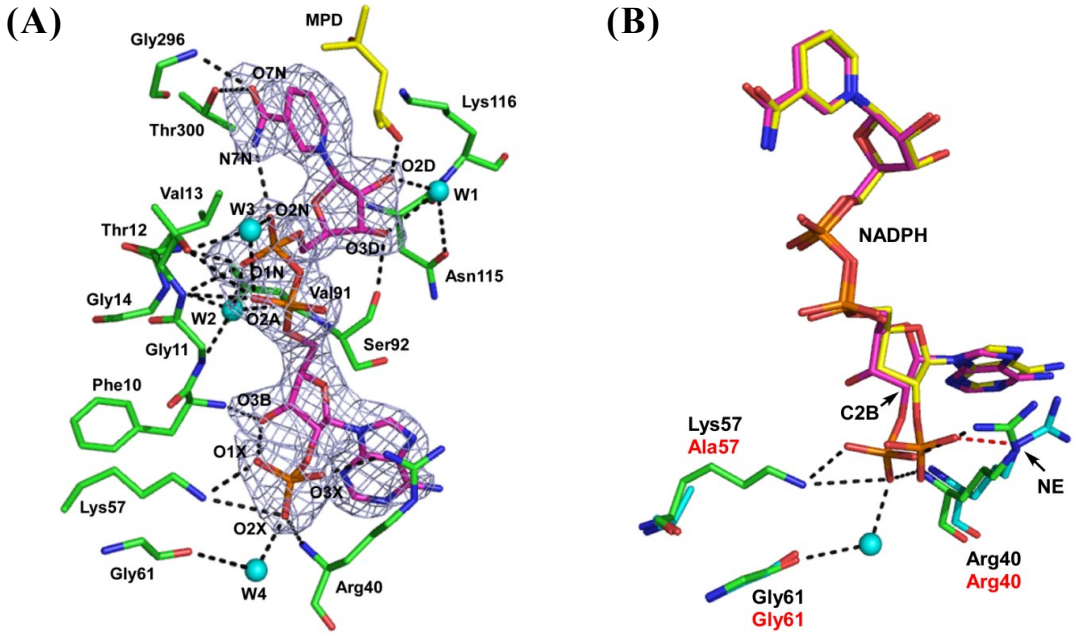

Figure 1. (A) Close-up of NADPH bound to *Pc. horikoshii* HseDH. The networks of hydrogen bonds are shown as dashed lines. Residues that interact with NADPH are shown in green. NADPH is in magenta, and 2-metyl-2,4-pentanediol (MPD) is in yellow. W1-W4 indicate water molecules. The final σA-weighted Fo - Fc omit electron density map for NADPH is shown at the 3σ level. Oxygen, phosphate and nitrogen atoms are shown in red, orange and blue, respectively. (B) Comparison of the NADPH-binding site structures in *Pc. horikoshii* HseDH wild-type (green and black labels) and the K57A mutant (cyan and red labels). NADPH molecules in wild-type and K57A are shown in magenta and yellow, respectively. The hydrogen bonds around the C2'-phosphate group of the adenine ribose are shown as black dashed lines in the wild-type protein and a red dashed line in the K57A mutant. The C2B atom in NADPH and NE atom in Arg40 are labeled.

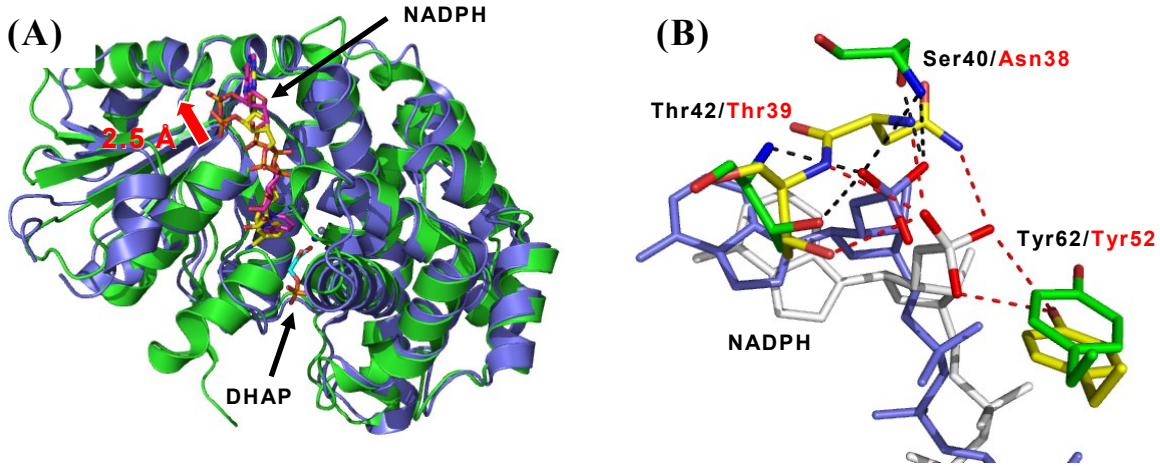

Figure 2. (A) Superposition of the structures of *Pb. calidifontis* G1PDH (green) and *M. jannaschii* G1PDH (light blue). NADPH molecules in *Pb. calidifontis* G1PDH and *M. jannaschii* G1PDH are shown in magenta and yellow, respectively. DHAP molecule in *M. jannaschii* G1PDH is shown in cyan. (B) Comparison of C2'-phosphate group of the adenine ribose binding site structures in *Pb. calidifontis* G1PDH (green and black labels) and *M. jannaschii* G1PDH (yellow and red labels). NADPH molecules in *Pb. calidifontis* G1PDH and *M. jannaschii* G1PDH are shown in light blue and white, respectively.