Structure-based conversion of coenzyme specificity in polysaccharide-metabolizing reductases and their application in biofuel production

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Research aims

To build a sustainable society and reduce dependence on fossil fuels such as petroleum and coal, biofuels are now being investigated as an alternative energy source. An issue in Japan is that that there is limited land available for biomass production. To overcome this, the Japanese government encourages the use of marine biomass (e.g. seaweed) for energy production. Acidic polysaccharides, alginate, and fucoidan, all abundant in brown algae, are likely to be used for biofuel production in the future[1)]. To highlight the potential of such technology, we have previously demonstrated bacterial production of bioethanol from alginate[2)]. NADH or NADPH-dependent reductases are essential for the metabolism of uronate-containing acidic polysaccharides such as alginate and pectin. These reductases are classified as short-chain dehydrogenase/reductases (SDR) and have a common scaffold (three layer α/β/α). Cellular coenzyme balance is also important for efficient microbial production of valuable products. Since NADPH is ten times more expensive than NADH, adding NADH dependence to NADPH specific enzymes is a valuable cost saving option. Although NADH and NADPH are structurally similar, there has been limited success in converting coenzyme specificity while maintaining high enzyme activity. Our study therefore aimed to clarify structural determinants for coenzyme specificity in bacterial reductases using X-ray crystallography. These included compounds crucial for the metabolism of acidic polysaccharides, such as alginate, fucoidan, and pectin. Understanding these structural determinants will contribute to improvements in biotechnology by modifying coenzyme specificity in reductases, increasing productivity,

and reducing costs in genetically engineered microbes or enzymes.

Methods

Bacterial reductases for the metabolism of acidic polysaccharides were crystallized in the presence of coenzymes by multiple screening in 96-well plates. Diffraction data were collected at SPring-8 (Hyogo, Japan) and processed using *HKL2000* software. Molecular replacements for structural determinations were calculated using *Molrep*, using coordinates from coenzyme-free enzymes to create the initial models. Structural refinement was performed using *Refmac5*.

Structure-based conversion of coenzyme specificity was performed using protein engineering.

Results

(1) Alginate-metabolizing reductases

Two alginate-metabolizing reductases (NADPH-specific A1-R and NADH-specific A1-R′) are present in the alginate-assimilating *Sphingomonas* sp. strain A1. The enzymes are similar in primary structure. The crystal structure of A1-R′ in complex with NAD^+ was determined and compared to $NADP^+$-bound A1-R[3]. Significant differences in space (A1-R, 44.0 $Å^3$ and A1-R′, 1.15 $Å^3$) and charge (A1-R, positive and A1-R′, negative) were observed. The residues important for structural determinants were located in two loops, one short and one long (Fig. 1). Coenzyme specificity in both enzymes was successfully converted through the exchange of the two loops.

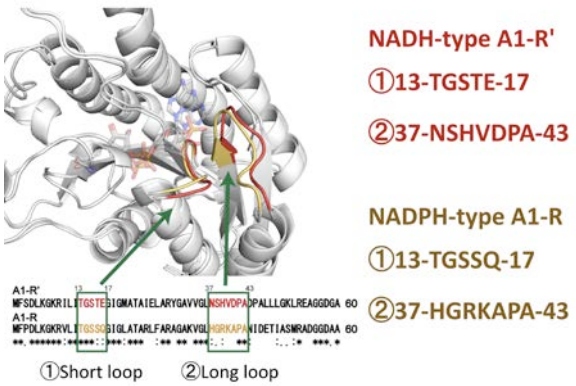

Fig. 1. Two loops for coenzyme recognition

(2) Fucoidan-metabolizing reductase

Fucoidan contains several glucuronic acid residues and reductases acting on α-keto acid derived from glucuronic acid. They are required for metabolism of polysaccharides[4]. We determined the crystal structure of the NADH-specific reductase DhuD from *Streptococcus pyogenes* (Fig. 2, left), both in and out of complex with

coenzyme. Although no crystal structure in complex could be obtained, DhuD showed a scaffold similar to NADPH-specific A1-R and NADH-specific A1-R′. Comparisons of coenzyme-binding sites across DhuD, A1-R, and A1-R′ revealed that DhuD has two A1-R′-type loops (space, 1.05 Å^3; charge, negative) for NADH specificity.

(3) Pectin-metabolizing reductase

The reductase KduD involved in metabolizing pectin can utilize both NADH and NADPH. The crystal structure of KduD from *Pectobacterium carotovorum* subsp. *carotovorum* in complex with NAD^+ was determined (Fig. 2, right)[5]. This demonstrated that KduD features a space of 15.9 Å^3 located at the coenzyme (2′ site of adenylate ribose)-binding site. This space has no charge at pH 7.0.

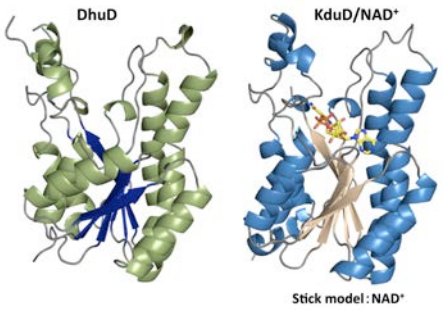

Fig. 2. Structure of DhuD and KduD

Conclusions

The structural determinants (space volume and surface charge) of coenzyme specificity in bacterial reductases were identified through analysis of their structural and functional relationships (Fig. 3). These enzymes are central to the metabolism of acidic polysaccharides. Understanding these determinants will contribute to the establishment of structural-based conversion of coenzyme specificity in reductases to be used in biotechnology.

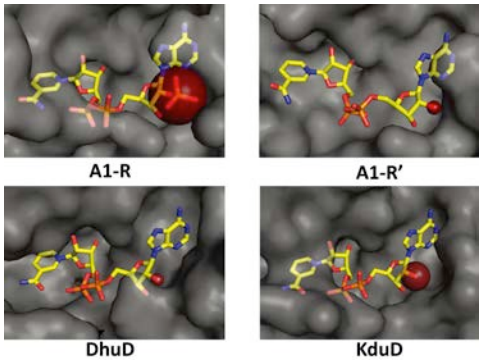

Fig. 3. Coenzyme-binding site

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5) Takase, R., Maruyama, Y., Oiki, S., Mikami, B., Murata, K., and Hashimoto, W. (2016) Structural determinants in bacterial 2-keto-3-deoxy-D-gluconate dehydrogenase KduD for dual-coenzyme specificity. *Proteins* **84:** 934-947.