S6-1

Development of New Biocatalysts and Their Uses in Organic Synthesis

- Selection, Fine Tuning, and Mechanisms -

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In screening environmental samples for microbes whose enzymes have applications in the field of biocatalysis, a key consideration is the design and evolution of novel isolation strategies. Such strategies are especially essential given that the physiology and growth conditions of many microorganisms are poorly understood. By showing a few examples of screening new enzymatic reactions and their applications, I should like to show that we encounter various cryptic mechanisms which could not be easily solved by the current genome studies. These catalyze typical chemo-, region- or stereo-selective reaction. Since natural enzymes are sometimes not suitable for production of useful chemicals, I will also show an example of a "fine-tuning" of one of the enzymes. These examples indicate the bright future of post-genomic screening for new biocatalysts (1).

1. Discovery and application of the "aldoxime-nitrile pathway" in microorganisms (1-9). [Chemo-selectivity]

Discovery of high accumulation of amides by use of a bacterial enzyme "nitrile hydratase" has led to successful application of the enzyme to the industrial production of various amides. Now, the enzyme has become one of the most important industrial enzymes. Recently, we are successful in the enzymatic synthesis of nitriles from aldoximes by using a new microbial enzyme aldoxime dehydratase, which functions in "Aldoxime-Nitrile" pathway (Fig. 1). We focused on aldoxime-dehydration enzyme, not only to study the microbial metabolism of aldoximes in nature, but also to apply the enzyme to organic synthesis.

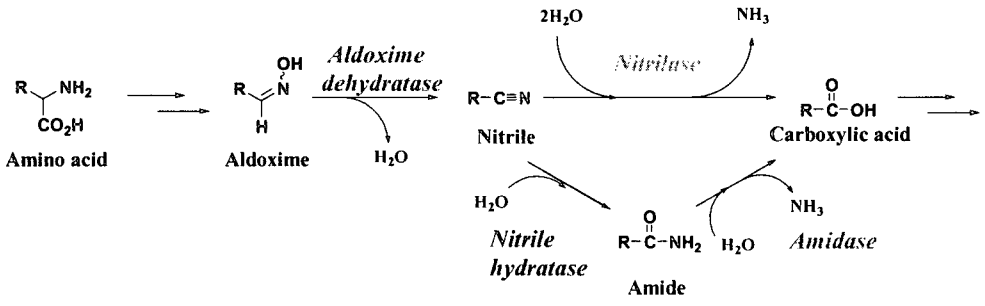

Fig. 1. The "Aldoxime-Nitrile" pathway.

We isolated aldoxime-degrading microorganisms from soil and screened for the activity among them. Bacterial strains OxB-1 and YH3-3, which degrade *Z*-phenylacetaldoxime (Z-PAOx) and *E*-pyridine-3-aldoxime (E-PyOx), respectively, were isolated by an acclimation culture technique and they were identified as *Bacillus* sp. and *Rhodococcus* sp, respectively. The cells of the strains grown in a medium containing aldoximes and nitriles catalyzed the stoichiometric dehydration reaction of aldoxime to form nitrile.

Aldoxime dehydratase induced in *E*-PyOx assimilating bacterium, *Rhodococcus* sp. strain YH3-3, catalyzed a dehydration reaction of various aryl- and alkyl-aldoximes to form the corresponding nitriles, but did not act on arylalkyl- and substituted alkyl- aldoximes. Various nitriles, such as 3-cyanopyridine, 2-cyanopyridine, cyanopyrazine, etc, were synthesized in preparative scales from 10 -100 mM of aldoximes under the optimized reaction conditions. This is the first report on the microbial synthesis of nitriles from aldoximes. A new enzyme aldoxime dehydratase was purified from a cell-free extract of *Z*-PAOx degrading bacterium, *Bacillus* sp. strain OxB-1. Its *Mr* was about 40,000, and it was composed of a single polypeptide chain. Results from an absorption spectrum, heme analysis, and atomic absorption spectrum analysis suggest that the enzyme contains loosely bound protoheme IX. The enzyme was active against several *E*/*Z*-arylalkyl-aldoximes and *E*/*Z*-alkyl-aldoximes to give the corresponding nitriles. Based on the results, we named the enzyme "phenylacetaldoxime dehydratase (EC 4.2.1.-)". We also optimized conditions for large-scale synthesis of aryl- and alkyl-nitriles in high yields from the corresponding aldoximes by *E. coli* JM109/poxd90F cells expressing phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1. We synthesized various aryl- and alkyl-nitriles from the corresponding aldoximes by the transformant cells.

We also found that the occurrence of aldoxime dehydratase is as wide as that for nitrile-degrading enzymes such as nitrile hydratase, amidase and/or nitrilase. All of the nitrile degraders hitherto isolated contained aldoxime dehydratase activities. The author proposes that the pathway in which aldoximes are successively degraded via nitrile could be named as the 'aldoxime–nitrile pathway'.

2. A new enzymatic method of selective phosphorylation of nucleosides (10-18) [Fine-tuning, Regio-selectivity]

Nucleotides are often used as food additives and as pharma intermediates. Among them, inosine-5'-monophosphate (5'-IMP) and guanosine-5'-monophosphate (5'-GMP) are important nucleotides, because they have characteristic taste and are used as a flavor. We have investigated a new nucleoside phosphorylation reaction using the food additive pyrophosphate (PPi) as the phosphate source. At first, microorganisms that phosphorylated nucleosides using PPi as the phosphate donor were screened (Fig. 2).

Nucleoside-5'-monophosphate-nucleoside phosphotransferase activity was found to be widely distributed among the bacteria examined. The biological activity of nucleotides is related to the position of the phosphate group, though the regioselectivity of phosphotransferase was very low in bacteria. Among them, *Morganella morganii* that produced the highest level of 5'-IMP with high regiospecificity, was selected as 5'-nucleotide producer. A selective nucleoside phosphorylating enzyme was purified to homogeneity from

M. morganii crude extract. The purified enzyme exhibited not only phosphotransferase activity but also phosphatase activity that hydrolyzed phosphate ester to release phosphate. By the purified enzyme, the synthesized 5'-IMP was hydrolyzed to inosine and phosphate as the reaction time was extended due to its phosphatase activity.

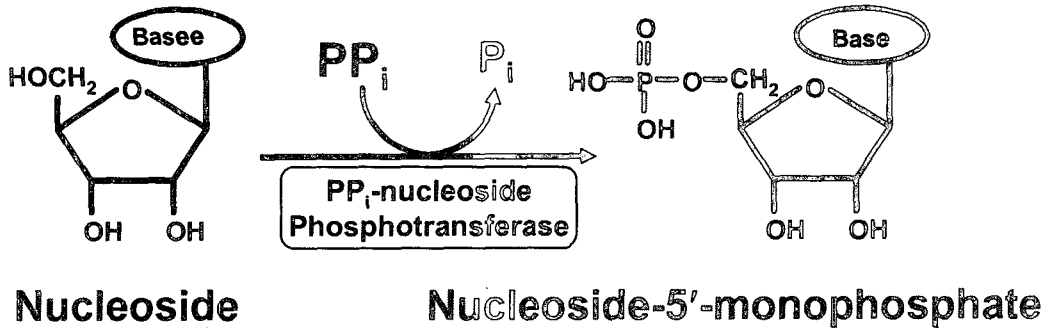

Fig. 2. The phosphorylation of nucleoside with pyrophosphate as a phosphoryl donor.

In order to achieve more efficient nucleotide production, directed evolutionary approach was carried out. The gene coding phosphotransferase activity was isolated from *M. morganii* genomic DNA. Then, sequential *in vitro* random mutagenesis on the gene was performed by error-prone PCR to construct a mutant library. The mutant library was introduced to *E. coli* and the transformants were screened for their productivity of 5'-IMP. One mutated acid phosphatase that increased in phospho-transferase reaction yield, was obtained. With the *E. coli* overproducing the mutated acid phosphatase, 101 g/l (191 mM) of 5'-IMP was synthesized from inosine in 85% molar yield. PPi is a safe and cheap compound that is easily synthesized from phosphate. A novel process for producing 5'-nucleotides could therefore be achieved, which consists of fermentation of nucleosides such as inosine and guanosine, and enzymatic phosphorylation of the nucleosides using PPi as the phosphate source.

3. Methylaspartate ammonia-lyase from soil Enterobacteria –Structure and function– (19-24) [Reaction mechanism, Stereo-selectivity]

A group of enzymes called amino acid ammonia-lyases has intrigued enzymologists and organic chemists, since the reactions cannot be done non-enzymatically, and the enzymes catalyze the addition of ammonia to achiral olefinic acids to form chiral L-amino acids. These enzymes include histidine ammonia-lyase (HAL; EC 4.3.1.3), phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), aspartate ammonia-lyase (AAL; EC 4.3.1.2), and 3-methylaspartate ammonia-lyase (3-methylaspartase, MAL; EC 4.3.1.1), etc. HAL and MAL catalyze the α, β-elimination of ammonia from (*S*)-His to yield urocanic acid, and (*S*)-*threo*-(2*S*,3*S*)-3-methylaspartic acid to mesaconic acid, respectively. In 1960, a Japanese company Tanabe Seiyaku started the industrial production of (*S*)-Asp from fumaric acid by immobilized cells of *Escherichia coli* containing AAL as one of the examples of the industrial use of the enzyme. It has also been discovered by Japanese researchers that PAL catalyzes not only the degradation of (*S*)-Phe, but also

the synthesis of (*S*)-Phe from *trans*-cinnamic acid only in a high concentration of ammonia up to 5 M. These enzymes pose a mechanistic challenge as to how they remove ammonia from L-amino acids by trans elimination with an abstraction of non-acidic and inactive β hydrogen.

I will outline how the structures have been elucidated and the possible reaction mechanisms of amino acid ammonia-lyases. Rétey et al have studied the structure and reaction mechanism of the superfamily of ammonia-lyases which include histidine (HAL) and phenylalanine (PAL) ammonia-lyase, and proposed that it involves an electrophile at the active center formed by the post translational modification of a serine residue to form an electrophilic group 4-methylidene-imidazole-one (MIO).

On the other hand, we isolated several Enterobacteria, including *Citrobacter amalonaticus*, expressing methylaspartate ammonia-lyase activity (MAL; EC 4.3.1.2) from soil samples, by anaerobic enrichment culture with (*S*)-glutamate containing medium. MAL catalyzes a reversible addition and elimination of ammonia to fumaric acid derivatives and using the cell-free extracts, optically active *threo*-(2*S*, 3*S*)-3-methyl- aspartate, *threo*-(2*S*, 3*S*)-3-ethylaspartate, and *threo*-(2*R*, 3*S*)-3-chloroaspartate were synthesized. To understand the mechanism and specificity of MAL, the X-ray structure of the *C. amalonaticus* enzyme was determined at 1.3Å resolution. Analysis of the structure of MAL shows that it is based on a TIM barrel, a fold that is completely different to that of HAL and PAL. Structure comparisons show that the fold of MAL is most closely related to enzymes of the enolase superfamily. This has allowed us to propose a mechanism for MAL which is distinct from that of the other ammonia lyases and which involves the initial abstraction of a proton α to the 3-carboxyl of the substrate to yield an enolic intermediate, which would then collapse to eliminate ammonia (Fig. 3). The structure has also revealed the molecular basis for the specificity of MAL which can now be manipulated for the chiral synthesis of novel aspartic acid derivatives.

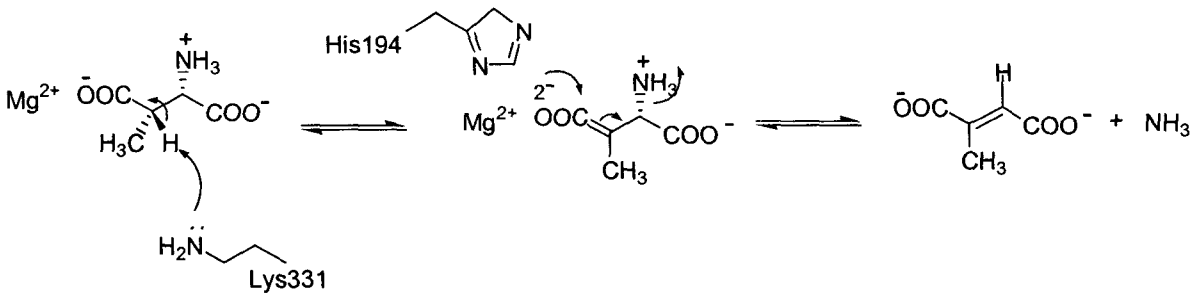

Fig 3. Proposed reaction mechanism of MAL.

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