

Award Accounts

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Structural-Based Engineering for Transferases to Improve the Industrial Production of 5′-Nucleotides

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The existence of a flexible region for substrate recognition by an enzyme was first discovered through a structure comparison study among ribonuclease T1–related molecules, including intact molecules, intact molecules complexed with 2′-guanylic acid (GMP), and carboxymethylated molecules complexed with 2′-GMP. Next, it was demonstrated that transglutaminase activity could be enhanced by identifying the flexible residues that affect the active site using nuclear magnetic resonance. Finally, such an approach was used to increase the production of 5′-inosinic acid (5′-IMP) and 5′-GMP. The enzyme is a nonspecific acid phosphatase with pyrophosphate-nucleoside phosphotransferase activity that is C-5′ position-selective. The crystal structure of G74D/I153T with a reduced K_M is virtually identical to that of the wild type, and neither of the side chains that were introduced Asp74 and Thr153, directly interact with the nucleoside, although both residues are situated near a potential nucleoside binding site. In addition, the two regions around residues 70 and 140 have the most flexible conformational rearrangement upon phosphate analogue binding. On the basis of three-dimensional (3D) structural information, some practical, high-performance enzymes for 5′-nucleotides production were discovered, and new varieties of microorganisms were developed.

One of the most important fundamental problems in biology and biotechnology is how enzymes interact with their substrates and act as catalysts. According to a lecture by Brian W. Matthews at the 45th Biophysical Society Meeting held in Boston, Massachusetts, U.S.A. in 2002, “Structure isn’t everything but sure helps.” His statement was true and appropriate as an academic observation; however, especially in industry, it was not accepted because structural biology is much more expensive than other sciences. Therefore, we have been challenged ourselves to do something more useful towards the elucidation and enhancement of enzymatic activity based on stereostructure. We show that structure-based engineering involving transferases can be used to improve the industrial production of 5′-nucleotides.

Nucleotides are often used as food additives and as pharmaceutical synthetic intermediates. Worldwide production of nucleotides is 16000 t/yr, and it increases around 7% annually. Ajinomoto Co., Inc. has a 40% share of the market. Among the nucleotides, 5′-inosinic acid (5′-IMP) and 5′-guanylic acid (5′-GMP) are important because they have a characteristic taste and are used as flavor potentiators in various foods. Their synergistic effect is several times stronger than monosodium glutamate (MSG), which is matched by just a few percent of the 5′-nucleotides. However, MSG is extremely important for the umami taste[1] in foods, worldwide, and the amount of production is about 100 times greater than 5′-nucleotides. Because purine nucleosides, such as guanosine[2] and inosine,[3] can be efficiently produced by fermentation, the progress of large-scale 5′-nucleotide production mainly depends on improving the nucleoside-phosphorylation process.

Some bacterial, class A nonspecific acid phosphatases (NSAPs) exhibit phosphotransferase activity in addition to their intrinsic phosphatase activity.[4–6] The phosphotransferase activity of *Morganella morganii* NSAP (MM-NSAP) was exploited to produce 5′-nucleotides by selectively phosphorylating nucleosides at the C-5′ position using diphosphate (PPi), as shown in the upper portion of Scheme 1.[7,8] The region-selective phosphotransferase activity is considered one of the merits of the enzyme when it is used in the nucleoside-phosphorylation process.

Unfortunately, the phosphatase activity of the wild-type NSAPs dominates the phosphotransferase activity, and consequently, a considerable amount of the 5′-IMP that is synthesized by the phosphotransferase activity is dephosphorylated into inosine via dominating phosphatase activity, as shown in the lower portion of Scheme 1. However, if the phosphotransferase activity is enhanced in comparison to the phosphatase activity, then the process using NSAPs would be useful in

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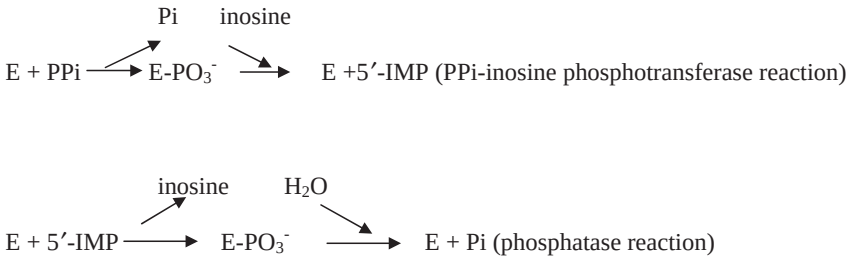

Scheme 1.

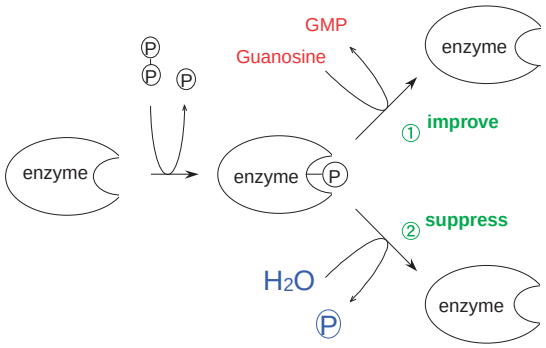

Fig. 1. Comparison of the active-site geometry in CM-RNase T1 with 2′-GMP (white), intact RNase T1 with 2′-GMP (blue), and free intact RNases (red). (top) The base binding site. (bottom) The catalytic site.

industry and could replace the current chemical[9] and enzymatic[10] processes to produce 5′-IMP. Mihara et al.[4] randomly mutated MM-NSAP and successfully generated the G74D/I153T mutant with enhanced PPi-inosine phosphotransferase activity, which is accounted for by the reduction in the Michaelis constant (K_M) value for inosine. K_M is often used as the measure of the tightness of binding or the affinity of an enzyme to a substrate. K_M decreased for from 117 mM for the wild type to 42 mM for G74D/I153T. However, the structure–activity relationship of the improved enzyme is not clear, and the K_M value for guanosine is not small enough for the industrially practical production of 5′-GMP. As its solubility is approximately 10 times lower than that of inosine at room temperature, its K_M value should be one-tenth of that for inosine. This is because, in Michaelis–Menten kinetics,

$$v = V_{max}[S]/(K_M + [S]), \quad (1)$$

where v is the rate of the reaction, V_{max} is maximum velocity, and $[S]$ is the substrate concentration.

When $[S]$ and K_M have the same order of magnitude or smaller, v can be written as $V_{max}/2$ or higher, and 5′-GMP production becomes industrially practical. It is necessary to improve the upper phosphorylate reaction in Fig. 1 and to suppress the lower hydrolysis reaction by reducing the K_M value for guanosine.

In this review, we first explain how basic research on ribonuclease T1 was helpful in determining that mutation at the flexible region is effective if a large enough number of mutations are screened. Second, we show how this idea was effective in the case of transglutaminase. Finally, we apply this approach in order to enhance the activity of transphosphorylase for production of 5′-nucleotides from nucleosides and phosphates.

1. Existence of a Flexible Region for Substrate Recognition as Revealed by Structure Comparison among Ribonuclease T1–Related Molecules

Ribonuclease T1 (RNase T1; EC 3.1.27.3) from the fungus *Aspergillus oryzae* is an enzyme that specifically hydrolyzes a phosphate diester bond on the 3′ side of guanosine in single-stranded RNA.[11] RNase T1 is a globular protein with 104 amino acid residues ($M_r = 11085$) in a single polypeptide chain.[12] Because RNase T1 is a small, stable enzyme, a number of studies, including chemical modifications,[13] protein engineering,[14,15] nuclear magnetic resonance (NMR),[16] and X-ray crystallography,[17] have been performed on this protein. These studies have elucidated that His40, Glu58, Arg77, and His92 are involved in the active site. In various crystallographic studies, many crystal structures, including the free form[18] and the complexed forms with 2′-GMP,[19,20] 3′-GMP,[21,22] guanylyl-2′,5′-guanosine (2′,5′-GpG),[23] guanosine-3′,5′-bisphosphate (3′,5′-pGp),[24] and vanadate ($H_2VO_4^-$),[25] have been determined. These crystal structures have played especially important roles in elucidating the enzymatic mechanisms and the nucleotide binding modes. Furthermore, some mutant proteins of RNase T1 have been subjected to crystallographic studies, which allow more detailed discussions on this enzyme, e.g., Tyr45Trp complexed with 2′-AMP and 2′-GMP.[26]

Takahashi et al.[27] reported that RNase T1 is completely deactivated by selective carboxymethylation of the γ-carboxyl group of Glu58 ($-COO^- \rightarrow -COOCH_2COO^-$) with iodoacetate at pH 5.5. The Glu58 carboxylate has been proposed to act as the base in a general acid–base catalysis.[21,28] The carboxymethylation of RNase T1 at the γ-carboxyl group of Glu58 leads to a complete loss of the enzymatic activity, although it retains almost the same level of substrate binding ability. Accompanying carboxymethylation, RNase T1 is thermally stabilized, i.e., the melting temperature (T_m) is increased by 9 °C. To clarify the inactivation and stabilization mechanisms of RNase T1 by carboxymethylation, the crystal structure of carboxymethylated RNase T1 (CM–RNase T1) complexed with 2′-GMP was determined at 1.8 Å resolution.[29] The carboxyl group of CM-Glu58, which is located in the active site, occupies almost the same position as the phosphate group of 2′-GMP in the crystal structure of the intact RNase T1–2′-GMP complex. Therefore, the phosphate group of 2′-GMP protrudes toward the solvent. This forces 2′-GMP to adopt an *anti*-form, which contrasts with the *syn*-form in the crystal of the intact RNase T1–2′-GMP complex. Thus, because the phosphate group cannot access the active site, CM–RNase T1 has no enzymatic activity. One of the carboxyl oxygen

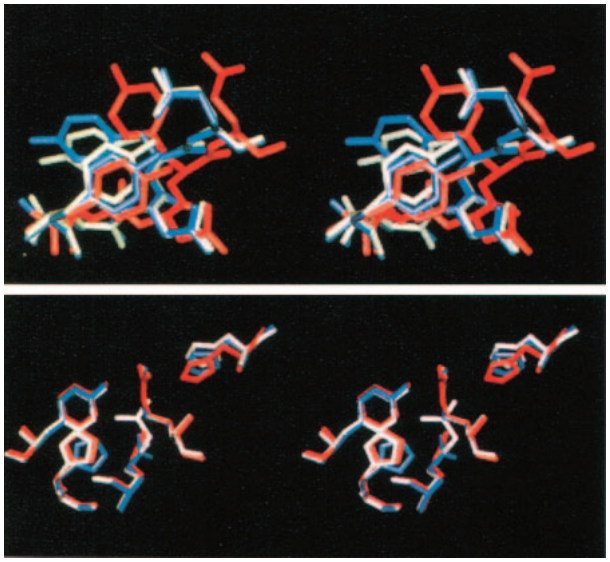

Fig. 2. Schematic of the enzymatic reaction mechanism of (top) guanosine phosphorylation and (bottom) enzyme hydrolysis.

atoms of CM-Glu58 forms two hydrogen bonds with the side chains of Tyr38 and His40. These hydrogen bonds are considered to contribute to the higher thermal stability of CM–RNase T1. Another carboxyl oxygen atom of CM-Glu58 is near His40 and Arg77 which may provide additional electrostatic stabilization.

Despite the complete loss of enzymatic activity, CM–RNase T1 retains almost the same binding ability towards guanosine and 10% of its binding ability towards 2′-GMP compared to intact RNase T1 in terms of the dissociation constant.[13] This suggests that the carboxymethylation of Glu58 affects only the binding of the phosphate portion and barely affects guanosine. To compare the thermal stability of CM–RNase T1 with that of intact RNase T1, a ^{1}H NMR spectra measurement was performed at various temperatures, and the changes in the peak areas were plotted for two specific protons.[30] It turned out not only that the T_m value of CM–RNase T1 is higher than that of intact RNase T1 by 9 °C ($\Delta\Delta G = 5.25\,\mathrm{kcal\,mol^{-1}}$), but also that this remarkable stabilization effect is due to the formation of a strong salt bridge between CM-Glu58 and its proximal residue, Arg77.

On the other hand, the structural aspects are totally different from these functional observations. In other words, when intact RNase T1 only, its complex with 2′-GMP, and the CM-RNase complex with 2′-GMP were superimposed, there was no disorder at the catalytic site, and remarkable flexibility was found at the base binding site (Fig. 2). Thus, artificial, site-directed mutations are worthy of investigation at the base binding site because it is a flexible region.

2. Enhancement of Transglutaminase Activity by Nuclear Magnetic Resonance Identification of Its Flexible Residues Affecting the Active Site[54]

Transglutaminases (TGase, protein-glutaminase, γ-glutamyltransferase; EC 2.3.2.13) are a family of enzymes that catalyze the displacement of the amide ammonia at the γ position in glutamine residues by replacing it with another amine, usually an ε-amino group from a suitable lysine residue.[31–34] The formation of ε-(γ-glutamyl)lysine isopeptide bonds results in both intramolecular and intermolecular cross linking of proteins, leading to polymerization. The incorporation of intermolecular or intramolecular covalent cross links into food proteins with microbial transglutaminase (MTG) improves the physical and textural properties of many food proteins, such as tofu, boiled fish paste, and sausage.[35–37]

It has been reported that the polymerization of several proteins with MTG progressed rapidly in comparison to the reaction rate with guinea pig liver transglutaminase (GTG).[38] Moreover, we have already shown that GTG and fish (red sea bream liver) transglutaminase (FTG) have limited usefulness in industry because they have high substrate specificities.[39] On the other hand, the reaction rate for Ser-MTG, which is an MTG variant lacking the N-terminal aspartic acid residue, was higher than that observed for MTG, even though their substrate specificities are identical.[39] At the present time, MTG and its variant are the preferred enzymes for industrial use. Therefore, MTG variants with higher activity are not only desired, but also will make them useful for other industrial applications, such as biological materials, drugs, and so on. Because the amount of the enzyme to change food texture can be decreased, leading to cost cutting, it is possible to prepare other proteins that are not possible with the present enzyme.

The proposed TGase reaction mechanism is based on the crystal structure of factor XIII.[40,41] At first, the γ-carboxyamide groups in glutamine residues interact with TGases, and subsequently, the primary amino groups of a variety of amines or the ε-amino groups of lysine residues are involved in the formation of new covalent bonds. The reactivity of a glutamine residue depends on the type of TGase and the microenvironment formed by its surrounding residues in the substrate protein. On the basis of the proposed reaction mechanism, we have developed an enzymatic labeling technique (ELT), in which ^{15}N nuclei are incorporated into the γ-carboxyamide groups of the glutamine residues in arbitrary proteins.[42] In addition, we have reported a novel method using ELT and NMR detection techniques to determine the substrate specificities for glutamine residues and the reaction rates of TGases simultaneously.[39] The method is useful for comparing the existing TGases and for screening new TGases or TGase variants and is hereafter referred to as the NMR-based screening method.

The calorimetric hydroxamate procedure is another method for measuring TGase activity; it uses a small peptide including a glutamine residue, *N*-carbobenzoxy-L-glutaminylglycine, *N*-benzyloxycarbonyl.[43] This method is convenient for estimating the reaction rate for small peptides but is not adequate for an analysis of the substrate specificity.

We are also studying the structure of MTG by NMR spectroscopy in an effort to understand the process of substrate recognition. We are focusing on the experimental results that suggest that the thiol group of Cys64 in MTG is essential for the enzymatic activity and that the reaction rate for Ser-MTG is higher than that for MTG.[39,44] To obtain as much relevant information concerning MTG structure and function as possible, we have chosen strategies that rely on the use of existing mutants and stable isotope labeling. High-level expression sys-

tems for MTG variants with an additional methionine residue and with an aspartic acid residue deletion in the N-terminus were reported previously.[45] Using these systems, we embarked on research to understand the structural role of the N-terminal residues and to obtain more efficient enzymes by site-directed mutagenesis.

Accordingly, dynamic filtering by the use of a Carr–Purcell–Meiboom–Gill (CPMG) pulse train[46] was applied for the extraction of resonances originating from flexible parts of the MTG molecule. In addition, the effects of the flexible region on the active site, Cys64, in MTG were determined by heteronuclear two-dimensional NMR experiments of MTG selectively labeled with ^{15}N cysteine. On the basis of these NMR structural studies, we obtained several mutants, including some with higher activity than the wild-type MTG. Finally, we briefly discuss a strategy for enzyme engineering based on the NMR study.

First, assignments of the ^{1}H resonances originating from the N-terminal region were made on the basis of spectral comparisons between Met-MTG and Ser-MTG. The four resonances at 2.08, 2.12, 2.59, and 2.70 ppm, observed in the difference spectrum, were assigned to Met(−1)-Hε, Met(−1)-Hβ, Met(−1)-Hγ, and Asp1-Hβ, respectively, in the N-terminal region which Ser-MTG does not have. For the selective detection of resonances originating from flexible parts of the Met-MTG molecule, the CPMG pulse train with a delay time of 10.5, 21, or 31.5 ms was used. The line widths of the ^{1}H resonances for Met-MTG are usually of the order of 50 Hz with an observation frequency of 600 MHz. Using the CPMG pulse train with these delay times, most of the resonances originating from the nonflexible portions are suppressed by the use of the CPMG pulse train. In the CPMG spectrum of Met-MTG, several resonances, including those from Met(−1)-Hε, Met(−1)-Hβ, Met(−1)-Hγ, and Asp1-Hβ, survived. These results indicate that the residues exhibiting relatively high levels of flexibility, in this case, of the Met-MTG molecule, are localized in the N-terminal region.

Next, the effect of the deletion of the N-terminal region was examined with regard to the active site of the enzyme. Figure 3 shows ^{1}H–^{15}N HSQC spectra of Met-MTG and Ser-MTG labeled with ^{15}N cysteine. We have assigned these resonances to Cys64 in Met-MTG and Ser-MTG, because only one cysteine residue, i.e. Cys64, is included in each molecule. The removal of the Met(−1) and Asp1 residues in the N-terminal region induced a slight chemical shift change in the peak for Cys64, in which the thiol group is essential for enzymatic activity. These results show that the N-terminal region influences the static or dynamic structure of the active site in MTG.

Taking the flexibility of the N-terminal region into consideration, we noticed that the N-terminal region is not of primary importance for the global fold. In other words, the global fold does not seem to be destroyed by the replacement and deletion of several N-terminal residues. On the other hand, the N-terminal region has a slight effect on the structure of the active site, suggesting that the N-terminal region is involved in the approach or binding (or both) of the substrate. As a practical matter, the reaction rates of wild-type MTG and Ser-MTG have already been compared, using ovalbumin as the substrate protein.[39,45] The reaction rate for Ser-MTG is higher than that

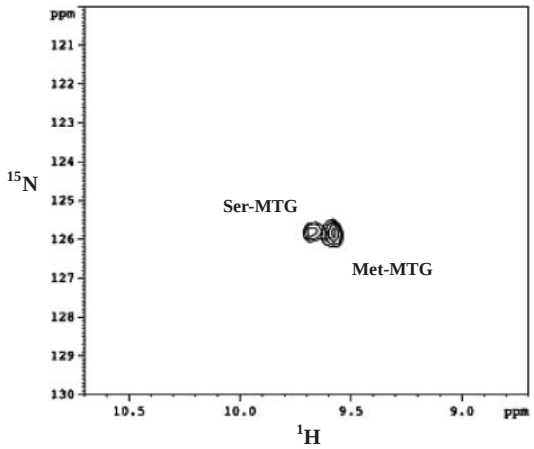

Fig. 3. ^{1}H–^{15}N HSQC spectra of Met-MTG and Ser-MTG labeled with ^{15}N cysteine. Resonances in both spectra are assigned to Cys64 in Met-MTG and Ser-MTG, respectively.

observed for MTG, at least for some glutamine residues in ovalbumin, even though their substrate specificities are identical. Therefore, to increase the TGase activity, the N-terminal residues were chosen as candidates for replacement or removal, which does not disturb the substrate binding or the global fold. To minimize the volume of the N-terminal region, the following four mutants were designed: del1-2, del1-3, del1-4, and del1-5 (Table 1). In addition, we designed three mutants, Ser2 → Arg (S2R), Ser2 → Asp (S2D), and Ser2 → Tyr (S2Y), to change the charged state of the N-terminal region (Table 1). The reaction rates for del1-2, del1-3, and S2R are higher than the rate observed for wild-type MTG, and especially, the reaction rate for del1-3 exceeds that for Ser-MTG (Table 1). This result is consistent with the reaction rates estimated using the NMR-based screening method.

The increased activities of del1-2 and del1-3 suggest that these flexible N-terminal residues are not involved in the substrate binding. On the other hand, the other deletion mutants, such as del1-4 and del1-5, lost the TGase activity, which suggests that the MTG variants lacking more than three N-terminal residues were not correctly folded. The third residue and its subsequent residues are important for the global fold or for the TGase activity, although the flexible region exists in the N-terminus. The S2R mutant, which was designed to reduce the number of negative charges in the N-terminal region, also displayed higher activity. The activities of Ser-MTG and del1-2, which have reduced negative charges by the deletion of Asp1, were also increased. These results suggest that an excess of negative charges in the N-terminal region is not preferable for substrate binding.

In conclusion, the mutants with higher activity, del1-2, del1-3, and S2R, in addition to Ser-MTG, were produced by site-directed mutagenesis on the basis of extracting flexible residues affecting the active site based on NMR screening. This suggests that a strategy for identifying flexible regions in enzymes using NMR would be effective for protein engineering. It is possible that the highly flexible regions are not primarily important for the global fold. Therefore, these regions can be chosen as primary candidates for replacement and deletion, as long as they

Table 1. Amino Acid Sequences of the N-Terminal Residues in MTG and Its Variants and Their Transglutaminase Activities Evaluated by the NMR-Based Screening Method

	Amino acid residues in the N-terminus (by number)								
	−1	1	2	3	4	5	6	7	NMR-based screening method $/\times10^{-2}$ U mg^{-1} [a]
Wild-type MTG		D	S	D	D	R	V	T	5.5 ± 0.2
Met-MTG[b]	M	D	S	D	D	R	V	T	5.6 ± 1.0
Ser-MTG[c]			S	D	D	R	V	T	8.9 ± 0.9
Del1-2			M	D	D	R	V	T	10.3 ± 0.5
Del1-3				M	D	R	V	T	14.7 ± 1.3
Del1-4					M	R	V	T	<2.0
Del1-5						M	V	T	<2.0
S2R	M	D	R	D	D	R	V	T	6.4 ± 0.6
S2D	M	D	D	D	D	R	V	T	<2.0
S2Y	M	D	Y	D	D	R	V	T	<2.0

a) Activities were assayed as described in the literature.[42] RMS deviations were calculated on the basis of two or three experiments. b) MTG in which N-terminal residue is serine. c) N-Terminal residue is methionine.

are close to the active sites of enzymes. The MTG mutants thus obtained are expected to improve the physical and textural properties of many food products more effectively than wild-type MTG.

Here, we should note that a similar concept, of which we were not previously aware, was put forth by a group at the California Institute of Technology[47] and that we could also succeed in engineering *Escherichia coli* L-serine-*O*-acetyltransferase on the basis of the above-mentioned "flexible site mutation" for desensitization to feedback inhibition by L-cysteine.[48]

3. Enhancement of Nucleoside Phosphorylation Activity in an Acid Phosphatase Based on the Devised Strategy[55]

5′-IMP and 5′-GMP are important nucleotides because they give the umami taste in foods, as stated earlier. A greener and newer enzymatic method was sought. Microorganisms that phosphorylated nucleosides using PPi as the phosphate donor were screened. The phosphorylation of inosine in the 5′ position to produce 5′-IMP was studied in a number of microorganisms from our culture collection. Although many of the microorganisms screened were able to phosphorylate inosine, phosphotransferase activity specific to the 5′ position was found to be distributed among the bacteria belonging to the family Enterobacteriaceae. *Morganella morganii* NCIMB 10466 (MM) was selected as a 5′-IMP producer.[7] A selective nucleoside phosphorylating NSAP enzyme was purified to homogeneity from MM crude extract.[8] The enzyme appeared to consist of six subunits identical in molecular weight ($M_r = 25000$; 150000 as a total). It phosphorylated various nucleosides at the 5′ position to produce nucleoside-5′-monophosphates, using PPi as the phosphate source, and energy-rich compounds, such as carbamoylphosphate and acetylphosphate, were also very effective phosphate donors. The enzyme also exhibited phosphatase activity and dephosphorylated various phosphate esters. The MM gene encoding a nucleoside phosphorylating enzyme was isolated by a shotgun-cloning strategy. It was identical to the MM PhoC acid phosphatase gene.[49] Using the purified enzyme, 32.6 mM 5′-IMP was synthesized from inosine with a 41% molar yield, but the synthesized 5′-IMP was hydrolyzed back to inosine as the reaction time was extended due to the enzymes phosphatase activity.

To suppress the dephosphorylation reaction and increase the efficiency of the transphosphorylation reaction, a random mutagenesis approach was used. By using error-prone polymerase chain reaction (PCR), one mutated acid phosphatase with increased phosphotransferase reaction yield was obtained.[4] With the *E. coli* overproducing the mutated acid phosphatase, 101 g/L (191 mM) of 5′-IMP were synthesized from inosine in an 85% molar yield. This improvement was achieved with two mutations: Gly to Asp at position 92 and Ile to Thr at position 171. A small K_M value for inosine was responsible for the increased productivity.

Bacterial, class A NSAPs with region-selective phosphotransferase activity have also been isolated from *Escherichia blattae*,[6] *Enterobacter aerogenes*, *Providencia stuartii*, *Klebsiella planticola*,[5] and so on. Mihara et al.[5] investigated the phosphotransferase activity of these NSAPs and found that the amounts of 5′-IMP produced by the NSAPs are also closely related to their K_M values for inosine. This finding is plausible because the PPi-inosine phosphotransferase activity prevails over the phosphatase activity if the phosphoenzyme intermediate prefers an attack by inosine to one by water (Scheme 1).

First, the G74D and I153T mutations were introduced to EB-NSAP because the previous random mutagenesis corresponding to G92D and I171T, i.e., the numbering gap is 18, of MM-NSAP showed that these mutations were effective in reducing the K_M value for inosine.[5] The productivity of wild-type EB-NSAP was very low, and only 13.6 g/L of 5′-IMP was produced with a maximum molar yield of only 22% from inosine using *E. coli* JM109 overproducing the wild-type enzyme.[5] In contrast, the productivity of the resultant EB-NSAP mutant was greatly improved, as expected. Using *E. coli* JM109 overproducing G74D/I153T, 103 g/L of 5′-IMP was produced with a molar yield of 52% from inosine, and the dephosphorylation of the produced 5′-IMP was considerably supressed. The G74D/I153T mutant enzyme was purified from a crude extract of the *E. coli* transformant

Table 2. Kinetic Constants for the PPi-Inosine Phosphotransferase Reaction[a)]

	K_M/mM	V_{max}/U mg^{-1}
Wild type	202	1.83
G74D/I153T	109	1.39
S72F/G74D/I153T	37	3.46

a) The enzyme activities were assayed as described in the literature.[4] The initial velocities were determined, and the steady state kinetic constants were calculated by using a Hanes–Woolf plot.

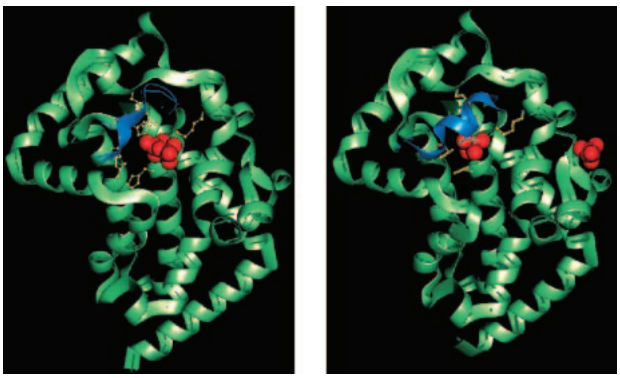

Fig. 4. Ribbon models of the enzyme (left) without phosphate and (right) with phosphate, which was modeled from the molybdate binding transition-state analog.

and was analyzed. As shown in Table 2, the K_M value of the mutant for inosine was 109 mM, which was approximately half that of the wild type. However, it was still higher than that of the G92D/I171T mutant of MM-NSAP and also was above the achievable inosine concentration of 80 mM.

Accordingly, further improvement was necessary for industrial production of 5′-GMP especially. We have embarked on research to understand the structure–activity relationship of the improved enzyme and to obtain an even more efficient enzyme by rational, site-directed mutagenesis because further improvement of the enzyme by random mutation was time consuming. We selected the *E. blattae* NSAP (EB-NSAP) as the target of rational mutagenesis, despite its rather high K_M value (200 mM) for inosine, because the crystal structure of the enzyme has been determined at high (1.9 Å) resolution,[6] whereas no structural data are available for the other NSAPs. EB-NSAP is a 150-kDa homohexamer and has 77% sequence identity to MM-NSAP. Because, fortunately, the crystal structure of EB-NSAP complexed with the transition-state analog, molybdate, has also been solved,[6] a hypothetical binding mode of inosine to the phosphoenzyme intermediate could be modeled using the enzyme–molybdate complex structure, in which the bound molybdate was replaced by phosphate (Fig. 4 (right)) and the inosine was placed in a depression surrounded by Leu16, Ser71, Ser72, and Glu104, with the 5′-O atom of the ribose situated close to the phosphorus atom. Because EB-NSAP is not specific to inosine, it was not possible to place the inosine in a unique conformation. However, the position of the phosphate and the structure around it indicated that the ribose must be situated near His150. Although the position of the base is affected by the conformation and the position of

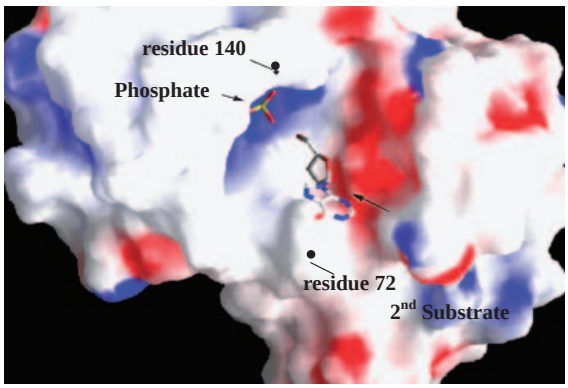

Fig. 5. One of the possible inosine binding modes.

the ribose, it seems most plausible to place it near Ser71 and Glu104. One of the possible inosine binding modes is shown in Fig. 5. In this model, Ile153 is situated close to the ribose, and therefore, this residue can be considered as a part of the inosine binding site. An automatic docking of the inosine to EB-NSAP has been hampered by a lack of force field parameters for the covalent phosphohistidine, although the development of empirical force constants for the phosphoimidazole has been studied by Kosinsky et al.[50] recently.

The wild-type and G74D/I153T proteins crystallize into different crystal forms, although only two residues are different. The asymmetric unit of the wild-type crystal has one subunit, whereas that of the mutant crystal accommodates one hexamer. Despite the different crystal forms, the overall structure of G74D/I153T is essentially identical to that of the wild type, as revealed by the root-mean-square (RMS) deviations between the equivalent Cα positions of 0.30–0.32 Å, calculated when the wild-type subunit structure was superimposed with each of the six subunits of G74D/I153T, using 214 Xα atoms (Gly7-Asn133 and Ser141-Gln227).

In the EB-NSAP structure, Gly74 and Ile153 are situated on the α4 helix and α10 helix, respectively.[6] In the G74D/I153T structure the carboxyl group of Asp74 forms a hydrogen bond with the Oγ of Ser71, and the side chain Oγ of Thr153 hydrogen bonds to the main-chain carbonyl oxygen of Ile103. Despite these newly formed hydrogen bonds, the overall structure of G74D/I153T remains unchanged. This structural similarity clearly indicates that the difference in the K_M value can be ascribed to the local structural differences around the two mutated residues.

In the inosine binding model, Thr153 is situated near the bound inosine. Because the mutation at position 153 causes a subtle structural change in the inosine binding site, the reduced K_M value could be ascribed to a difference in the interaction between the inosine and the inosine binding site. On the other hand, Asp74 is not situated close enough to the bound inosine to be considered as a part of the inosine binding site. A comparison of the B factors between the G74D/I153T and the wild-type structures revealed that the flexibility of the region ranging from Asn69 to Val75 is significantly increased in the G74D/I153T structure. The average B factors of the main-chain atoms in this region of G74D/I153T and the wild type are 51.8 and 21.8 Å^2, respectively, whereas those in the more extended region (Gly7-Asn133 and Ser141-Gln227) are

Table 3. K_M Values for Inosine and Relative Activities of the PPi-Inosine Phosphotransferase Reaction[a)]

	K_M/mM	Relative activity
L16W/G74D/I153T	33	0.21
S71W/G74D/I153T	75	0.26
S72W/G74D/I153T	30	1.71
E104W/G74D/I153T	67	0.26
S72F/G74D/I153T	20 (37)	2.80
S72Y/G74D/I153T	30	2.04
S72D/G74D/I153T	38	1.59
S72E/G74D/I153T	40	3.19
S72N/G74D/I153T	124	0.43
S72K/G74D/I153T	78	1.53
S72V/G74D/I153T	41	2.46
S72M/G74D/I153T	46	1.94
S72P/G74D/I153T	109	1.34
S72T/G74D/I153T	50	1.91
S72A/G74D/I153T	115	0.78
S72G/G74D/I153T	137	0.43
G74D/I153T	77 (109)	1.00

a) The K_M values were calculated by using a Hanes–Woolf plot. The relative activities were estimated from the crude extracts of mutant enzymes. The activity of G74D/I153T was defined as 1.00, and those of the other mutated enzymes are expressed as relative activities. No significant changes in the levels of production of each mutated enzyme in the crude extract prepared from each *E. coli* transformant were observed on SDS-PAGE. The K_M values in the parentheses for S72F/G74D/I153T and G74D/I153T were determined from purified enzymes.

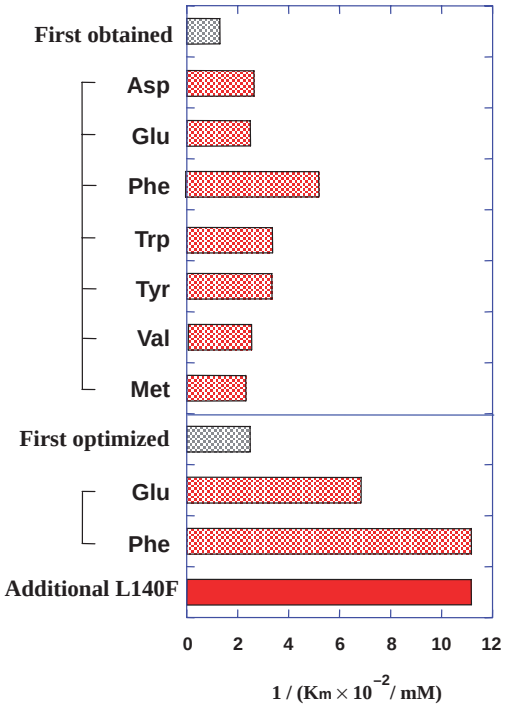

Fig. 6. Comparison among the transphosphorylase activities of mutants substituted at the 72nd residue from alanine. "First obtained" means G74D/I153T, "first optimized" means the 10-amino-acid substituted mutant (L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/T135K/E136D/I153T), and "additional L140F" means the mutant with additional L140 mutation on the right above mutant A72F of the 10-amino-acid substituted mutant.

35.5 and 22.9 Å^2, respectively. The increased flexibility of this region may help to enhance the enzyme's affinity for inosine because the inosine–enzyme binding model (Fig. 5) suggests that this region is likely to interact with inosine. The finding that the Gly74 → Asp and Ile153 → Thr mutations reduce the K_M value for inosine without either a notable structural change or a new direct interaction suggests that any amino acid substitution around the inosine binding site could decrease the K_M value.

While building the inosine binding model, we noticed that there is little interaction between the enzyme and the inosine base. To introduce base–enzyme interactions, we searched for residues that, if replaced with aromatic residues, would possibly lead to the formation of aromatic–aromatic interactions without perturbing the enzyme structure. Such interactions between the base of a nucleotide/nucleoside and the side chain of an aromatic residue are observed in most nucleotide/nucleoside-recognizing enzymes, such as ribonuclease T1[51] and purine nucleoside phosphorylase.[52] Consequently, Leu16, Ser71, Ser72, and Glu104 were chosen as candidates for replacement. A typical aromatic–aromatic interaction between residues, such as phenylalanine, tyrosine, and tryptophan, has a nonbonded potential energy of between −1 and −2 kcal mol^{-1}, which was determined from 34 protein structures from the Protein Data Bank.[53] To maximize the possibility of the formation of an aromatic–aromatic interaction, the introduction of the largest amino acid, tryptophan, to the above four positions seemed promising, and thus, the following four mutations were designed: Leu16 → Trp, Ser71 → Trp, Ser72 → Trp, and Glu104 → Trp. The K_M values and the relative activities of crude extracts of the resultant mutants are shown in Table 3. Among the four mutants, S72W/G74D/I153T had the lowest K_M value. In addition, this mutant showed higher activity than that of the parent. In the inosine binding model, the introduced tryptophan side chain at position 72 is not close enough to the inosine base to interact ideally; the distance between them is estimated to be 5.5 Å. However, it is likely that the loop containing Trp72 undergoes a slight conformational change on inosine binding, and thus, an ideal aromatic–aromatic interaction is formed because this loop is relatively flexible and significantly changes its conformation on the formation of the phosphoenzyme intermediate.[6] Actually, when the structures of the enzyme–molybdate complex and the native enzyme are superimposed, Ser72 in the native enzyme structure is situated very close to Ser71 in the enzyme–molybdate complex structure, which is located near the inosine base in the inosine–enzyme binding model (Fig. 5).

The reduced K_M value and the increased activity of S72W/G74D/I153T prompted us to substitute other amino acids for Ser72. Surprisingly, 7 of the 12 resultant mutants showed improved performance over G74D/I153T (Table 3 and Fig. 6). The best-performing mutant was S72F/G74D/I153T (K_M for inosine, 9 mM), suggesting that phenylalanine is more suited to form an aromatic–aromatic interaction with the inosine base than tryptophan and tyrosine. Except for aromatic residues, re-

placements by acidic residues, such as Asp and Glu, resulted in low K_M values and high activities. It is probable that the introduced carboxyl group forms an electrostatic interaction with the base of the inosine. S72F/G74D/I153T has around 5.4-fold improvement in the K_M values of both inosine and guanosine as compared to the wild type. To characterize S72F/G74D/I153T further, we purified the mutant protein and measured its kinetic parameters. The K_M and V_{max} values of the mutant were 2.9-fold lower and 2.7-fold higher than those of the parent, respectively. Next, the time course of 5′-IMP production using *E. coli* JM109 overproducing the mutant was measured. The productivity of the S72F/G74D/I153T mutant was superior to that of the G74D/I153T mutant, and 140 g/L of 5′-IMP was obtained with a molar yield of 71% from inosine. The mutations that were effective in EB-NSAP in this study can be applied to the other NSAPs. It is expected that the MM-NSAP mutant bearing three mutations corresponding to S72F/G74D/I153T could be superior to the S72F/G74D/I153T mutant of EB-NSAP as the former G74D/I153T mutant has better K_M and relative activity.

However, additional S72F mutation for some mutants resulted in less enzyme activity. Accordingly, further study was necessary, and it was found that additional mutation at the 140th residue gave us more than 10 times as high activity as the original enzyme, while keeping the same K_M, as shown in Fig. 6. It is possible to consider this activity enhancement by comparing free and molybdate binding structures. Thr135 and Glu136 are disordered or flexible in the native enzyme but become ordered in the molybdate complex. Consequently, the loop ranging from Asn133 to Asn143, which is highly flexible in the native enzyme, forms a less flexible structure that contains an α helix ranging from Glu136 to Ser141. This newly formed α helix extends over the active site and covers the bound molybdate. Accompanying the α helix formation, the side chain of Leu140 moves 6.2 Å toward the molybdate and is situated near the metal oxyanion; the distance between CD1 of Leu140 and one of the equatorial oxygen atoms of the molybdate is 3.3 Å. The existence of the hydrophobic side chain near the covalent phosphohistidine is thought to make the approach of a water molecule less frequent. When the phosphoenzyme intermediate is formed, the α helix probably stabilizes the intermediate by protecting the covalent phosphohistidine from an attack by a water molecule. The phosphoenzyme intermediate would be more susceptible to hydrolysis if the conformational change did not occur, and the substituted phenylalanine should be more helpful for covering the phosphohistidine than leucine. This mutation is also an example of "flexible site mutation."

In summary, the highly improved mutants, S72F/G74D/I153T, and so on, were produced by site-directed mutagenesis based on the G74D/I153T mutant, which was generated using the knowledge derived from the random mutagenesis of the homologous enzyme from MM. This suggests that the combined use of rational, site-directed mutagenesis and random mutagenesis is effective and thus amenable to other enzymes. Furthermore, our results demonstrate that rational, site-directed mutagenesis based on an examination of the three-dimensional structure should be tried, even when a substrate–enzyme binding mode is difficult to predict.

4. Concluding Remarks

Through all of the developmental studies mentioned above, the most impressive experience was the discussion one of the authors (E.S.) had with Richard Ernst in 1991, the year before he received receiving the Nobel Prize. At that time, E.S. asked him a question, "When you discovered two-dimensional NMR, did you imagine it would become successful without special efforts?" He answered, "A good question. My opinion was two-dimensional FT NMR should be possible theoretically; however, natural fluctuation is bigger than the detection limit of correlation signals. So it may be impossible to get two-dimensional FT NMR spectra in practical meaning. But it's very difficult to demonstrate something is impossible as we have to prepare the experiment very carefully. In reality, I used highest-quality chips for the electric circuit, etc., for those experiments, and the preparation needed a longer term than several months. As a result, the first trial experiment was successful. I'd like to advise you like that: when you try to overcome great difficulty, although it's very important for your scientific life, you should prepare an experiment in order to demonstrate it's impossible."

Some explanation may be necessary for better understanding, and a carefully prepared experimental setup is necessary to demonstrate that something is impossible as compared to demonstrating that something is possible. That is to say, when something is demonstrated to be possible, people do not ask why the experiment was successful, but when something cannot be demonstrated to be impossible, people ask if the experimental setup was insufficient or suppose that the reason for the unsuccessful experiment was an unskillful experimental technique. As a result, more experimental trials and endless effort are required. Accordingly, Ernst used available highest-performance electric circuits and carefully prepared two-dimensional Fourier transform data processing software along with a substantial period of time. On the basis of these preparations, his first experimental trial resulted in the successful observation of two-dimensional correlation cross peaks. This approach is a great challenge compared to trial and error.

When we began this work, people thought that protein engineering was important as a science but was not useful for an industrial purpose. Therefore, the development plan was not approved easily in our laboratories, despite the possibility of important scientific direction could not be denied. However, the research theme is so apparent in our company that if it turned out to be unsuccessful, people might be disappointed. Accordingly, remembering Ernst's words, E.S. explained that the purpose of this study was to demonstrate that it is impossible, and he succeeded in getting the approval of the director. This explanation was also understandable for other researchers because it had been stated by the Nobel laureate. Also, we considered over 100 mutations, which can be considered sufficient to show that further activity enhancement up to the level that is practical for industry should be impossible. We carried out these mutations with priority, and when the first 10 mutations were found to be useful, we succeeded in getting a higher-performance, industrially promising enzyme.

Although the structures of compounds that biochemists are dealing with can appear simple to synthetic organic chemists,

microorganisms display quite interesting biological phenomena involving enzymes, even the simple compounds. We have been discovering new reactions which, in the past, organic chemistry never described. Thus, more synthetic compounds will be used as substrates, with the "extrachemophiles" as the sources of enzymes. It is hoped that the isolation of extrachemophiles and their uses will expand the use of the enzyme in organic synthesis. The combination of microbial screening for a desirable enzyme and further modification and fine-tuning of the enzyme by directed evolution appears to be promising, and thus, the enzymes can be expected to be widely used in "green chemistry."

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