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Improvement of Oxidative and Thermostability of *N*-Carbamyl-D-Amino Acid Amidohydrolase by Directed Evolution

Ki-Hoon Oh, Sung-Hun Nam and Hak-Sung Kim[1]

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373–1, Kusung-dong, Yusung-Gu, Taejon, 305–701, South Korea

[1]To whom correspondence should be addressed. E-mail: hskim@mail.kaist.ac.kr

***N*-Carbamyl-D-amino acid amidohydrolase (*N*-carbamoylase), which is currently employed in the industrial production of unnatural D-amino acid in conjunction with D-hydantoinase, has low oxidative and thermostability. We attempted the simultaneous improvement of the oxidative and thermostability of *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 by directed evolution using DNA shuffling. In a second generation of evolution, the best mutant 2S3 with improved oxidative and thermostability was selected, purified and characterized. The temperature at which 50% of the initial activity remains after incubation for 30 min was 73°C for 2S3, whereas it was 61°C for wild-type enzyme. Treatment of wild-type enzyme with 0.2 mM hydrogen peroxide for 30 min at 25°C resulted in a complete loss of activity, but 2S3 retained about 79% of the initial activity under the same conditions. The K_m value of 2S3 was estimated to be similar to that of wild-type enzyme; however k_{cat} was decreased, leading to a slightly reduced value of k_{cat}/K_m, compared with wild-type enzyme. DNA sequence analysis revealed that six amino acid residues were changed in 2S3 and substitutions included Q23L, V40A, H58Y, G75S, M184L and T262A. The stabilizing effects of each amino acid residue were investigated by incorporating mutations individually into wild-type enzyme. Q23L, H58Y, M184L and T262A were found to enhance both oxidative and thermostability of the enzyme and of them, T262A showed the most significant effect. V40A and G75S gave rise to an increase only in oxidative stability. The positions of the mutated amino acid residues were identified in the structure of *N*-carbamoylase from *Agrobacterium* sp. KNK 712 and structural analysis of the stabilizing effects of each amino acid substitution was also carried out.**

Keywords: D-amino acids/*N*-carbamyl-D-amino acid amidohydrolase/directed evolution/oxidative stability/thermostability

Introduction

Enzymes are remarkable catalysts, which can exclusively work on their substrate(s) in mild conditions, discharging little waste. The use of enzymes is considered a great alternative for industrial chemical processes, as environmental problems and the demands for new chemicals rise. However, low stability, narrow substrate specificity and high production costs often limit broad applications of enzymes. Hence improvement of enzyme properties for application to industrial processes or other fields has been a major goal of protein engineering. Despite significant advances in related areas, knowledge-based design of enzymes/proteins with desired properties has not been accomplished in many cases because the current understanding of structure–function relationships is limited. Over the years, the directed evolution technique has proven to be a powerful approach for improving the properties of enzymes/proteins even without information on structure–function relationships and a number of promising results have been reported (Petrounia and Arnold, 2000; Kurtzman *et al.*, 2001). In the directed evolution process, a mutant library is generated by various methods including *in vitro* random point mutagenesis and recombination of the genes (Stemmer 1994; Zhao *et al.*, 1998; Ostermeier *et al.*, 1999). Shuffling of multiple, related genes is considered the most effective method for generating genetic diversity and consequently accelerating *in vitro* evolution (Stemmer 1994; Ness *et al.*, 1999).

N-Carbamyl-D-amino acid amidohydrolase (*N*-carbamoylase), which catalyzes the hydrolysis of the *N*-carbamyl group from *N*-carbamyl-D-amino acid, is employed industrially in the production of optically active D-amino acids from D,L-5′-monosubstituted hydantoin in conjunction with D-hydantoinase. Optically active D-amino acids are widely used as intermediates in the synthesis of antibiotics, antifungal agents, pesticides and sweeteners (Aida *et al.*, 1986; Syldatk *et al.*, 1990). *N*-Carbamoylases from various sources have been isolated, cloned into *Escherichia coli* and used for the synthesis of D-amino acids (Buson *et al.*, 1996; Grifantini *et al.*, 1996; Ikenaka *et al.*, 1998a; Nanba *et al.*, 1998; Chao *et al.*, 1999; Wiese *et al.*, 2001). It was reported that *N*-carbamoylases of *Agrobacterium tumefaciens* has five cysteine residues, rendering them liable to inactivation under oxidizing conditions (Grifantini *et al.*, 1996). The topological mapping of five cysteines in *N*-carbamoylase revealed that the Cys172 of *N*-carbamoylase is located at the active site of the enzyme and is critical for the enzyme's activity. This observation was confirmed by the recently solved structure of *N*-carbamoylases of *Agrobacterium* sp. KNK712 (Nakai *et al.*, 2000) and *A.radiobacter* CCRC14924 (Wang *et al.*, 2001). The two enzymes share 97% of amino acid sequence identity with each other.

We have made an effort to develop an enzymatic process for the production of D-amino acids using D-hydantoinase of *Bacillus stearothermophilus* SD1 and the *N*-carbamoylase of *A.tumefaciens* NRRL B11291 (Kim and Kim, 1995; Kim *et al.*, 2000a,b; Park *et al.*, 2000). However, *N*-carbamoylase was found to have a low oxidative and thermostability compared with D-hydantoinase, which is considered one of the limiting factors in the process development. In this paper, we describe the evolution of *N*-carbamoylase from *A.tumefaciens* NRRL B11291 for improvement of both oxidative and thermostability. DNA shuffling was used to generate a mutant library and the best mutant after the second round of evolution was characterized. The stabilizing effects of each amino acid

mutation in the evolved enzyme were evaluated by site-directed mutagenesis. Structural analysis was carried out to interpret the influence of each mutation on the stability of the enzyme.

Materials and methods

Materials

N-Carbamyl-D-*p*-hydroxyphenylglycine (NC-HPG) was synthesized as described in our previous work (Kim and Kim, 1995). Hydrogen peroxide solution (3%, w/w) was purchased from Sigma, restriction enzymes and *Taq* DNA polymerase from KOSCO (Sungnam, South Korea) and *DNase I* and *Pfu* polymerase from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade.

Mutant library construction

N-Carbamoylase gene from *A.tumefaciens* NRRL B11291 was amplified by PCR with two primers, CAN (5′-CAAAGGTCC-ATGGCACGTCAGATG-3′) and CAC (5′-AAGGGATCC-TTATCGAATTCCGCGATCAG-3′) as N- and C-terminal primers, respectively. The gene was purified from 0.8% agarose gel using a Bio101gene clean kit II (Qbiogene, Carlsbad, CA) and then digested with *DNase I*. Fragments of 50–100 bp, purified from 2% agarose gel, were self-assembled by PCR (94°C, 1 min; 50°C, 1 min; 72°C, 1 min; total 30 cycles). The reassembled DNA fragments were amplified by PCR (94°C for 1 min; 50°C, 1 min; 72°C, 1 min; total 15 cycles) with two primers, CAN and CAC. Whole DNA shuffling steps were attempted by using *Taq* polymerase under the conditions offered by the provider. The amplified DNA fragments (0.9 kb) were purified from agarose gel and then digested with *Nco*I and *Bam*HI. The resulting genes were cloned into the expression vector pTrc99A and transformed into *E.coli* JM109 by electroporation (Gene Pulser II Electroporation System, Bio-Rad, Hercules, CA). Transformants were plated on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) and grown overnight at 37°C.

Screening of a mutant library

Primary screening of *N*-carbamoylase variants with improved oxidative and thermostability was developed based on the activity staining method as previously described (Park *et al.*, 2000). The agar plate contained 5 g/l *N*-carbamyl-D-*p*-hydroxyphenylglycine (NC-HPG), 1 mM EDTA, 0.01% phenol red and 1% bacto-agar. The final pH of the solution was adjusted to 6.3. *N*-Carbamoylase converts NC-HPG to ammonia and carbon dioxide, increasing the pH, and the yellowish color changes to red around the colonies with *N*-carbamoylase activity.

The transformants from a mutant library were transferred on to filter papers in duplicate and incubated with lysozyme (2 mg/ml) at 37°C for 30 min. The filters were washed with distilled, deionized water and subjected to enzyme inactivation steps. In the first round of evolution, one of the filters was incubated at 70°C for 2 h in a dry oven for the thermal inactivation step. The other was treated with 1.5 mM hydrogen peroxide at 37°C for 30 min for the oxidative inactivation step. After the inactivation steps, each filter paper was overlaid on the activity staining plate and incubated for 30 min at 37°C and colonies displaying the color change simultaneously on both agar plates were primarily selected from the master plate. Wild-type *N*-carbamoylase completely lost the activity after the above-mentioned inactivation steps and no color change occurred on the agar plates. In the second round, a library of mutant was incubated under conditions where the best mutant selected in the first round lost activity completely: 70°C for 4 h in a dry oven and 2.5 mM hydrogen peroxide for 30 min, respectively. Selected variants were cultured in 3 ml of LB broth and then induced by addition of 1 mM IPTG when the absorbance reached about 0.6. The cells were further grown for 3 h and then separated by centrifugation. Collected cells were disrupted by sonication and the soluble fraction was used for the confirmation of oxidative and thermostability.

Site-directed mutagenesis

Site-directed mutagenesis was performed by the overlap extension method using polymerase chain reaction (PCR) (Sambrook and Russell, 2001). The sequences of synthetic oligonucleotides for site-directed mutagenesis were as follows: 5′-ACACGCG-AACTGGTGGTTGGC-3′ and 5′-GCCAACCACCAGTTCG-CGTGT-3′ for the substitution of Gln23 with Leu, 5′-CCG-GGGCGCGAACTTCATC-3′ and 5′- GATGAAGTTCGCGC-CCCGG-3′ for the substitution of Val40 with Ala, 5′- CCG-CGCTGGTATTTCACCGAC-3′ and 5′-CGGTGAAATAC-CAGCGCGG-3′ for the substitution of His58 with Tyr, 5′-GAAATGCCCAGCCCGGTG-3′ and 5′-CACCGGGCTG-GGCATTTC-3′ for the substitution of Gly75 with Ser, 5′-TGGCGGGTGCTGGGACTTAAG-3′ and 5′- CTTAAGT-CCCAGCACCCGCCA-3′ for the substitution of Met184 with Leu and 5′- CGTTGCCCTGGCCACGACGTTG-3′ and 5′-CCAACGTCGTGGCCAGGGCAACGATTT-3′ for the substitution of Thr262 with Ala (Genotech, Taejon, Korea). The mutated gene was cloned into the expression vector, pTrc 99A, and expressed in *E.coli* JM109 by adding 1 mM IPTG. The mutations were confirmed by DNA sequencing.

Enzyme purification

N-Carbamoylase and variants were purified as follows. Cells were grown in 300 ml of LB broth and induced by addition of 1 mM IPTG when the absorbance at 600 nm reached ~0.6. Cells were further grown for 3 h and collected by centrifugation. The pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethanesulfonyl fluoride and disrupted by sonification (VC 750, Sonics & Materials, Newtown, CT). After addition of 0.1% protamine sulfate and incubation on ice for 1 h, cell debris was removed by centrifugation at 12 000 *g* for 30 min. The soluble fraction was collected and loaded on to a Sepharose Q resource column (Amersham Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The column was eluted with a linear gradient (0–1 M) of NaCl. The concentrated protein solution was loaded on to a Phenyl Superose column (Amersham Pharmacia Biotech) equilibrated with 20 mM potassium phosphate buffer containing 4% sodium sulfate. The column was eluted with a reverse linear gradient (4–0%) of sodium sulfate. Fractions exhibiting *N*-carbamoylase activity were analyzed using SDS–PAGE and samples showing a single band were used in further studies. All purification steps were carried out using an FPLC system (Amersham Pharmacia Biotech).

Oxidative and thermostability of enzyme

Purified enzyme solution was incubated with 1 mM DTT for 1 h at 25°C and the remaining DTT was removed by passing through a D-salting column (Pierce, Rockford, IL) prior to the enzyme inactivation steps. For determination of the thermostability of the enzyme, purified enzyme (0.25 mg/ml) was incubated for 30 min at different temperatures and the residual

activity was measured. For oxidative stability, purified enzyme (0.25 mg/ml) was incubated for 30 min with various levels of hydrogen peroxide at 25°C and the residual activity was measured. Stability was estimated by measuring the ratio of residual activity to the initial activity of the enzyme.

Enzyme assay and analysis

The *N*-carbamoylase was assayed in 100 mM potassium phosphate buffer (pH 7.0) containing 25 mM NC-HPG as substrate. The reaction mixture was incubated at 40°C for 30 min and the D-HPG produced was determined by HPLC (Shimazu, Kyoto, Japan) as described in our previous work (Kim and Kim, 1995). Kinetic constants of the enzyme were determined from double reciprocal plots of initial activity and substrate concentration. Analytical SDS–PAGE was performed according to the method of Laemmli (Laemmli, 1970). Acrylamide gels were stained with Coomassie Brilliant Blue R250. The protein concentration was determined by the method of Bradford (Bradford, 1976).

Structural analysis of mutations was carried out by PDB viewer (v.3.7b2) obtained from the ExPASy molecular biology server of the Swiss Institute of Bioinformatics. The *N*-carbamoylase structure was constructed by using MolScript v.2.1 (Kraulis, 1991).

Results and discussion

Enzyme evolution

From the mutant library generated by the first round of evolution, about 10 000 clones were subjected to the enzyme inactivation steps mentioned in the Materials and methods section. After the enzyme inactivation steps, wild-type *N*-carbamoylase gave no color change on the activity staining agar plate, thus the variants with improved oxidative and thermostability compared with the parental enzyme could easily be screened. The stability of the selected variants was verified by comparing the residual activity after incubation at various temperatures and levels of hydrogen peroxide. Of the positive variants, 1S15 was found to have the highest oxidative and thermostability. In the second round of evolution, the 10 best clones from the first round library were recombined as parental genes to create a mutant library by DNA shuffling. Screening of 8500 clones after the enzyme inactivation steps on the activity staining plate resulted in a mutant (2S3) with more improved stability than 1S15. After the inactivation steps used for the second round screening, 1S15 did not show any color change on the agar plates. In the same manner, the third round library was generated by using the best 10 clones from the second round library as parental genes. However, no variant with more improved oxidative or thermostability than 2S3 was screened. Therefore, the mutants 1S15 and 2S3 were selected for further study in terms of oxidative and thermostability.

Mutant libraries were analyzed to obtain some insight into the correlation between oxidative and thermostability of the enzyme. In the primary screening of a library on the activity staining plate, more than 50% of clones with improved oxidative stability also displayed enhanced thermostability and vice versa (data not shown) by eye. This result implies that these two properties are at least positively correlated, if not directly correlated.

Characterization of the selected variants

The selected 1S15 and 2S3 along with wild-type *N*-carbamoylase were purified according to the procedures described in the Materials and methods section and their oxidative and thermostability were determined. In the case of thermostability, purified enzymes were incubated at various temperatures and residual activities were measured. As shown in Figure 1A, 2S3 retained about 50% of the initial activity after heat treatment at 73°C for 30 min, showing clearly improved thermostability compared with both wild-type enzyme and 1S15. For oxidative stability, for 2S3 ~80% of the initial activity remained even after incubation with 0.2 mM hydrogen peroxide for 30 min (Figure 1B). Wild-type enzyme almost completely lost its activity under the same conditions. The increment in both oxidative and thermostability was distinctive for the evolved variants 1S15 and 2S3, which confirms that the oxidative and thermostability of *N*-carbamoylase were simultaneously improved by directed evolution.

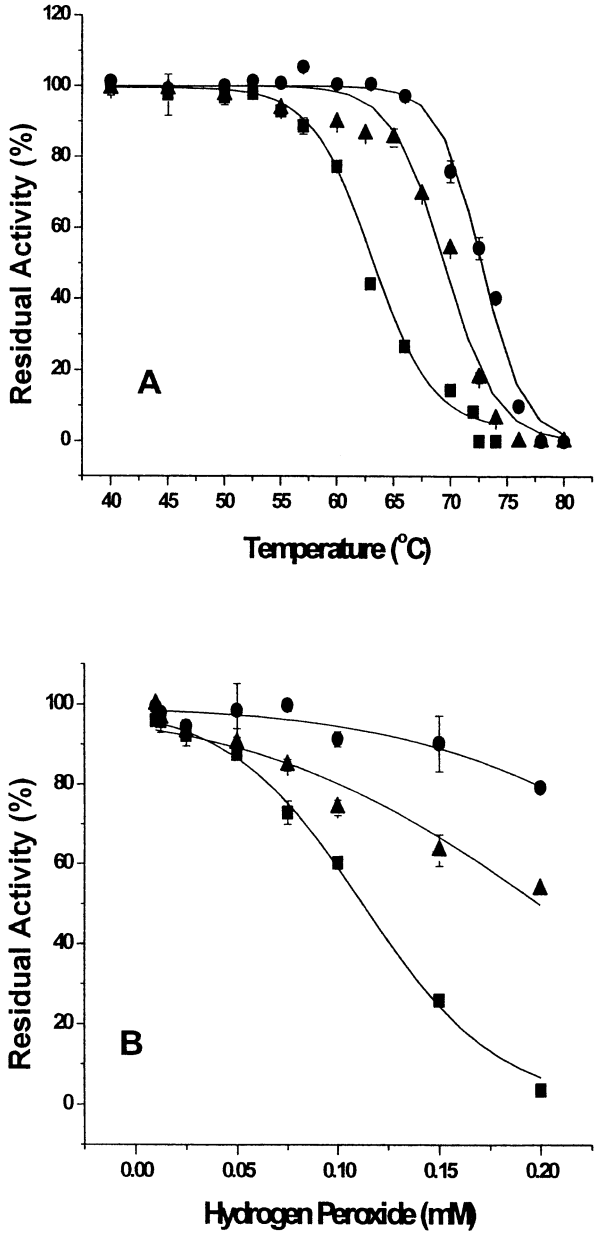

Fig. 1. (**A**) Thermostability of the wild-type and evolved *N*-carbamoylases. (■) Wild-type; (▲) 1S15; (●) 2S3. Data represent the averages of duplicate experiments. (**B**) Oxidative stability of the wild-type and evolved *N*-carbamoylases. Symbols as in (A). Data represent the averages of duplicate experiments.

Table I. Kinetic parameters of the wild-type and evolved *N*-carbamoylases

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	9.5 ± 0.2	1.1 ± 0.0	8.7 ± 0.2
1S15	12.3 ± 0.1	2.4 ± 0.1	5.1 ± 0.3
2S3	6.9 ± 0.1	1.1 ± 0.1	6.3 ± 0.3

Each value represents the average and mean of triplicate experiments.

There has been an argument about the correlation between activity and thermostability of enzymes. It has been reported that an improvement in thermostability is obtained at the cost of enzyme activity such as an increase in K_m and a decrease in k_{cat} (Shoichet *et al.*, 1995). It has been suggested that the activity and thermostability of enzymes are not inversely correlated and that it is possible to evolve enzymes to higher thermostability, maintaining activity at low temperatures comparable to that of the wild-type counterpart (Serrano *et al.*, 1993; Giver *et al.*, 1998). Kinetic constants of the evolved enzymes 1S15 and 2S3 were determined and compared with those of the wild-type (Table I). In the case of 1S15, the K_m and k_{cat} values increased about 2.5- and 1.3-fold, respectively, compared with the wild-type enzyme, but k_{cat}/K_m decreased to 60% of the level of the wild-type. For 2S3, a slight increase in k_{cat}/K_m was observed compared with 1S15, even though K_m recovered to the level of the wild-type enzyme, which was due to a decrease in k_{cat}. From these results, one cannot conclude whether the two properties (activity and thermostability) are inversely correlated or not correlated, but it appears that some thermostabilizing mutations in mutant 2S3 came at the cost of activity.

Amino acid substitutions in the evolved enzymes

Mutated amino acid residues in 1S15 and 2S3 were identified by sequencing the corresponding genes. In mutant 1S15, Thr262 and Met184 were substituted for Ala and Leu, respectively. Mutant 2S3 possessed six mutations, Q23L, V40A, H58Y, G75S, M184L and T262A, and two mutations (M184L and T262A) had occurred in 1S15. It was reported previously that the substitution of histidine at position 58 for tyrosine improved the thermostability of *N*-carbamoylase from *Agrobacterium* sp. KNK712 (Ikenaka *et al.*, 1998b,c).

In order to evaluate the contribution of each amino acid change to an increase in oxidative or thermostability, mutations were incorporated individually into wild-type enzyme by site-directed mutagenesis and the resulting mutants were subjected to a stability test. As can be seen in Table II, both M184L and T262A, which were found in 1S15, led to an increase in oxidative and thermostability and these mutations were considered almost additive. In particular, T262A resulted in a significant improvement in both oxidative and thermostability of 1S15. In mutant 2S3, Q23L and H58Y both showed increased stabilities to some extent. Especially the mutation Q23L resulted in the highest contribution to oxidative stability in the mutations found in 2S3. The other two mutations, V40A and G75S, mainly gave rise to increments in oxidative stability rather than thermostability. The thermostability of 2S3 was reinforced as these single thermostabilizing mutations accumulated. However, the cumulative effect on oxidative stability was not observed even though the improvement in oxidative stability by a single mutation was considerable.

Methionine, cysteine, tryptophan, tyrosine and histidine residues are susceptible to oxidation and modification of these residues might lead to inactivation of enzymes. Oxidation of methionine to the sulfoxide form is considered to result in the generation of a larger and more polar side chain, which disrupts the structure and stability of enzymes (Kim *et al.*, 2001). Thus, substitution of methionine residues in the evolved variants (1S15 and 2S3) seems to confer resistance against oxidation, leading to an improvement in oxidative stability of the enzymes. *N*-Carbamoylase from *A.tumefaciens* has five cysteine residues and, of these, Cys172 was revealed to be essential for catalysis of the enzyme (Grifantini *et al.*, 1996). However, none of the cysteine residues were found to be changed in the evolved enzymes. An increase in oxidative stability by substitution of non-oxidizible amino acids was observed in the case of peroxidases (Cherry *et al.*, 1999; Morawski *et al.*, 2001). In contrast, it was reported that the oxidative stability of enzymes is enhanced through direct alteration of oxidizable to non-oxidizable amino acids (Johnsen *et al.*, 2000; Ju *et al.*, 2000; Slusarczyk *et al.*, 2000; Yang *et al.*, 2000). Therefore, it is noteworthy that substitution of even the non-oxidazable amino acid residues such as Q23, V40, G75 and T262 also made a significant contribution to the increase in oxidative stability of the enzyme, and this demonstrates an advantage of directed evolution in the improvement of enzyme stability.

Table II. Oxidative and thermostability of the wild-type enzyme and each mutant

	Thermostability[a] (%)	Oxidative stability[b] (%)
WT	10.0 ± 0.8	5.0 ± 1.2
M184L	19.7 ± 2.0	20.0 ± 2.3
T262A	50.5 ± 3.4	40.9 ± 2.2
1S15 (M184L, T262A)	54.3 ± 0.5	54.8 ± 1.2
Q23L	19.1 ± 1.4	63.9 ± 2.3
V40A	7.0 ± 2.3	20.6 ± 1.2
H58Y	20.0 ± 1.9	20.6 ± 1.2
G75S	3.5 ± 1.1	42.5 ± 0.8
2S3	78.5 ± 3.1	79.3 ± 1.2

Each value represents the average and mean of triplicate experiments.
[a]Residual activity after heat treatment for 30 min at 70°C.
[b]Residual activity after incubation with 0.2 mM hydrogen peroxide for 30 min at 25°C.

It is known that mutations leading to the simultaneous improvement of multiple properties of enzymes such as activity and stability are rare (Giver *et al.*, 1998). Unless multiple constraints are imposed simultaneously, an improvement of any one property is likely to be obtained at the cost of another one. However, in the case of the evolved mutant 2S3, single mutations such as Q23L, H58Y, M184L and T262A resulted in an enhancement of both oxidative and thermostability. This might be explained by the fact that the screening of positive clones with improved stability proceeded by imposing two constraints (oxidative and thermostability) on a mutant library throughout the directed evolutionary process of *N*-carbamoylase. Alternatively, there might be a close correlation between the oxidative and thermostability of enzymes. It has been reported that the oxidation of amino acid residues causes the disruption of protein conformation and, as a result, a decrease in protein thermostability (Usami *et al.*, 1996; Gao *et al.*, 1998; Komsa-Penkova *et al.*, 1999). Hence it is plausible that the enhancement of oxidative stability might lead to an improvement in thermostability. An increase in thermostability is considered to result from the increased interactions between amino acid residues and consequently increased rigidity of

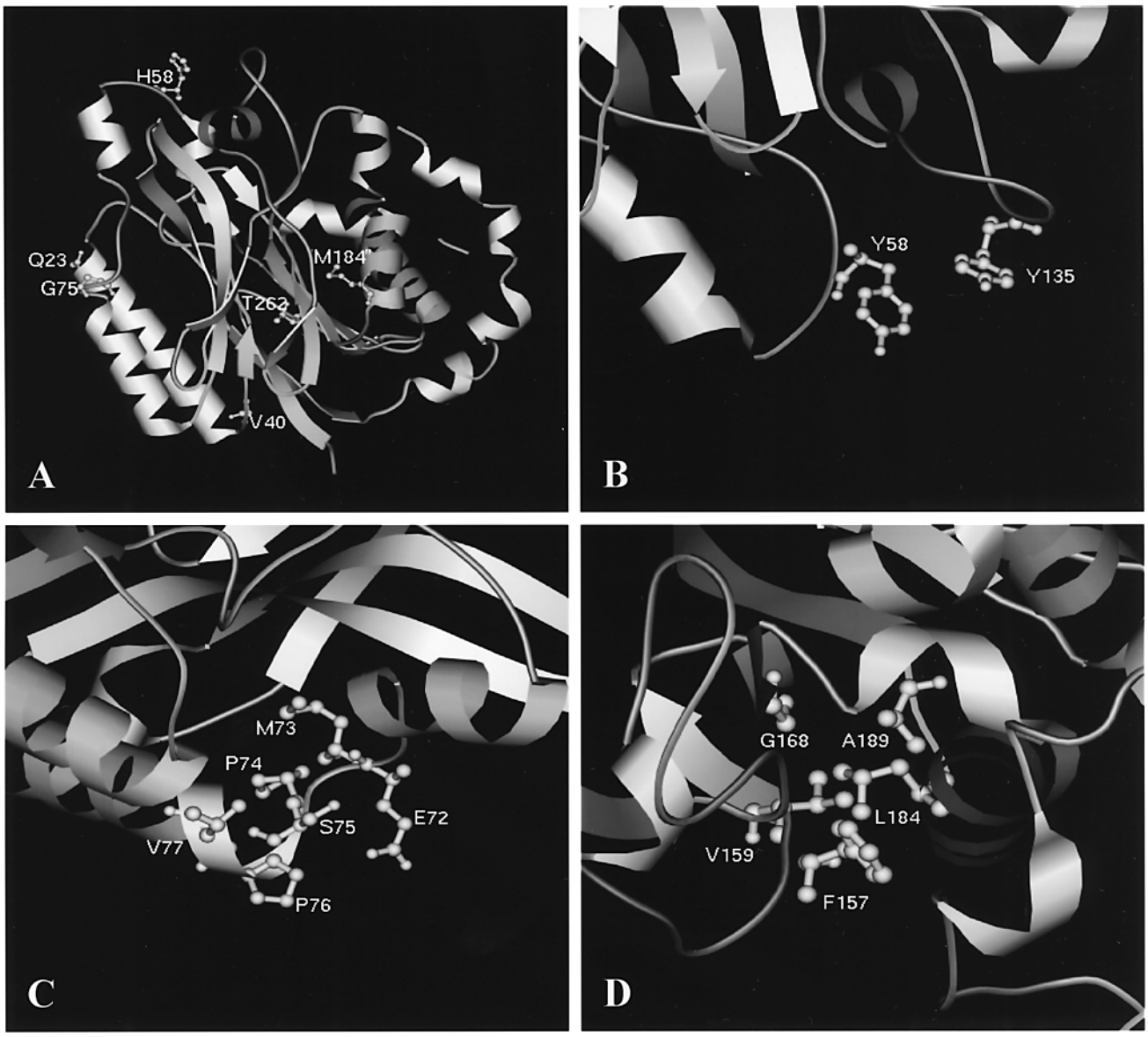

Fig. 2. (**A**) Ribbon representation of monomeric structure of *N*-carbamoylase from *Agrobacterium* sp. KNK712. The amino acid residues substituted in 2S3 are highlighted. (**B**) Partial view of loops between β2/α2 and β5/α4 in which the mutation sites of H58Y and Tyr136 are located, respectively. (**C**) Mutation site of Gly75 to Ser and the neighboring amino acid residues. (**D**) Location of M184L and the neighboring amino acid residues.

protein, which might contribute to a reduction of oxidative inactivation of protein by oxidizing agents. As mentioned above, more than 50% of the mutants with improved thermostability also exhibited enhanced oxidative stability and vice versa. This strongly suggests that there is at least a positive correlation between oxidative and thermostability and that the simultaneous improvement of oxidative and thermostability of enzymes is possible.

Structural analysis of the evolved N-carbamoylase

Recently, the structures of *N*-carbamoylases from *Agrobacterium* sp. KNK712 and *A.radiobacter* CCRC14924 were resolved (Nakai *et al.*, 2000; Wang *et al.*, 2001). The overall folds of these two *N*-carbamoylases were revealed to be identical. Each monomer of *N*-carbamoylase folds into a four-layer sandwich structure and the quaternary structure is a tetramer. *N*-Carbamoylase from *A.tumefaciens* NRRL B11291 shares 97 and 100% amino acid sequence identity with those from *Agrobacterium* sp. KNK712 (PDB ID: 1ERZ) and *A.radiobacter* CCRC14924 (PDB ID: 1FO6), respectively. It is reasonable that the structure of *N*-carbamoylase from *A.tumefaciens* NRRL B11291 is almost the same as those from the above two sources. Currently, the structural coordinate of *N*-carbamoylase from *Agrobacterium* sp. KNK712 is available from the Protein Data Bank (PDB). Amino acid residues, which were changed in 2S3, are the same as in *N*-carbamoylase from *Agrobacterium* sp. KNK712, except the V40; in *Agrobacterium* sp. KNK712, alanine is located at this position. Thus, the stabilizing effects of each amino acid residue in the evolved 2S3 were assessed based on the structural data for *N*-carbamoylase of *Agrobacterium* sp. KNK712.

All the mutations occurred on the protein surface or near the surface, except M184L, which is in the core region (Figure 2A). Three mutation sites, V40, H58 and G75, are located in the loop region between α1/β2, β2/α2 and α2/α3, respectively. Two mutation sites, Q23 and M184, are placed in α1 and α5 helices, respectively. T262 is found in the edge of the b13

strand. M184 located in α5 helix seems to constitute an inter-subunit interface, but not directly involved in the inter-subunit association.

There are many different types of interactions within proteins that affect stability, including hydrophobic interactions, hydrogen bondings, electrostatic interactions, disulfide bonds and dispersion forces. It is generally accepted that the enhancement of these interactions leads to an increase in protein stability. The replacement of Q23, H58, M184 and T262 resulted in an improvement in the thermostability of 2S3 (Table II). Deamidation of Asn and Gln was reported to destabilize the protein conformation (Wright, 1991) and substitution of these amino acid residues led to an increase in the thermostability of protein (Giordano *et al.*, 1999; Declerck *et al.*, 2000). The increase in the thermostability of 2S3 by mutation of Gln23 might be attributed to a reduction in deamidation of amino acid residues.

The substitution of His58 for Tyr seems to increase the hydrophobic interaction with Tyr136, which is in the vicinity of His58 (Figure 2B), enhancing the oxidative and thermostability of the enzyme. The 134–142 region constituting the loop between the β5 strand and α4 helix is known to be very flexible and the average *B*-factor for atoms was estimated to be 43 Å^2, being highest for Tyr139 (50 Å^2), compared with an average *B*-factor of 26 Å^2 for all atoms in *N*-carbamoylase (Wang *et al.*, 2001). Thus, the creation of a hydrophobic interaction between Tyr58 and Tyr136 might enhance the rigidity of the protein structure and consequently the thermostability of the enzyme. The loop between β5/α4 forms the active site cleft with other loops and His129 and His144 in the loop is closely involved in the maintenance of a stable conformation of the catalytic cleft of *N*-carbamoylase. The low k_{cat}/K_m of 2S3 compared with the wild-type enzyme might be caused by the increase in the rigidity of this region.

Gly75 is located between Pro74 and -76 and flexible amino acid residue is favored in this position (Figure 2C). The decrease in thermostability on replacement of Gly75 with Ser is likely to be due to the unfavorable protein conformation. However, a substituted serine residue with hydroxyl side chain might lead to hydrogen bonding with a neighboring amine group, which appears to compensate for the adverse structural conformation to some extent.

As mentioned above, oxidation of methionine residues is known to disrupt the protein structure. Replacement of Met184, which is located near the enzyme core, with Leu resulted in an increased stability of the enzyme. This stabilizing effect can also be explained by increased hydrophobic interaction. Analysis using PDV viewer suggests that the side chain of Met184 of wild-type enzyme might interact with Phe157 and Val159. Thus, it is expected that substitution of Met 184 with the more hydrophobic leucine might cause a stronger interaction with Phe157, Val159 and Ala189, which leads to an increase in stability of the enzyme. Especially, it is likely that the additional methyl group of leucine enhances hydrophobic interactions with Phe157 and Val159 as depicted in Figure 2D.

The mutation of Thr262 to Ala exhibited the most significant effect on stabilization of *N*-carbamoylase. We tried to explain the stabilizing effect of T262A based on the structural analysis, but it was difficult to elucidate the possible effects. This residue is located in the interfacing region of the loosely packed, so-called A and D subunits and there exist 56 interactions including four hydrogen bonds (Wang *et al.*, 2001). It was reported that the oligomeric structure also contributes to the thermal and chemical stability of proteins (Salminen *et al.*, 1996; Rietveld and Ferreira, 1998). Hence it seems that the replacement of Thr262 with Ala might induce the oligomeric structure of the enzyme in such a way that the enzyme is stabilized.

In conclusion, our studies have shown that the oxidative and thermostability of *N*-carbamoylase can simultaneously be improved by directed evolution even without structural information. The screening system attempted here allowed the rapid selection of the simultaneously evolved enzymes from a mutant library. Analysis of the positive clones suggested that the oxidative and thermostability of the enzyme are at least positively correlated, if not directly correlated. The full range of structural features that confer an increase in oxidative and thermostability of the enzyme remains to be demonstrated.

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