Journal of Bioscience and Bioengineering
VOL. 133 No. 4, 309–315, 2022

www.elsevier.com/locate/jbiosc

Thermostability enhancement of L-glutamate oxidase from *Streptomyces* sp. NT1 by full consensus protein design

Yuka Hayashi,[1] Masaya Nakamura,[1] Shogo Nakano,[2] Sohei Ito,[2] Yasuhisa Asano,[3] and Daisuke Sugimori[1,*]

Materials Science Course, Faculty of Symbiotic Systems Science and Technology, Fukushima University, 1 Kanayagawa, Fukushima 960-1296, Japan,[1] Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-Ku, Shizuoka 422-8526, Japan,[2] and Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan[3]

Received 4 August 2021; accepted 16 December 2021
Available online 19 January 2022

Thermostable L-glutamate oxidases (LGOXs) are desirable for use in L-glutamate (L-Glu) assay kits, enzymatic synthesis of α-ketoglutarate and for biosensor development. However, protein engineering efforts to improve thermostability often lead to a decrease in enzymatic activity. In this report, we aimed to enhance the thermostability (melting temperature, T_m) of a mesophilic LGOX from *Streptomyces* sp. NT1 ($LGOX_{NT1}$) without a reduction in activity by a sequence-based protein design approach, termed full consensus (Fc) protein design. Among the 690 amino acids of $LGOX_{NT1}$, 104 amino acids were substituted by the Fc protein design. The mutant gene was artificially synthesized and expressed in *Escherichia coli* BL21(DE3) cells. The T_m of the purified, recombinant LGOX mutant (FcLGOX) was determined to be ~72 °C, which is an increase on the T_m of 65 °C for $LGOX_{NT1}$ and the highest among known LGOXs. Importantly, purified FcLGOX showed no loss of specific activity or substrate specificity after a 30-min incubation at 70 °C. Our findings provide a new approach to improve the thermostability of enzymes.

[**Key words:** L-Glutamate oxidase; Thermostability enhancement; Artificial protein design; Consensus protein design; *Streptomyces* sp.]

L-Glutamate oxidase (LGOX, EC 1.4.3.11) catalyzes oxidative deamination of L-glutamate (L-Glu) to α-ketoglutarate (αKG), is a FAD-dependent enzyme and a member of the L-amino acid oxidase family. LGOX is a useful enzyme for αKG production (1) and in L-Glu quantification kits and has been used in biosensor design (2). Currently, many LGOXs have been reported but the only commercially available LGOX is from *Streptomyces* sp. strain X-119-6 ($LGOX_{X119}$) (3,4). However, the low thermal stability of $LGOX_{X119}$ with no treatment of metalloprotease (4) limits its use in industrial applications. Thus, an LGOX with high specific activity and thermostability is required for industrial applications. There have been numerous efforts to improve the thermostability of proteins using various approaches such as directed evolution (5), consensus design (6) and ancestral design (7) with many reported improvements in the thermostability of enzymes (5,6). Increasing the internal hydrophobicity or reducing the number of neutral amino acids (5), formation of ion-pairs (salt bridge) (7) and introducing a disulfide bridge (8) are approaches to improve protein thermostability effectively. However, these successful efforts are on a case-by-case basis and a general set of rules for enhancing protein thermostability remains unresolved. Moreover, it is difficult to improve thermostability of an enzyme without reducing enzyme activity, i.e., without an activity–stability trade-off. In many cases, an improvement in thermostability through mutation is accompanied by a concomitant decrease in the specific activity and catalytic efficiency of the mutant enzyme (7–10). Only the thermostabilities of *Rhodopseudomonas palustris* 5-aminolevulinic acid synthase (11) and the archaeal and bacterial nucleoside diphosphate kinase (12) were improved without loss of enzyme activity.

In this study, we aimed to improve the thermostability of a mesophilic LGOX by an original artificial protein design without a trade-off between stability and activity. Among the 690 amino acids of LGOX ($LGOX_{NT1}$) from *Streptomyces* sp. strain NT1, introduction of 104 amino acid mutations yielded a mutant LGOX with a melting temperature (T_m) elevated from ~65 °C to ~72 °C without a reduction in enzyme kinetics and other enzymatic properties such as substrate specificity and optimum pH when compared with the wild-type enzyme (WT); however, there might be a trade-off between the thermal stability and pH stability. Our findings demonstrated that introducing amino acid substitutions on the surface of the enzyme, such as loops on the molecular surface including subunit interfaces is an effective approach for thermoadaptation. We believe artificial protein design performed by INTMSAlign (13) and an original python script (14) offers a new approach and new era in artificial protein evolution.

MATERIALS AND METHODS

Materials Bacto tryptone and Bacto malt extract were purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan). Yeast extract and peroxidase (POD) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Kanamycin sulfate was from Nacalai Tesque, Inc. (Kyoto, Japan). *N,N*-Bis(4-sulfobutyl)-3-methylaniline, disodium salt (TODB) was purchased from Dojindo Laboratories (Kumamoto, Japan). 4-Aminoantipyrine (4AA) was purchased from Sigma–Aldrich Japan Co., LLC (Tokyo, Japan). HisTrap HP, Resource Q, Mono Q and Superdex200 10/300 GL

* Corresponding author.
E-mail address: sugimori@sss.fukushima-u.ac.jp (D. Sugimori).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2021.12.008

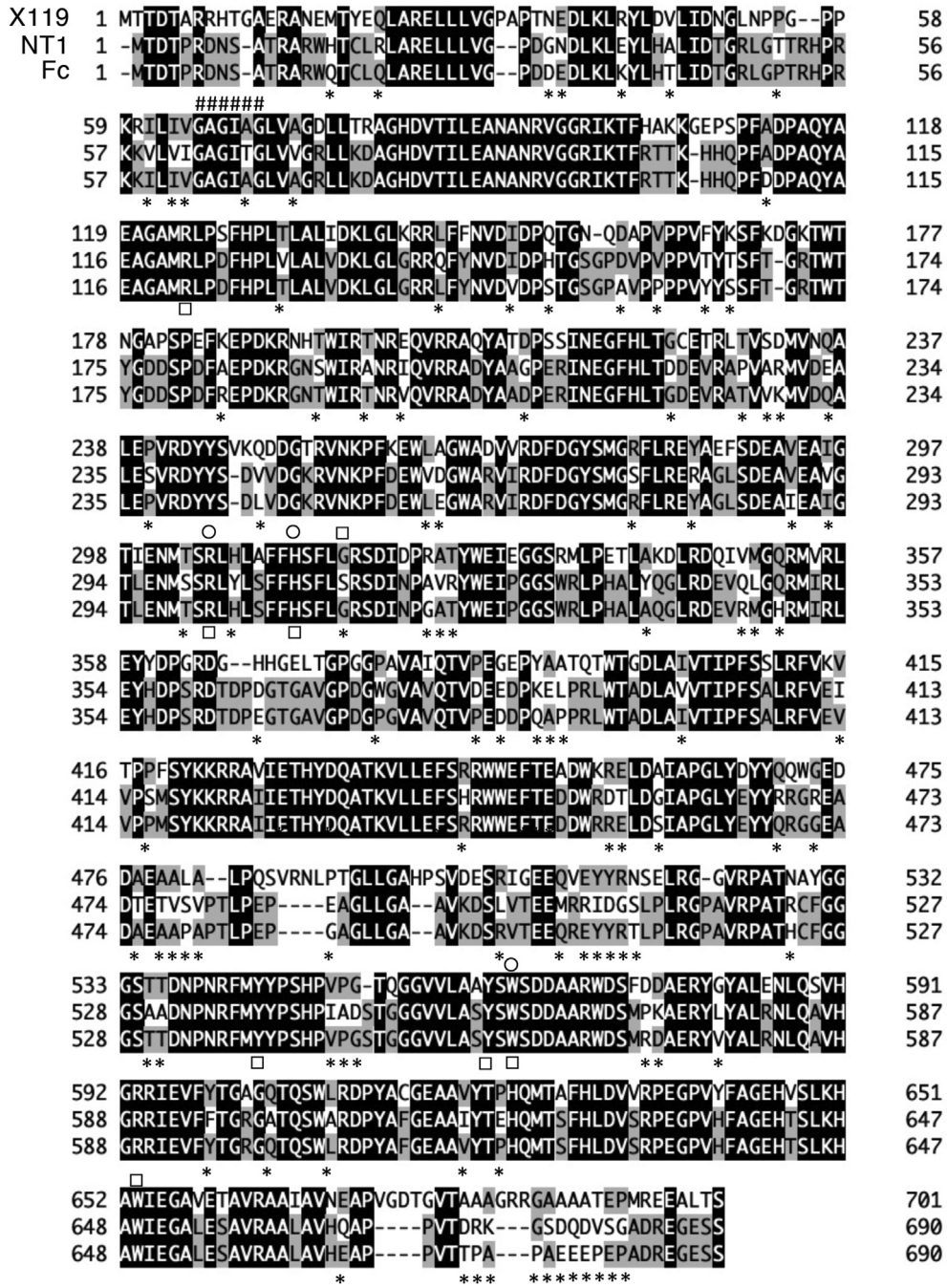

FIG. 1. Sequence alignment of $LGOX_{NT1}$, FcLGOX and $LGOX_{X119}$. X119, LGOX of *Streptomyces* sp. strain X-119-6; NT1, LGOX of *Streptomyces* sp. strain NT1; Fc, full-consensus designed LGOX. Black- and grey-shaded letters indicate identical or similar amino acids, respectively. Asterisks, amino acid substituted between NT1 and Fc; number signs, FAD binding motif (GxGxxG); open squares, FAD binding site; open circles, amino acid involved in substrate recognition.

columns were purchased from Cytiva (Tokyo, Japan). Toyopearl DEAE-650M was from Tosoh Co. (Tokyo, Japan). The restriction enzyme, DpnI, *Escherichia coli* HST08 premium competent cells and Tks Gflex DNA polymerase were purchased from Takara Bio Inc. (Shiga, Japan). Ligation high (ver. 2) was purchased from Toyobo Co., Ltd. (Osaka, Japan). All other chemicals were of the highest or analytical grade from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan) or Nacalai Tesque, Inc.

Microorganisms and gene identification of $LGOX_{NT1}$ About 112 strains of actinomycetes from stock cultures were screened for LGOX activity. *Streptomyces* sp. strain NT1 exhibiting the highest specific LGOX activity was selected and cultured in 6 L of International Streptomyces Project-2 (ISP2) medium (10 g malt extract, 4 g yeast extract and 4 g glucose per liter, pH 7.2) containing 0.1% L-Glu for 5 days at 28 °C using a 10-L fermentor (500 rpm agitation, 1 vvm). Fifty wet-grams of cells harvested by centrifugation were suspended in 20 mM Bis-Tris HCl (pH 7.0) and disrupted by sonication (20 kHz, 100 W). Following centrifugation of the cell lysate, $LGOX_{NT1}$ was purified from the cell free extract by 75% (v/v) acetone precipitation followed by Toyopearl DEAE-650M (column volume (CV), 36 mL), 2nd round DEAE-650M (CV, 36 mL), Resource Q (CV, 1 mL), Mono Q (CV, 1 mL), Superdex 200 10/300 GL (CV, 24 mL) with 0.15 M NaCl/20 mM Bis-Tris HCl (pH 7.0) at 0.5 mL/min and 2nd Mono Q (CV, 1 mL) chromatography steps. Buffers used for the anion exchange chromatography (DEAE-650M, Resource Q, and Mono Q) were 20 mM Bis-Tris HCl buffer (pH 7.0), and the proteins were eluted with a linear gradient (10 CV) of 0–1 M NaCl in the same buffer. The purified enzyme sample (11.3 U/mg-protein) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the four bands, 21.2-, 50.2-, 75.6- and 111-kDa (Fig. S1A), on the gel were analyzed by nanoLC-MS/MS using a Xevo QTOF MS system (Waters Corp., Milford, MA, USA) as described previously (15) to determine the amino acid sequences. The obtained peptide sequences showed high homology with other microbial LGOXs and L-amino acid oxidases as well as amine oxidases in a BLAST search and the obtained LGOX sequence was

used as a probe. Draft genome sequencing of strain NT1 was performed using Ion PGM (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The $LGOX_{NT1}$ gene, *lgox*, was identified in the predicted ORF database as 2073 bp encoding a protein 690 amino acids in length.

In silico analyses The amino acid sequence of LGOX from *Streptomyces nodosus* NBRC12895 (SnLGOX) was used as the template for Blastp search. The sequence library was prepared by submitting the SnLGOX sequence to Blastp as a query, and 10,000 sequences were obtained. The database was set to non-redundant. Sequences bearing >90% identity with other sequences and exceeding the length of SnLGOX by ± 5% were omitted by the original python script used in previous studies (14,16–19). Here, the python scripts were available from GitHub (https://github.com/shognakano/Library_curation). A total of 4919 sequences were obtained. Further, the key residues were assigned using the following procedure which was already reported in previous study (14,16–19): (i) Dozens of sequences were selected randomly from the library and aligned by multiple sequence alignment (MSA) software. (ii) Correlatively mutated residues were predicted by analyzing the MSA result; the selected residues were regarded as candidates for key residues. Consequently, seven residues (N88, W173, M274, H308, Y576, R606 and E613) were assigned as key residues. Through selection of sequences from the library bearing the key residues, 28 sequences were obtained. Using these sequences, full-consensus LGOX (FcLGOX) was designed by the python script (14). The resulting FcLGOX amino acid sequence had 104-point mutations (Figs. 1 and 2). The FcLGOX gene (*fclgox*) was codon optimized for expression in *E. coli* and synthesized by Genewiz Japan Corp. (Saitama, Japan).

Construction of expression systems for WT and artificially designed enzymes *lgox* was amplified by PCR using the genome of strain NT-1 as a template and primer set 5'-aattatacatatgaccgacactccac-3' and 5'-ttaaagctttcacgaggactcc-3' (underlined letters represent NdeI and HindIII sites), which were designed according to the predicted ORF. The PCR reaction (25 μL) contained Gflex PCR buffer, 7.5 pmol of each primer, 0.625 U Tks Gflex DNA Polymerase (Takara Bio) and 500 ng chromosomal DNA as the template. The thermal cycling parameters were 94 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 2 min. The obtained PCR fragment was digested with NdeI and HindIII, purified by electrophoresis on a 0.7% agarose gel and ligated into the NdeI and HindIII digested pET24a (+) vector (Merck KGaA, Darmstadt, Germany) to give pET24a/*lgox*. *E. coli* HST08 competent cells (Takara Bio) were transformed by pET24a/*lgox*, the competent cells grown in Luria–Bertani (LB) medium, the plasmid extracted and purified from these cells and transformed into *E. coli* BL21(DE3) cells. An inverse PCR with the primer set 5'-caccaccaccaccaccactgag-3' and 5'-cgaggactccccttcccggtc-3' (underlined sequence represents a His_6 tag) using pET24a/*lgox* as the template was performed to link the His_6 tag to the C-terminus of WT, as described previously (20), to yield pET24a/*lgox_his*. Additionally, *lgox* was introduced between the NdeI and HindIII sites of pCold II (Takara Bio) to give a WT construct with an N-terminal His_6 tag, which was designated as pCold II/*his_lgox*. *E. coli* BL21 cells were transformed with pCold II/*his_lgox*. The synthesized *fclgox* carried on pUC57 was amplified via PCR with primer set 5'-aattatacatatgaccgataccccgcgtg-3' and 5'-attaagctttagctttcaccttc-3' (underlined text represents NdeI and HindIII sites), and the PCR product was ligated into the NdeI and HindIII digested pET24a (+) vector to give pET24a/*fclgox*. *E. coli* BL21(DE3) cells were transformed with pET24a/*fclgox*.

The recombinant *E. coli* BL21(DE3) cells carrying pET24a/*lgox* and pET24a/*fclgox* produced active LGOX; however, the recombinant cells carrying pET24a/*lgox_his* showed no LGOX activity. The recombinant cells carrying pColdII/*his_lgox* exhibited higher activity than the recombinant cells carrying pET24a/*lgox*. Thus, only active recombinant LGOXs (rLGOXs) produced from pET24a/*lgox*, pColdII/*his_lgox* and pET24a/*fclgox* were investigated.

Expression and purification of rLGOXs The rLGOXs were prepared by liquid cultivation of recombinant *E. coli* cells. A 5-mL culture of recombinant *E. coli* BL21(DE3) carrying pET24a/*lgox* was incubated in a test tube (18 × 180 mm) at 37 °C for 17 h (160 strokes per min), and 1% (v/v) culture was transferred to 100 mL terrific broth containing 30 μg/mL kanamycin in a 500-mL flask and incubated at 37 °C for 6 h (160 rpm). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 10 μM and the culture incubated for a further 24 h (160 rpm) at 20 °C. An average of 4 g-wet cells were obtained from 200 mL cultures of two 500-mL flasks. The harvested cells were resuspended in 10 mL of 20 mM MES-NaOH buffer (pH 6.0) per g-wet cells. The cells were disrupted using a sonicator (100 W, 20 kHz, 5 min), and the cell free extract was prepared by centrifugation at 21,800 × *g* for 10 min.

The cell free extract from recombinant cells carrying pET24a/*fclgox* was prepared as described above with an incubation step after sonication of 65 °C for 30 min and centrifugation at 21,800 × *g* for 10 min. The obtained supernatants were loaded onto a DEAE-650 M column (17.5 mL) equilibrated with 20 mM MES-NaOH buffer (pH 6.0) and the column washed with 3 CV of the same buffer. Subsequently, the proteins were eluted with a linear gradient (10 CV) of 0–1 M NaCl in the same buffer at 1 cm/min. The active fractions were collected, and the buffer exchanged to 20 mM Tris–HCl (pH 7.0) using a 50 kDa Amicon Ultra centrifugal filtration unit (Merck). The enzyme sample was then loaded onto a Resource Q column (1 mL) equilibrated and washed with 3 CV of the same buffer. Proteins were eluted with a linear gradient (40 CV) of 0–1 M NaCl in the same buffer at 3.11 cm/min. Fractions with high specific LGOX activity were pooled and buffer exchanged to the same buffer using the 50 kDa centrifugal filtration unit and then used for subsequent investigation.

A 5-mL culture of recombinant *E. coli* BL21 cells carrying pCold II/*his_lgox* were incubated in a test tube (18 × 180 mm) at 37 °C for 17 h (160 strokes per min), and 1% (v/v) of the culture was transferred to 100 mL LB broth containing 30 μg/mL ampicillin in a 500-mL flask and incubated at 37 °C for 5 h (160 rpm). IPTG was added to the culture to a final concentration of 10 μM and the culture incubated for a further 24 h (160 rpm) at 20 °C. Cells (~5 g-wet) obtained from 200 mL of culture were suspended in 50 mL buffer (5 mM imidazole, 0.5 M NaCl/50 mM MES-NaOH, pH 6.0), and the cell free extract and the supernatants were prepared as above, except the incubation step at 65 °C for 30 min was excluded. The supernatants were applied to a HisTrap HP column (CV, 5 mL) equilibrated with 5 mM imidazole, 0.5 M NaCl/50 mM MES-NaOH (pH 6.0). After washing, the proteins were eluted with a linear gradient (16 CV) of 5–500 mM imidazole, 0.5 M NaCl/50 mM MES-NaOH (pH 6.0) at 1.5 cm/min. Fractions displaying the highest specific LGOX activity were pooled, and the buffer was exchanged to 20 mM MES-NaOH (pH 6.0) using a 50 kDa Amicon Ultra centrifugal filtration unit.

Protein analyses The protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) with BSA as the standard. Protein samples were analyzed by SDS-PAGE (21) and native/PAGE using the NativePAGE Bis-Tris Gel System (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 or silver. The molecular mass of the purified enzyme was estimated using a gel filtration (Superdex 200 10/300 GL, Cytiva) column and dynamic light scattering (DLS) (Malvern Panalytical Ltd., Malvern, UK)

LGOX activity assay The standard reaction mixture (125 μL) consisted of 0.16 M MES-NaOH (pH 6.0) for HisLGOX, 0.16 M Bis-Tris–HCl (pH 6.5) for WT or 0.16 M Tris–HCl (pH 7.0) for FcLGOX with 0.016% (w/v) TODB, 4 U/mL POD, 1.6×10^{-3} % (w/v) sodium azide and 5 μL of enzyme solution. After preincubation (37 °C, 5 min), the reaction was initiated by adding 50 mM L-Glu and 0.12% (w/v)

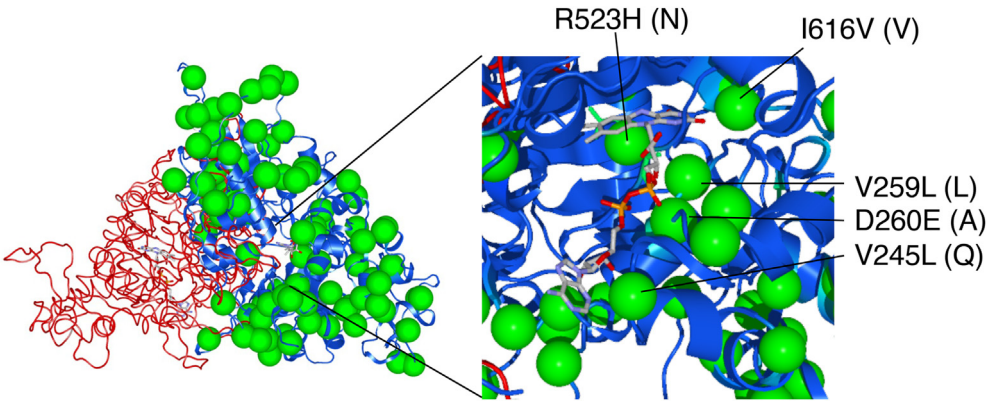

FIG. 2. Substituted amino acids in $LGOX_{NT1}$. Homology-model of $LGOX_{NT1}$ presented as a homodimer (red string and blue ribbon). In one subunit, the 104 amino acids substituted are indicated by green balls. FAD is presented in stick representation. V245L, V259L, D260E, R523H, and I616V were within ~5 Å of bound FAD. The amino acids in parentheses indicated the corresponding amino acids of $LGOX_{X119}$.

4AA in a total volume of 25 μL. The reaction mixture was incubated at 37 °C for 2 or 5 min. The reaction was stopped by addition of 150 μL ethanol. Absorbance at 550 nm based on a quinone dye generated by the coupling reaction of H_2O_2 with 4AA and TODB was measured with a Multiskan FC microplate reader (Thermo Fisher Scientific). The concentration of produced H_2O_2 was determined using a calibration curve generated from known concentrations of H_2O_2. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of H_2O_2 per min.

For the kinetic study, the initial velocity (v_0) of the enzyme reaction was determined in a 2-min reaction using the above assay at each optimum condition (pH 6.0 for HisLGOX or 7.0 for FcLGOX at 60 °C) and by varying the L-Glu concentration ([L-Glu]) from 0.2 to 2 mM. Data of v_0 versus [L-Glu] were subjected to nonlinear curve fitting using nonlinear regression (KaleidaGraph, Synergy Software, Reading, PA, USA) and kinetic parameters were determined.

Characterization of rLGOXs The optimum pH was investigated by measuring enzyme activity at 37 °C for 5 min, as described above using each buffer. The optimum temperature was determined by measuring the enzyme activity at each temperature in 0.16 M MES-NaOH (pH 6.0) for HisLGOX or 0.16 M Tris–HCl (pH 7.0) for FcLGOX. Thermostability was estimated by incubating the enzyme samples in 20 mM MES-NaOH (pH 6.0) and Tris–HCl (pH 7.0) for 30 min at selected temperatures and then left for 5 min on ice. Subsequently, without centrifugation the residual activity of the enzyme samples was measured by incubation under each standard assay condition (pH 6.0 for HisLGOX or 7.0 for FcLGOX at 37 °C). The effect of the incubation temperature on the protein situation such as thermal maturation, denaturation or aggregation/precipitation of FcLGOX was examined by measuring the residual activity in the centrifugal supernatant and precipitate after a 30-min incubation at each temperature. The purified FcLGOX samples in 20 mM Tris–HCl (pH 7.0) were incubated for 30 min at selected temperatures followed by 5 min cooling on ice and then centrifugation at 21,800 × *g* for 10 min at 4 °C. The supernatants were collected, and the residual precipitates were suspended in equivolume of the same buffer. The residual activities of the supernatant and precipitate were determined under the standard assay condition (2-min assay at pH 7.0, 37 °C) with preincubation (37 °C, 5 min). All experiments were performed in triplicate, and data are represented by the mean and standard deviation (SD).

Structural model The homology structural models of WT and FcLGOX were created using HHPRED search and Modeller 9.11 (22), after which VERIFY3D (23) was used to estimate the quality of the predicted model. The templates used in structure modeling were L-lysine oxidase from *Trichoderma viride* (PDB ID: 3X0V), L-amino acid oxidase from *Calloselasma rhodostoma* (PDB ID: 2IID), lysine-specific histone demethylase 1 (PDB ID: 2Z5U), cyclohexylamine oxidase from *Brevibacterium oxydans* IH-35A (PDB ID: 4I59), L-amino acid oxidase from *Rhodococcus opacus* (PDB ID: 2JAE) and L-amino acid oxidase from *R. opacus* in complex with L-alanine (PDB ID: 2JB1).

Nucleotide and peptide sequence accession numbers The nucleotide and peptide sequence data of the $LGOX_{NT1}$ gene from *Streptomyces* sp. strain NT1, designated *lgox*, are available in the DDBJ/EMBL/GenBank databases under the accession number LC564886.

RESULTS

Characterization of WT and rLGOXs $LGOX_{NT1}$ induced by L-Glu was yielded from *Streptomyces* sp. strain NT1 by a screening study. The intracellular $LGOX_{NT1}$ was partially purified from the cell free extract (Table S1) and 21.2-, 50.2-, 75.6- and 111-kDa bands were observed on the SDS-PAGE gel (Fig. S1A). The 75.6-kDa band is close to molecular mass calculated using the predicted amino acid sequence. The molecular mass of $LGOX_{NT1}$ was estimated to be approximately 140 kDa based on gel filtration results. These results indicated that the enzyme functioned as a homodimer composed of two 75.6-kDa polypeptides. The partially purified $LGOX_{NT1}$ exhibited optimal activity at pH 6.0 and 50 °C (data not shown). The partially purified $LGOX_{NT1}$ and FcLGOX showed high substrate specificity toward L-Glu, with no activity observed toward other amino acids, including L-Asp, L-Asn, L-Gln and D-Glu.

The amino acid sequence (690 amino acids) of $LGOX_{NT1}$ shares 63.7% and 84% identities with those of $LGOX_{X119}$ (Uniprot Q8L3C7) and FcLGOX, respectively (Fig. 1). FcLGOX was designed by introducing 104-amino acid mutations into $LGOX_{NT1}$ (Figs. 1 and 2). A homology search by using BLAST demonstrated that the amino acid sequence of $LGOX_{NT1}$ shared high identity with flavin-containing superfamily amine oxidases. Distance-based phylogenetic analysis placed FcLGOX at the center of homologous LGOXs and indicated that FcLGOX may be an ancestral-like enzyme of $LGOX_{X119}$ (Fig. S2). Most of the 104 amino acid substitutions were at the interface of the subunits, in loops and distal from the active center. Five amino acids substituted (V245L, V259L, D260E, R523H and I616V) are within ~5 Å of bound FAD. Substitutions V259L and I616V are identical to the corresponding amino acids of $LGOX_{X119}$ (Figs. 1 and 2). Moreover, R305, H312 and W564 in $LGOX_{X119}$, which are associated with L-Glu binding and located at the active site, and R305, which plays a role in substrate specificity (24), were conserved in FcLGOX and $LGOX_{NT1}$.

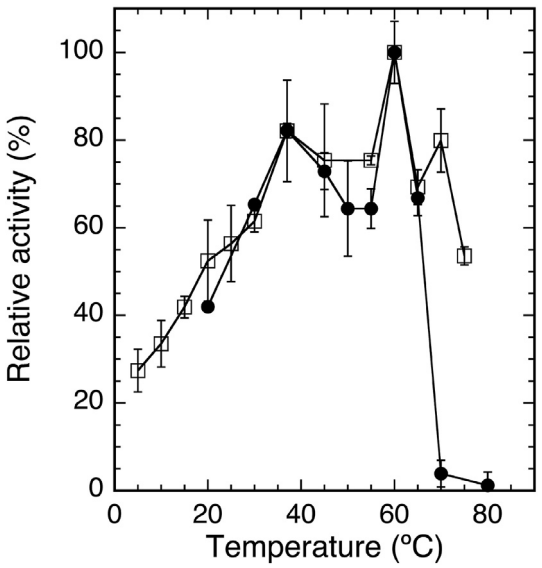

FIG. 3. Effect of the reaction temperature on HisLGOX and FcLGOX activity. The enzyme activity was determined for 2-min incubation at each temperature under each standard assay condition at pH 6.0 (HisLGOX, open squares) and pH 7.0 (FcLGOX, filled circles).

HisLGOX was purified from the cell free extract of the recombinant cells to electrophoretic homogeneity with a single band of 78.6-kDa observed on an SDS-PAGE gel (Fig. S1B). BlueNative-PAGE and gel filtration analysis demonstrated that HisLGOX functioned as a homodimer of 78.6-kDa (data not shown). The optimal conditions of HisLGOX activity were pH 6.0 and 60 °C (Figs. 3 and S3A). FcLGOX was purified to electrophoretic homogeneity showing a single band of 76.4-kDa on an SDS-PAGE gel (Table S2 and Fig. S4). The enzyme sample gave a band at 638 kDa on BlueNative-PAGE and a molecular mass of 691 ± 24 kDa at 25 °C by DLS analysis, suggesting that FcLGOX formed an homooctamer. The optimal conditions of FcLGOX activity were pH 7.0 and 60 °C (Figs. 3 and S3B).

Thermostability of HisLGOX and FcLGOX The heat treatment of part of FcLGOX caused precipitation; however, the precipitates retained its activity (Fig. 4C). The thermostability of HisLGOX at both pH 6.0 and 7.0 exhibited lower than that of the recombinant $LGOX_{X119}$ digested with *Streptomyces griseus* metalloprotease (Sgmp) which was maintained at 65 °C for 30 min (Fig. 4A) (4). On the other hand, FcLGOX activity was maintained at pH 7.0 and 70 °C for 30 min; however, at pH 6.0, the thermostability decreased significantly. Notably, the thermostability of FcLGOX was remarkably enhanced when compared with those of HisLGOX and $LGOX_{X119}$, and no reduction in kinetic properties as well as other characteristics such as substrate specificity were observed for FcLGOX when compared with WT. The residual activity of the FcLGOX sample without centrifugation and the centrifugal supernatant after 30-min incubation decreased as the temperature increased from

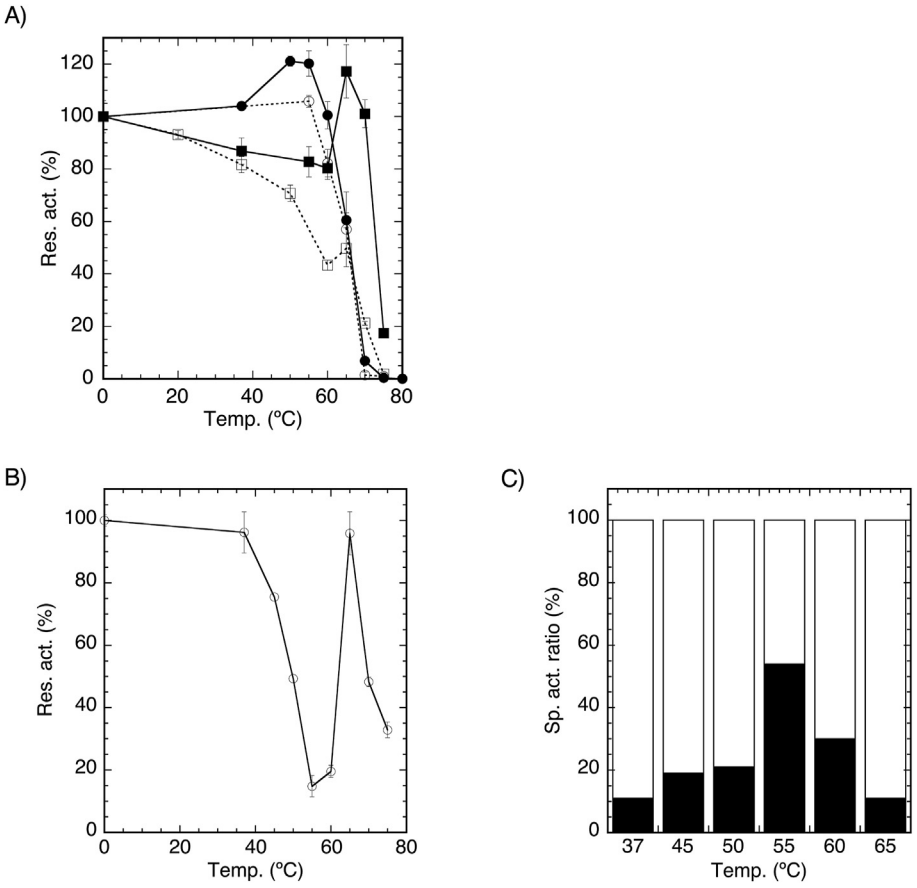

FIG. 4. Thermostability of Fc- and HisLGOX (A) and effect of the incubation temperature on the protein situation such as thermal maturation, denaturation or aggregation/precipitation of FcLGOX (B, C). (A) FcLGOX was incubated in 20 mM MES-NaOH buffer (pH 6.0) (open squares) or 20 mM Tris–HCl buffer (pH 7.0) (filled squares), and HisLGOX was incubated in 20 mM MES-NaOH buffer (pH 6.0) (open circles) or Tris–HCl buffer (pH 7.0) (filled circles) at each temperature for 30 min. The residual LGOX activity was determined directly without centrifugation by each standard assay condition (HisLGOX, pH 6.0; FcLGOX, pH 7.0 at 37 °C). (B) The residual FcLGOX activity in the centrifugal supernatants after 30-min incubation at each temperature is shown. (C) The residual specific activity ratios in the supernatants and precipitates after 30-min incubation at each temperature of purified FcLGOX are shown. The precipitates were suspended in equivolume of 20 mM Tris–HCl buffer (pH 7.0) with the supernatant sample. The residual activities of the supernatants and precipitates were determined under the standard assay condition (2-min assay, pH 7, 37 °C) with preincubation (37 °C, 5 min). Data are the means of experiments performed in triplicate. Error bars represent the standard deviation.

37 °C to 60 °C (Fig. 4A and B), whereas the residual activity of the precipitant increased (Fig. 4C). In addition, as shown in Fig. 4, HisLGOX and FcLGOX activity decreased between 45 and 55 °C, respectively. HisLGOX also exhibited ~50–80% relative activity between 70 and 75 °C, whereas FcLGOX showed weak relative activity at temperatures above 70 °C.

Kinetics of HisLGOX and FcLGOX Kinetic parameters of LGOXs toward L-Glu are summarized in Table 1. Although not measured under the same conditions, V_{max} and k_{cat} of $LGOX_{X119}$ were higher when compared with those of HisLGOX and FcLGOX. On the contrary, K_m values of HisLGOX and FcLGOX were lower than that of $LGOX_{X119}$. The k_{cat}/K_m values of FcLGOX and HisLGOX were much higher than that of $LGOX_{X119}$ because of the higher affinity between FcLGOX, HisLGOX and L-Glu.

TABLE 1. Steady-state kinetic parameters of LGOXs.

	$LGOX_{X119}$ [a]	HisLGOX	FcLGOX
Condition	pH 7.4, 30 °C	pH 6.0, 37 °C	pH 7.0, 60 °C
V_{max} (μmol/min/mg-protein)	26	12.1 ± 1.1	19.5 ± 0.9
K_m (mM)	5.0	1.25 ± 0.20	0.649 ± 0.068
k_{cat} (s^{-1})	33	14.9 ± 1.4[b]	25.9 ± 1.1[c]
k_{cat}/K_m (mM^{-1} s^{-1})	6.6	12.0 ± 1.0[b]	40.1 ± 2.5[c]

The kinetic parameters were determined under each standard assay condition. Data are represented by the means and standard deviations of experiments performed in triplicate.
[a] Data of paper published by Arima et al. (2).
[b, c] Calculated as a homodimer and a homooctamer, respectively.

DISCUSSION

We successfully enhanced the thermostability of $LGOX_{NT1}$ by the artificial protein design without a reduction in enzymatic properties, i.e., no activity–stability trade-off. The thermostability of $LGOX_{NT1}$ was elevated from 55 to 70 °C. There are numerous reports describing increases in the T_m of proteins by 10–32 °C with the success rate of enhancing thermostability ~50% (6,9). However, these reports often observe an improvement in thermal stability with a concomitant reduction in enzyme activity, i.e., an activity–stability trade-off (7–10). This trade-off depends on the protein, even when using ancestral or consensus design (7,12). Only the thermostability of *R. palustris* 5-aminolevulinic acid synthase (11) and archaeal and bacterial nucleoside diphosphate kinase (12) were improved without an activity–stability trade-off.

The improved enzyme, FcLGOX, was created by artificial design with 104 amino acid substitutions to $LGOX_{NT1}$. Compared with $LGOX_{NT1}$, the number of Pro, Thr, Tyr and Glu residues in the protein sequence increased by 12, 4, 3 and 4, respectively, whereas the number of Val, Ser and Asp residues decreased by 6, 7 and 6, respectively. An increase in the number of Pro residues by

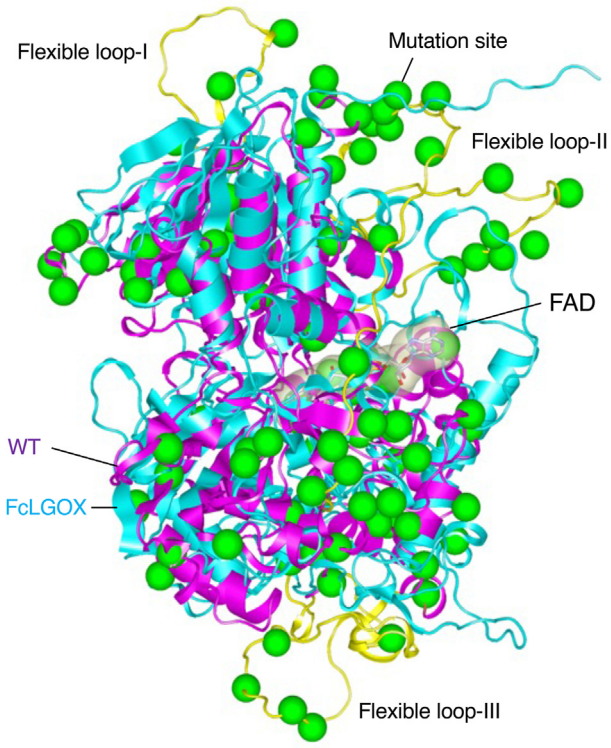

FIG. 5. Homology models of WT and FcLGOX. Only one subunit of WT (cyan) and FcLGOX (magenta) are shown in ribbon representations. In WT, the 104 amino acid substitutions are indicated by green balls. FAD is shown in stick representation. Long and unstructured loops in WT are compared with the corresponding loops in FcLGOX and are shown in yellow.

consensus mutations is unlikely according to Sternke et al. (25) in many proteins, the number of Pro residues introduced would be invariable or decrease slightly by consensus design. Substitution to a consensus Pro may have improved the thermostability of FcLGOX. The optimal activity of WT and FcLGOX was observed at pH 6.0 and pH 7.0, respectively. In addition, the preference for buffer composition has changed (Fig. S3). These results demonstrate that it might be due to the difference of the protein folding between WT and FcLGOX. In fact, the loops and the molecular size of FcLGOX appeared to be differed from those of WT (Fig. 5). In contrast, the optimal temperature of WT and FcLGOX activity was identical at 60 °C.

Notably, the thermostability of FcLGOX was enhanced without a decrease in kinetic properties as well as the other enzymatic properties when compared with those of WT. However, there might be a trade-off between the thermal stability and pH stability. Only the quaternary protein structure was found to change. Unexpectedly, most of the 104 amino acid substitutions were at the interface of the subunits, in loops and distal from the active site. Sakasegawa et al. (26) reported that the thermostability of glycerol kinase from *Flavobacterium meningosepticum* increased by 3.1 °C by substituting Ser329 located at the subunit interface with Asp. Recently, local flexible loops at the protein surface were reported to be important for protein function and for improving the function (27,28). Results using artificial protein evolution have shown recently that it is important to introduce amino acid substitutions to regions surrounding the active center (13,16). In this study, loops I–III of WT were absent in FcLGOX and the molecular size of FcLGOX appeared to be smaller when compared with that of WT (Fig. 5).

The quaternary protein structure of FcLGOX was different to $LGOX_{NT1}$ and HisLGOX. The quaternary structure of LGOX seems to be very complex and purification of LGOX from *Streptomyces* sp. was difficult because of its stable structure and tendency to aggregate. Arima et al. (4) reported that recombinant $LGOX_{X119}$ was an active and mature enzyme following Sgmp digestion. $LGOX_{NT1}$ is an inducible enzyme and likely to be composed of $\alpha\beta\gamma$ subunits. The $LGOX_{NT1}$ amino acid sequence is similar to $LGOX_{X119}$ and SDS-PAGE analysis of purified $LGOX_{NT1}$ digested with Sgmp gave bands at 10-, 17- and 42-kDa. In addition, *Streptomyces* sp. NT1 showed protease activity. We originally hypothesized that the mature form of $LGOX_{NT1}$ is composed of $\alpha_2\beta_2\gamma_2$; however, HisLGOX and FcLGOX were determined to be α_2 and α_8, respectively, based on the results presented herein.

In the 2-min assay, HisLGOX and FcLGOX activity decreased at 45–55 °C; moreover, FcLGOX was remarkably inactivated at >70 °C, whereas HisLGOX maintained >50% activity till 75 °C. Furthermore, the LGOX activity with possibly refolding or repairing the aggregation form was detected in the precipitate samples of the 30-min incubation at 37–65 °C. Especially, approximately 50% of FcLGOX sample precipitated once with the 30-min incubation at 55 °C showed the enzyme activity again in the assay period (5-min preincubation and 2-min enzyme reaction at pH 7.0, 37 °C). These observations indicated that HisLGOX and FcLGOX might be produced as misfolded or immature states, and the population of correctly folded or non-aggregated state HisLGOX may be higher than that of FcLGOX. Nonetheless, all constructs were unable to adopt an active state because of the short assay time. Namely, the assay period was too short to allow the protein to refold and repair the aggregation form. Thus, the protein fold of HisLGOX may refold faster than FcLGOX following incubation at 45–75 °C. In fact, SDS-PAGE analysis demonstrated that the quaternary protein structures of WT and FcLGOX were complex, and they appeared to form aggregates.

In summary, we have created a thermostable LGOX by the artificial protein design with kinetic properties and other enzymatic properties that are more than or equal to WT. Although additional work is required to investigate the quaternary protein structure of FcLGOX, this thermostable L-amino acid oxidase may be suitable for use in various industrial applications. This report presents artificial protein evolution as a new approach in protein engineering.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2021.12.008.

ACKNOWLEDGMENTS

This work was supported by a grant from the Japan Foundation for Applied Enzymology to D.S. This work was supported by JSPS KAKENHI Grant Numbers 16K18688 and 18K14391 to S.N., 17K06931 to S.I. and 17H06169 to Y.A. The authors declare no conflict of interest. We thank Edanz for editing a draft of this manuscript.

References

1. **Niu, P., Zhang, Z., and Liu, L.:** Enzymatic production of α-ketoglutaric acid by L-glutamate oxidase from L-glutamic acid, J. Biotechnol., **30**, 1318–1322 (2014).
2. **Upadhyay, S., Ohgami, N., Kusakabe, H., Mizuno, H., Arima, J., Tamura, T., Inagaki, K., and Suzuki, H.:** Performance characterization of recombinant L-glutamate oxidase in a micro GOT/GPT sensing system, Sens. Actuators B Chem., **119**, 570–576 (2006).
3. **Kusakabe, H., Midorikawa, Y., Fujishima, T., Kuninaka, A., and Yoshino, H.:** Purification and properties of a new enzyme, L-glutamate oxidase, from *Streptomyces* sp. X-119-6 grown on wheat bran, Agric. Biol. Chem., **47**, 1323–1328 (1983).
4. **Arima, J., Tamura, T., Kusakabe, H., Ashiuchi, M., Yagi, T., Tanaka, H., and Inagaki, K.:** Recombinant expression, biochemical characterization and stabilization through proteolysis of an L-glutamate oxidase from *Streptomyces* sp. X-119-6, J. Biochem., **134**, 805–812 (2003).

5. **Imanaka, T., Shibazaki, M., and Takagi, M.:** A new way of enhancing the thermostability of proteases, Nature, **324**, 695–697 (1986).
6. **Porebski, B. T. and Buckle, A. M.:** Consensus protein design, Protein Eng. Des. Sel., **29**, 245–251 (2016).
7. **Nguyen, V., Wilson, C., Hoemberger, M., Stiller, J. B., Agafonov, R. V., Kutter, S., English, J., Theobald, D. L., and Kern, D.:** Evolutionary drivers of thermoadaptation in enzyme catalysis, Science, **355**, 289–294 (2017).
8. **Yokoyama, K., Ogaya, D., Utsumi, H., Suzuki, M., Kashiwagi, T., Suzuki, E., and Taguchi, S.:** Effect of introducing a disulfide bridge on the thermostability of microbial transglutaminase from *Streptomyces mobaraensis*, Appl. Microbiol. Biotechnol., **105**, 2737–2745 (2021).
9. **Kurahashi, R., Tanaka, S. I., and Takano, K.:** Activity-stability trade-off in random mutant proteins, J. Biosci. Bioeng., **128**, 405–409 (2019).
10. **Fukuda, Y., Abe, A., Tamura, T., Kishimoto, T., Sogabe, A., Akanuma, S., Yokobori, S., Yamagishi, A., Imada, K., and Inagaki, K.:** Epistasis effects of multiple ancestral-consensus amino acid substitutions on the thermal stability of glycerol kinase from *Cellulomonas* sp. NT3060, J. Biosci. Bioeng., **121**, 497–502 (2016).
11. **Tan, Z., Zhao, J., Chen, J., Rao, D., Zhou, W., Chen, N., Zheng, P., Sun, J., and Ma, Y.:** Enhancing thermostability and removing hemin inhibition of *Rhodopseudomonas palustris* 5-aminolevulinic acid synthase by computer-aided rational design, Biotechnol. Lett., **41**, 181–191 (2019).
12. **Akanuma, S., Nakajima, Y., Yokobori, S., Kimura, M., Nemoto, N., Mase, T., Miyazono, K., Tanokura, M., and Yamagishi, A.:** Experimental evidence for the thermophilicity of ancestral life, Proc. Natl. Acad. Sci. USA, **110**, 11067–11072 (2013).
13. **Nakano, S. and Asano, Y.:** Protein evolution analysis of *S*-hydroxynitrile lyase by complete sequence design utilizing the INTMSAlign software, Sci. Rep., **5**, 8193 (2015).
14. **Motoyama, T., Hiramatsu, N., Asano, Y., Nakano, S., and Ito, S.:** Protein sequence selection method that enables full consensus design of artificial L-threonine 3-dehydrogenases with unique enzymatic properties, Biochemistry, **59**, 3823–3833 (2020).
15. **Sugimori, D., Kano, K., and Matsumoto, Y.:** Purification, characterization, molecular cloning and extracellular production of a phospholipase A_1 from *Streptomyces albidoflavus* NA297, FEBS Open Bio, **2**, 318–327 (2012).
16. **Nakano, S., Motoyama, T., Miyashita, Y., Ishizuka, Y., Matsuo, N., Tokiwa, H., Shinoda, S., Asano, Y., and Ito, S.:** Benchmark analysis of native and artificial NAD^+-dependent enzymes generated by a sequence-based design method with or without phylogenetic data, Biochemistry, **57**, 3722–3732 (2018).
17. **Nakano, S., Minamino, Y., Hasebe, F., and Ito, S.:** Deracemization and stereoinversion to aromatic D-amino acid derivatives with ancestral L-amino acid oxidase, ACS Catal., **9**, 10152–10158 (2019).
18. **Nakano, S., Niwa, M., Asano, Y., and Ito, S.:** Following the evolutionary track of a highly specific L-arginine oxidase by reconstruction and biochemical analysis of ancestral and native enzymes, Appl. Environ. Microbiol., **85**, e00459-19 (2019).
19. **Kido, K., Yamanaka, S., Nakano, S., Motani, K., Shinohara, S., Nozawa, A., Kosako, H., Ito, S., and Sawasaki, T.:** AirID, a novel proximity biotinylation enzyme, for analysis of protein-protein interactions, eLife, **9**, e54983 (2020).
20. **Hirano, Y., Chonan, K., Murayama, K., Sakasegawa, S. I., Matsumoto, H., and Sugimori, D.:** *Syncephalastrum racemosum* amine oxidase with high catalytic efficiency toward ethanolamine and its application in ethanolamine determination, Appl. Microbiol. Biotechnol., **100**, 3999–4013 (2016).
21. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, **227**, 680–685 (1970).
22. **Šali, A., Potterton, L., Yuan, F., van Vlijmen, H., and Karplus, M.:** Evaluation of comparative protein modeling by MODELLER, Proteins, **23**, 318–326 (1995).
23. **Luthy, R., Bowie, J. U., and Eisenberg, D.:** Assessment of protein models with three-dimensional profiles, Nature, **356**, 83–85 (1992).
24. **Utsumi, T., Arima, J., Sakaguchi, C., Tamura, T., Sasaki, C., Kusakabe, H., Sugio, S., and Inagaki, K.:** Arg305 of *Streptomyces* L-glutamate oxidase plays a crucial role for substrate recognition, Biochem. Biophys. Res. Commun., **417**, 951–955 (2012).
25. **Sternke, M., Tripp, K. W., and Barrick, D.:** The use of consensus sequence information to engineer stability and activity in proteins, Methods Enzymol., **643**, 149–179 (2020).
26. **Sakasegawa, S., Takehara, H., Yoshioka, I., Takahashi, M., Kagimoto, Y., Misaki, H., Sakuraba, H., and Ohshima, T.:** Increasing the thermostability of *Flavobacterium meningosepticum* glycerol kinase by changing Ser329 to Asp in the subunit interface region, Protein Eng., **14**, 663–667 (2001).
27. **Kovacic, F., Mandrysch, A., Poojari, C., Strodel, B., and Jaeger, K. E.:** Structural features determining thermal adaptation of esterases, Protein Eng. Des. Sel., **29**, 65–76 (2016).
28. **Saavedra, H. G., Wrabl, J. O., Anderson, J. A., Li, J., and Hilser, V. J.:** Dynamic allostery can drive cold adaptation in enzymes, Nature, **558**, 324–329 (2018).