



Development of a protein-engineered cholesterol oxidase suitable for a rate assay

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Summary Cholesterol oxidase (Cho), widely used in clinical settings, exhibits extremely high substrate affinity; therefore, it is unsuitable for rate assays. In this study, we developed a protein-engineered Cho with reduced cholesterol affinity, making it suitable for rate assays. A thermostable mutant of *Streptomyces* Cho (ChoA-VE), a commonly used diagnostic enzyme, was genetically engineered for intracellular production. The K_m value of the purified intracellular Cho (ChoA-VEi) for cholesterol increased by approximately 9–11-fold compared with those of the wild-type and thermostable mutant enzymes. A tertiary structure model of ChoA-VEi predicted by AlphaFold suggested that conformational changes in a loop structure at the active site entrance contribute to the reduced substrate affinity. Furthermore, increasing the detergent concentration in the assay reagent further decreased substrate affinity for ChoA-VEi. In contrast, rationally designed glycine-to-cysteine mutations in three loop regions near the active site increased the K_m of ChoA-VEi by 2.5–5.1-fold. The glycine residue at position 375 was further optimized to alanine or valine, resulting in a K_m approximately tenfold higher than that of ChoA-VEi. Finally, a cholesterol rate assay reagent containing the G375V mutant was prepared, and its practical applicability was preliminarily demonstrated.

Key words: Cholesterol oxidase, Rate assay, Substrate affinity, Tertiary structure, Mutagenesis

1. Introduction

Cholesterol (cholest-5-en-3 β -ol) in blood and food is commonly measured enzymatically using endpoint assay methods¹⁻³. The cholesterol rate assay method expands the dynamic range and enables the application of double kinetic assays⁴⁻⁸. However, the cholesterol oxidase (Cho, EC 1.1.3.6; cholesterol:oxygen oxidoreductase) used in these measurements exhibits extremely high substrate

affinity, and therefore, it is unsuitable for rate assays⁹⁻¹².

Cho is a flavin adenine dinucleotide (FAD)-dependent enzyme that catalyzes the oxidation of cholesterol, producing equimolar amounts of cholest-4-en-3-one and hydrogen peroxide¹⁰. This enzyme is widely used in clinical applications in combination with related enzymes such as cholesterol esterase and peroxidase^{1,3,9}. The wild-type Cho isolated from *Streptomyces* sp. SA-COO (ChoA) and its thermostable mutant (ChoA-VE) have been

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developed previously and are still used as diagnostic reagents¹¹. Secretory production of ChoA and ChoA-VE in an *Escherichia coli* host-vector system has also been industrially implemented¹¹.

In this study, we report the development of a protein-engineered ChoA with reduced cholesterol affinity that is suitable for rate assays. A cholesterol rate assay reagent containing engineered ChoA was prepared, and its practical applicability was preliminarily confirmed.

2. Materials and Methods

Reagents, chemicals, bacterial strains, plasmids, and culture conditions.

All reagents and chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wakenyaku Co., Ltd. (Osaka, Japan). LabAssay Cholesterol (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was used as a reference for the cholesterol endpoint assay. *E. coli* BL21(DE3)pLysS (BioDynamics Laboratory Inc., Tokyo, Japan) and the plasmid pET-24b(+) (5,309 bp, Km^r) (Novagen, Madison, WI, USA) were used for recombinant strain preparation and plasmid construction, respectively. Bacterial cells were cultured in Luria-Bertani Broth (LB), Terrific Broth (TB), or on LB agar (LB supplemented with 1.5% agar) at 37°C. Kanamycin (30 µg/mL) and chloramphenicol (25 µg/mL) were used as selective antibiotics.

DNA manipulation and recombinant plasmid construction

Plasmid isolation, restriction enzyme digestion, and DNA ligation using T4 DNA ligase were performed as described previously¹³. *E. coli* cells were transformed using a chemical transformation method¹³.

The mature ChoA-VE gene, encoding amino acid residues 43 to 546 (DNA Data Bank of Japan Accession No. E60904), with an additional sequence at the N-terminus (total 516 residues), was artificially synthesized with codon optimization for *E. coli* generated by GenScript Biotech Corporation (Tokyo, Japan) (Fig. 1). The synthesized gene was cloned into *Nde*I and *Bam*HI restriction sites of pET-24b(+), and the resulting plasmid was designated pET-ChoA-VEi. The DNA sequence was verified using sequencing.

Site-directed mutagenesis

The intracellular ChoA-VE (ChoA-VEi) expression plasmid, pET-ChoA-VEi, was used as a template for site-directed mutagenesis. The ChoA-VEi gene encodes 504 mature ChoA-VE residues and 12 additional N-terminal residues, including a hexahistidine tag (Fig. 1)¹⁴. Expression plasmids for the mutants were generated using inverse polymerase chain reaction (PCR) with PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. The sequences of the PCR primers used in this work are listed in Table 1.

Enzyme expression and purification

The expression plasmids were transformed into

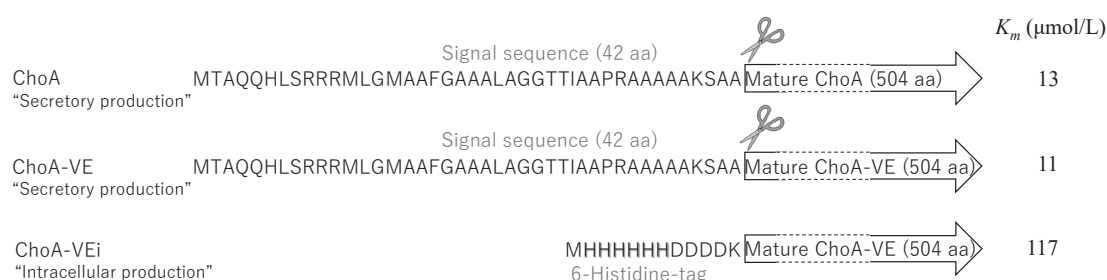


Fig. 1. Primary structures of ChoA, ChoA-VE, ChoA-VEi, and Michaelis constants (K_m) of each purified enzyme. ChoA, cholesterol oxidase; ChoA-VE, thermostable mutant of ChoA; ChoA-VEi, intracellular ChoA-VE.

Table 1 Specific PCR primers used for site-directed mutagenesis

G84C	Forward	5'-ACCGAGT <u>IG</u> CCCATTTGGGCTCTTTTCTGTGGC-3'
	Reverse	5'-CAATGGG <u>GCA</u> CTCGGTACGGTACGGTTTTTGAACCAAGAG-3'
G344C	Forward	5'-CCCACCT <u>IG</u> C GCCCACCAGTCCTCCATCCC-3'
	Reverse	5'-GTGGGC <u>GCA</u> AGGTGGGGTTCCACATGTGGTTGGCCC-3'
G375C	Forward	5'-CCGGCC <u>IG</u> CCTGGAGACGTGGGTCAGC-3'
	Reverse	5'-CTCCAGG <u>CAG</u> GCCGGCATGGGGGCGATCTC-3'
G441C	Forward	5'-CTGTTT <u>IG</u> CACGCAACTGAAAGCGTTTGC-3'
	Reverse	5'-TTGCGT <u>GCA</u> AAACAGGTCGTAAACGATAAATGGTTC-3'
G375P	Forward	5'-CCGGC <u>ACCG</u> CTGGAAACCTGGGTTT-3'
	Reverse	5'-TTCCAG <u>CGG</u> TGCCGGCCGGCATCGGG-3'
G375A	Forward	5'-CCGGCAG <u>CG</u> CTGGAAACCTGGGTTT-3'
	Reverse	5'-TTCCAG <u>CGC</u> TGCCGGCATCGCG-3'
G375V	Forward	5'-CCGGCAGT <u>TT</u> CTGGAAACCTGGGTTT-3'
	Reverse	5'-TTCCAG <u>AACT</u> GCCGGCATCGGGG-3'

Underlines indicate mutation sites.

E. coli BL21(DE3)pLysS cells. Recombinant cells were cultured in TB medium supplemented with 30 µg/mL kanamycin and 25 µg/mL chloramphenicol at 37°C with shaking at 200 rpm for 2.5 h. Protein expression was induced by adding 250 µmol/L isopropyl β-D-1-thiogalactopyranoside to the TB medium when the optical density at 590 nm reached approximately 0.6. The cells, cultured at 25°C for 22 h, were collected using centrifugation at 4°C and 13,000 ×g for 10 min and suspended in buffer P (50 mmol/L potassium phosphate, pH 7.0).

After sonication of the harvested cells on ice, the cell extracts were centrifuged at 13,000 ×g for 10 min. The supernatant was applied to a HiTrap Q HP anion-exchange chromatography column (Cytiva Life Sciences, Marlborough, MA, USA) equilibrated with buffer P. Bound proteins were eluted using buffer P containing 500 mmol/L NaCl. The flow-through sample used in subsequent experiments was applied to a His GraviTrap™ chelating column (Cytiva Life Sciences) equilibrated with buffer P containing 50 mmol/L imidazole. The bound proteins were eluted with buffer P containing 200-500 mmol/L imidazole. The protein solution was dialyzed against buffer P to remove imidazole. Finally, each enzyme was purified to homogeneity.

Molecular weights were estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was quantified using

the Bradford assay (Takara Bio Inc.).

Computational analysis

The tertiary structure of ChoA-VEi, produced intracellularly from *E. coli* BL21(DE3)pLysS(pET-ChoA-VEi), was predicted using AlphaFold2 (<https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb>).

Based on the ChoA tertiary structure (Protein Data Bank ID: 4XXG) and the ChoA-VEi structural model, *in silico* analysis and rational mutation design were performed using Molecular Operating Environment software (MOE, Chemical Computing Group Inc., Montreal, Canada)¹⁵⁻¹⁸. Before energy minimization, the Amber10:EHT force field was used to add hydrogen atoms and partial charges according to the manufacturer's instructions. Relaxation of the added hydrogen atoms via energy minimization was performed using a conjugate gradient/truncated Newton optimization algorithm with convergence criteria of 0.05 kcal/mol. Enzyme structure models were visualized using MOE software.

Cho activity assay

Cho activity was measured using a previously described method^{4,11,12} with slight modifications. The enzyme solution prepared by dilution with buffer P (35 µL) was added to the assay solution (1,000 µL)

containing 82 mmol/L sodium phosphate buffer (pH 7.0), 0.84 mmol/L ($\times$ 38.665 mg/dL) cholesterol, 0.34–2.1% Triton X-100, 6.4 mmol/L sodium cholate, 1.4 mmol/L 4-aminoantipyrine, 20 mmol/L phenol, and 6.5 U/mL peroxidase. The time-dependent spectral change at 500 nm was measured against a blank using a spectrophotometer (U-3900, Hitachi Corporation, Tokyo, Japan) to quantify the quinone-imine dye formed upon coupling with 4-aminoantipyrine, phenol, and peroxidase. One unit of activity was defined as the formation of 1 μ mol of hydrogen peroxide per minute at 37°C and pH 7.0. All data were averaged over three independent experiments.

Kinetic parameters

According to the Michaelis–Menten equation, the kinetic parameters of ChoA and its mutants were determined based on their enzymatic activities at various substrate concentrations at 37°C. All data were averaged over three independent experiments conducted on different days.

Cholesterol rate assay

The increase in quinone-imine dye absorbance was measured using a spectrophotometer (U-3900, Hitachi Corporation). The working solution (WS) contained 50 mmol/L sodium phosphate buffer (pH 7.0), 0.34% Triton X-100, 6.4 mmol/L sodium cholate, 1.0 mmol/L 4-aminoantipyrine, 0.45 mmol/L TOOS, 5.0 U/mL peroxidase, and 0.006 U/mL Cho (G375V mutant of ChoA-VEi). Next, 800 μ L of WS was preheated at 37°C for 2 min, and 30 μ L of cholesterol (50–400 mg/dL) was added. Changes in absorbance at 555 nm were measured in real time at 37°C for 5 min. Dilution linearity was determined by measuring the rates of absorbance increases at 555 nm.

Reference experiments for cholesterol endpoint assay were performed using a commercially available reagent (LabAssay Cholesterol, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), in accordance with the manufacturer's instruction. A total of 800 μ L of WS was preheated at 37°C for 5 min, and 30 μ L of cholesterol (50–400 mg/dL) was

added. Changes in absorbance at 600 nm were measured in real time at 37°C for 5 min. Dilution linearity was calculated from the absorbance at 5 min.

All data were averaged over three independent experiments.

3. Results and Discussion

K_m of intracellularly produced ChoA-VE (ChoA-VEi)

In a previous study¹¹, ChoA and ChoA-VE [a thermally stable valine (V)-to-glutamate (E) single mutant at position 115] were produced by secretory expression. In this study, intracellular production of ChoA-VE without membrane permeation, named ChoA-VEi, was attempted.

The ChoA-VEi-encoding gene with an N-terminal six-histidine tag (1,548 bp) was cloned into *E. coli* cells, as described in the Materials and Methods section. Subsequently, ChoA-VEi was intracellularly produced in recombinant *E. coli*¹⁴ and purified to homogeneity as described in the Materials and Methods section. The K_m value of purified ChoA-VEi for cholesterol was compared with those of ChoA and ChoA-VE, which were secreted and purified. The K_m of ChoA-VEi increased by approximately 9–11-fold compared with those of ChoA and Cho-VE (Fig. 1). This property makes it a desirable rate-assay enzyme, although this was unexpected.

Structural analysis of ChoA-VEi

To investigate the differences in substrate affinity, a tertiary structure model of ChoA-VEi was generated using AlphaFold2 and compared with the X-ray crystallographic structure of ChoA. The overall structures of ChoA and ChoA-VEi superposed well, with a root mean square deviation (RMSD) of 1.054 Å for atomic C α positions (498 residues). However, the RMSD for residues E80-arginine94 (R94) of ChoA-VEi (corresponding to residues E73-R87 of ChoA), which are part of one of the loop structures forming the active site entrance, exceeded 2.5 Å (Fig. 2A, dotted-line circle). In particular, the RMSD values for

tryptophan88 (W88) and leucine89 (L89) were 8.894 and 8.580 Å, respectively. This local conformational change affects the space and hydrophobicity of the active site entrance (Fig. 2B). The entrance in ChoA-VEi was clearly more expanded than that in ChoA, and the surrounding hydrophobic regions were enlarged. Both alterations likely contribute to reduced affinity toward hydrophobic substrates such as cholesterol. The expanded hydrophobic regions may interact with the substrate and hinder its transport to the active site. Moreover, the enlarged entrance likely reduces substrate retention at the active site.

These results suggest that conformational changes in the loop structure at the active site entrance are associated with decreased substrate affinity. Thus, the ChoA-VEi structural model developed in this study provides further insight into the structure-function relationship.

Effects of detergents on substrate affinity

Cholesterol was dissolved in the presence of two detergents (Triton X-100 and sodium cholate) and used as a substrate for the Cho assay, as described in the Materials and Methods section. Detergent concentrations were expected to affect substrate hydrophobicity and affinity for Cho. When

the Triton X-100 concentration in the assay solution was increased from 0.34%, the cholesterol concentration-dependent specific activity of ChoA-VEi decreased (Fig. 3A). Therefore, the K_m value increased with increasing Triton X-100 concentration (Fig. 3B). An increased detergent concentration in the reagent was effective in further reducing the substrate affinity of ChoA-VEi. However, measurement accuracy decreased with increasing Triton X-100 concentration (Fig. 3B), which is a major practical issue.

Mutational effects on loop structures around active sites

To further increase the K_m value of ChoA-VEi, an *in silico* mutation design targeting the active site entrance was performed. Glycine (G) residues in four loop structures constituting the entrance, G84, G344, G375, and G441, were selected as mutagenesis targets (Fig. 4). Each G-to-cysteine (C) mutation was predicted to affect the flexibility and charge of the corresponding loop.

Expression plasmids for the G84C, G344C, G375C, and G441C mutants were constructed using site-directed mutagenesis. Subsequently, the four ChoA-VEi mutants were purified from recombinant *E. coli* strains as described in the Materials and

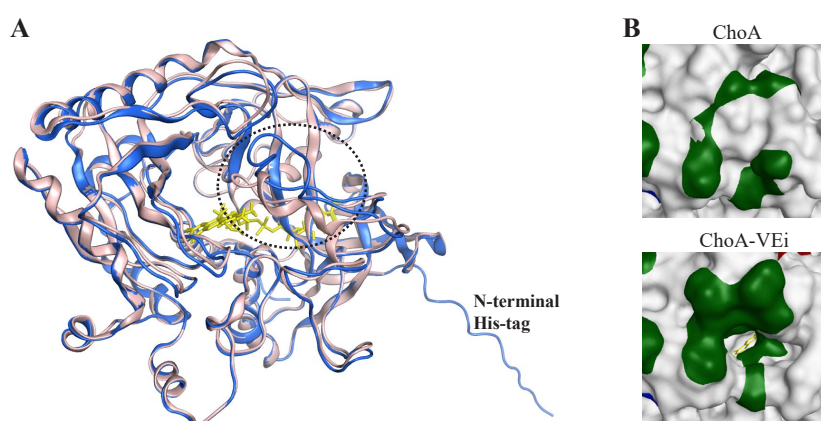


Fig. 2. Comparison between the predicted ChoA-VEi structure and the X-ray crystallographic structure of ChoA. A: Overall structures. The main chain is represented by a ribbon model. FAD is represented using a stick model. ChoA, ChoA-VEi, and FAD are indicated in pink, blue, and yellow, respectively. One of the loop structures forming the active site entrance is surrounded by a dotted-line circle. B: Active-site entrance. Each enzyme is represented using a surface model. Hydrophobic patches and FAD are shown in green and yellow, respectively. ChoA, cholesterol oxidase; ChoA-VE, thermostable mutant of ChoA; ChoA-VEi, intracellular ChoA-VE; FAD, flavin adenine dinucleotide.

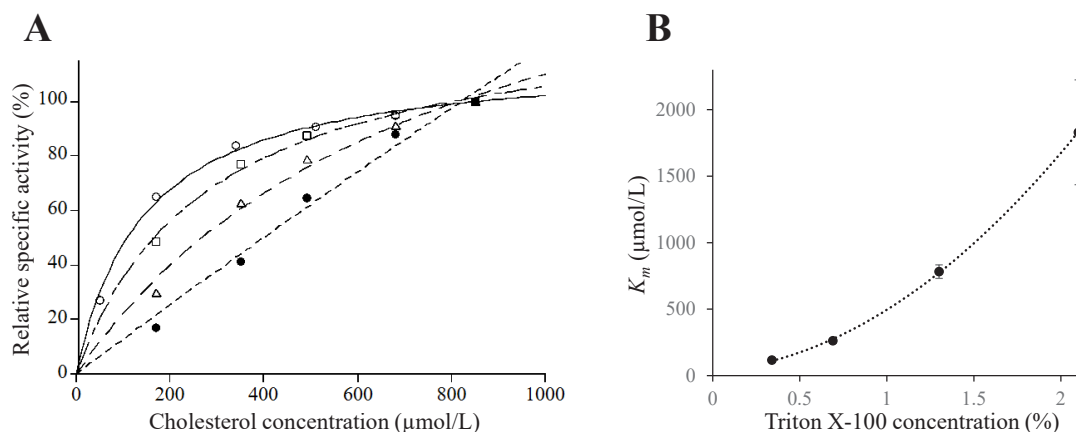


Fig. 3. Effects of Triton X-100 concentration on ChoA-VEi substrate affinity. A: Influence of Triton X-100 final concentrations on Michaelis-Menten plots. Open circles, open squares, open triangles, and closed circles indicate results at 0.34, 0.69, 1.3, and 2.1% Triton X-100, respectively. Specific activity data at each Triton X-100 concentration were normalized by setting the specific activity at a cholesterol concentration of 800 $\mu\text{mol/L}$ as 100% relative activity. B: Relationship between Triton X-100 concentration and K_m value. Error bars represent standard deviation ($n = 3$). ChoA-VEi, intracellular ChoA-VE.

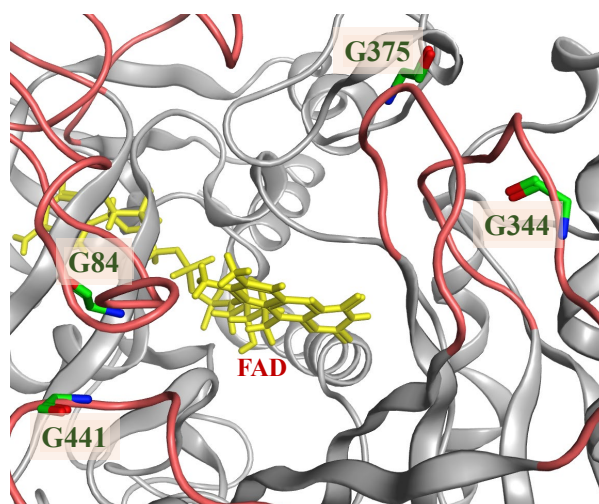


Fig. 4. Close-up view of the predicted ChoA-VEi structure around the active site entrance. The main chain is represented by a ribbon model, and the four loop structures are indicated in orange. The G residues (green) are used as mutagenesis targets and FAD (yellow) are represented using a stick model. Oxygen and nitrogen atoms of glycine are indicated in red and blue, respectively. ChoA-VEi, intracellular ChoA-VE; FAD, flavin adenine dinucleotide.

Methods section. The K_m values of the mutants were measured and compared with that of ChoA-VEi. Three of the rationally designed G-to-C mutations in loop structures near the active site increased the K_m value of ChoA-VEi by 2.5-5.1-fold (Fig. 5). However, the K_m of G84C was similar to that of ChoA-VEi (data not shown). As mentioned above, G84 is located within the loop undergoing a local

conformational change in ChoA-VEi (Fig. 2), suggesting that this site already contributes significantly to the reduced affinity for cholesterol.

Because the mutation at position 375 showed the strongest effect, it was further optimized. G-to-proline (P), alanine (A), and V mutations at position 375 were constructed, and their K_m values increased by approximately tenfold compared with that of

ChoA-VEi, except for G375P (Fig. 5). When the potential energies of the corresponding loop structures in ChoA-VEi and the mutants were calculated using MOE, only that of G375P increased significantly (Fig. 6). Therefore, this mutant is likely structurally unstable and less effective.

Based on the G375A and G375V mutants, which exhibited the highest K_m values, multiple mutants G375A+G344C, G375V+G344C, G375A+G441C, and G375V+G441C were also generated and purified. However, no Cho activity was detected in the G375A+G344C or G375V+G344C mutants. The K_m values of G375A+G441C and G375V+G441C could not be evaluated because their Cho activities were only 1.3% and 0.1% of that of ChoA-VEi, respectively. These results indicate that mutations in multiple loop structures significantly impair enzyme function.

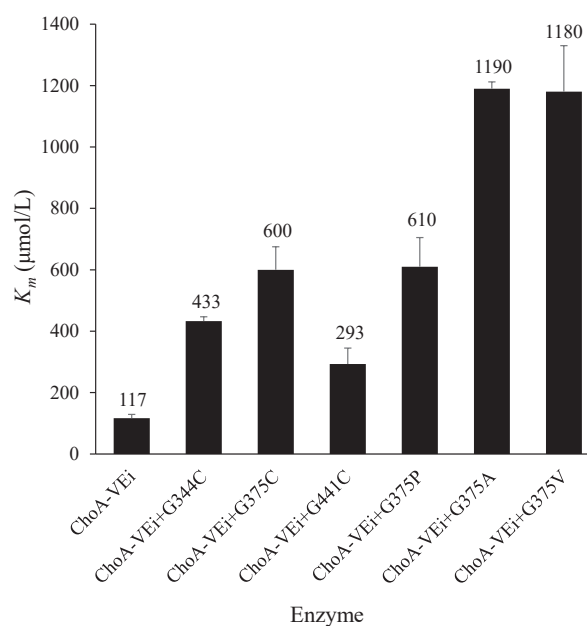


Fig. 5. K_m values of ChoA-VEi and its mutants. Error bars represent standard deviation ($n = 3$). ChoA-VEi, intracellular ChoA-VE.

Cholesterol rate assay using the G375V mutant

A rate assay reagent containing the G375V mutant was prepared and utilized in the cholesterol assay as described in the Materials and Methods section. Time-course measurements of cholesterol showed linear increases over 5 min at all concentrations (50–400 mg/dL) (Fig. 7A). In addition, high linearity at a cholesterol concentration of approximately 300 mg/dL was observed, with a correlation coefficient of 0.998 (Fig. 7B). In contrast, the commercially available endpoint cholesterol measurement reagent showed a narrower dynamic range under the same conditions (Figs. 7C and D). These results preliminarily confirm the practicality of the mutants developed in this study. Further evaluation using real samples will be important for future practical applications.

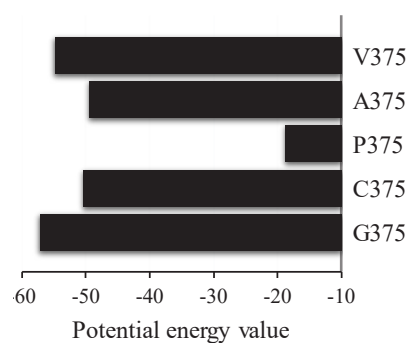


Fig. 6. Potential energies of loop structures at position 375. The potential energies of the loop structures in ChoA-VEi and its mutants at position 375 were calculated using MOE software. ChoA-VEi, intracellular ChoA-VE; MOE, Molecular Operating Environment.

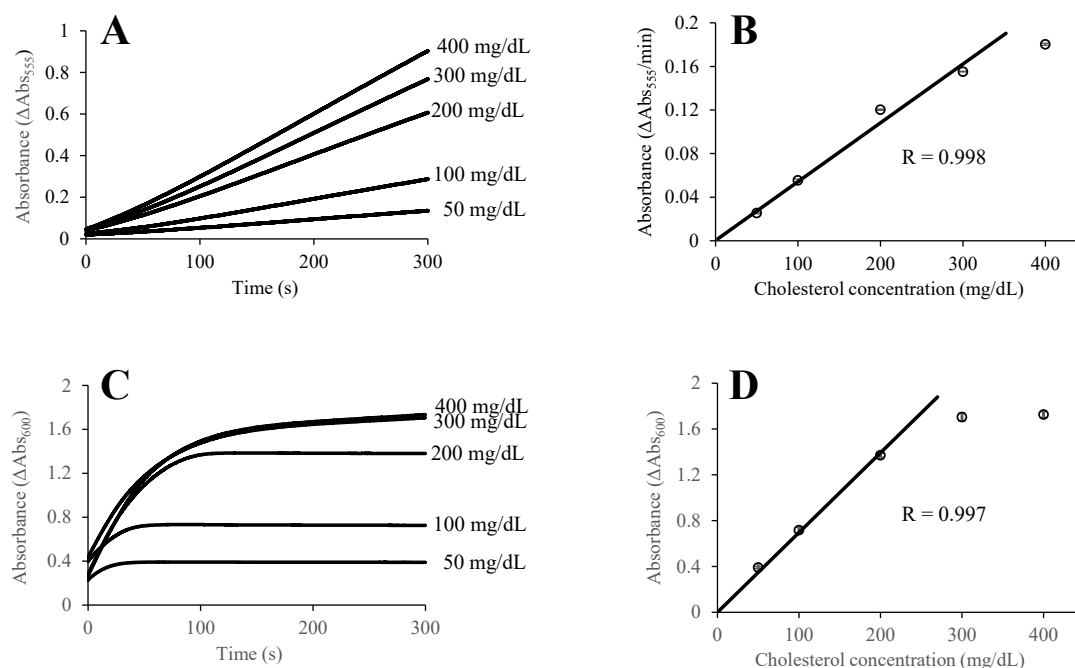


Fig. 7. Cholesterol rate assay using the G375V mutant. A: Reaction time course. Cholesterol concentrations are shown in the graph. B: Linearity. Error bars represent standard deviation ($n=3$). The R value represents the correlation coefficient. C: Reference experiments for cholesterol endpoint assay using a commercially available cholesterol measurement reagent (LabAssay Cholesterol). Endpoint assay time courses were measured in accordance with the manufacturer's instruction. Cholesterol concentrations are shown in the graph. D: Linearity of the endpoint assay. Error bars represent standard deviation ($n=3$). The R value represents the correlation coefficient.

Conflicts of interest

The authors declare no conflict of interest.

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